Studies to investigate a possible association between Polycystic Ovary Syndrome and Epithelial Ovarian Cancer

By

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Thesis submitted to University College London for the degree of MD (Res).

Declaration

I, Dr Essam El Mahdi confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated.

Dedication

This work is dedicated to my children Ahmed, Ayman, my mother and my late father.

Abstract

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders, affecting 5 % to 10 % of women of reproductive age. The Syndrome is associated with Type II diabetes and endometrial carcinoma but an association with epithelial ovarian cancer has also been suggested. The studies described in this thesis were designed to investigate this association at population, cellular and molecular levels.

In the first study, a cross sectional questionnaire survey was conducted of 121 women aged between the ages of 20 and 40, with or without PCOS. Analysis of the replies from 52 women with PCOS and 82 controls, showed that women with PCOS were significantly more likely to give a positive family history of breast cancer and myocardial infarction (20% vs 5%, $p<0.05$ and 35% vs 15%, p, 0.05, respectively).

The second study was performed on 102 formalin fixed, paraffin embedded ovarian biopsies. In this study the surface epithelium of PCOS ovaries was compared with controls. The results showed significant epithelial changes in the PCOS group, with a higher prevalence of Psammoma bodies and mitoses ($p< 0.01$ and $p < 0.02$, respectively). Expression of cell cycle and apoptotic (p53, Cyclin D, Ki67 and bcl2) proteins in the ovarian surface epithelium was assessed using immunohistochemistry in 15 PCOS subjects and 15 controls. P53 expression was significantly ($p= 0.003$) increased in the PCOS women compared with controls.

The third project was performed to identify gene expression in ovaries from women with PCOS, ovarian cancer and healthy controls (three ovaries from each group were utilized). 34(2%) genes consistently varied in abundance between normal and PCOS samples, 12 genes were over expressed in PCOS and 22 under

expressed. One of the over expressed genes identified is human alpha 2 smooth muscle actin. It was 15 fold higher in PCOS ovary**,** than in normal ovary $p< 0.001$. Conclusions: the results of these studies do not provide convincing evidence of a correlation between PCOS and ovarian cancer

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Statement of contribution to thesis

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Publications

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Atiomo WU, El-Mahdi E, Hardiman P. Familial associations in women with polycystic ovary syndrome. Fertility and Sterility. 2003: 80(1):143-5.

Lakhani K, Yang W, Dooley A, El-Mahdi E, McLellan S, Hardiman P. Aortic function is compromised in a rat model of polycystic ovary syndrome.Hum Reprod. 2006; 21:651-6.

Presentations

- Morphological changes and cell cycle expression in the ovarian surface epithelium of women with Polycystic Ovary Syndrome. The 9th RCOG International Scientific Meeting, a joint meeting with the Hellenic Obstetric & Gynaecological Society, was held 28 - 30 September 2011. Athens, Greece

- Morphological changes and cell cycle expression in the ovarian surface epithelium of women with Polycystic Ovary. 5TH Annual Meeting for the institute for Women's Health. UCL Elizabeth Garrett Anderson. 2010

- Fertility drugs and risk of ovarian cancer in PCOS patients, any possible link? 2nd International Congress on fallopian tube. London, 2009

- The influence of Polycystic Ovary Syndrome on Ovarian gene expression. 1st Annual Meeting for the Institute for Women's Health. UCL Elizabeth Garrett Anderson. 2005

- The influence of Polycystic Ovary Syndrome on Ovarian gene expression. 94th Meeting of the society for Endocrinology .Royal College of Surgeons, London.2003

- Familial associations in women with Polycystic Ovary Syndrome. Presented at the Blair Bell Research Society Meeting, Sheffield, 2002.

- Polycystic Ovary Syndrome and Cancer Breast. Presented at Whipps Cross Hospital. London. North East Thames Reproductive Medicine Society. 2002

Abbreviations

ASRM	American Society of Reproductive Medicine
AI	Androgen index
Abs	Antibodies
AP	Arbitrary primer
B-HCG	Beta human chorionic gonadotrophin
BMI	Body mass index
CKI	Cyclin kinase inhibitors
CC	Clomiphen citrate
CI	Confidence interval
CDK	Cyclin dependent kinases
Cox-2	Cyclooxygenase 2
cDNA	Complementary DNA
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DD	Differential display
DTT	Dithiothreitol
ESHRE	European Society of Human Reproduction and Embryology
ER	Oestrogen receptors
ER1	Epitope retrieval 1
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GT	Generation time
HMG	Human Gonadotrophins
H&E	Haematoxylin and eosin
hMG	Human Menopausal Gonadotropins

IVF	In-vitro-fertilisation
IHC	Immunohistochemistry
LH	Luteinising hormone
LB	Luria Bertani
mRNA	Messenger ribonucleic acids
M	Molar
MDM2	Murine double minute 2
NOS	Nitric oxide synthase
N	Normal
OSE	Ovarian surface epithelium
OR	Odds ratio
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase chain reaction
PAI	Plasminogen activator inhibitor
PCNA	Proliferating cell nuclear antigen
pRb	Retinoblastoma gene protein product
QI	Quetelet Index
UTND	Ultrasound-guided transvaginal needle ovarian drilling
rFSH	Recombinant FSH
RR	Rate ratio
RNA	Ribonucleic acid
RT	Reverse transcriptase
SIR	Standardized incidence ratio
TBE	Tris/Borate/EDTA buffer
T	Testosterone
UCL	University College London

WHR	Waist to hip ratio
µg	Micrograms
α –SMA	Alpha-smooth muscle actin

Chapter One

Introduction

Polycystic ovary syndrome (PCOS) is a complex multisystem disorder characterised by infrequent ovulation and androgen excess. These features together with morphological changes highlight the central role of the ovary in this Syndrome but more than 75 years after it was first described, the mechanism underlying the functional abnormalities in the ovary are still unknown. In this thesis, I will describe a series of studies which were designed to explore one aspect of this condition which has received less scientific attention; the possible association between PCOS and epithelial ovarian cancer. Firstly, I will describe the anatomy of the normal ovary followed be an overview of the diagnosis and management of PCOS. The different types of ovarian cancer will later be described including the pathogenesis and the staging of the disease and its progression. Emphasis will be placed on epithelial ovarian cancer. This will be followed by a description of the cell cycle and putative genetic factors which might contribute to PCOS and ovarian cancer.

1.1 Hypothesis

Women with PCOS are not at increased risk of developing epithelial ovarian cancer compared to controls that do not have PCOS.

1.2Aim

To establish whether or not there is an association between PCOS and ovarian cancer.

1.3 Objectives

1. Establish the history of ovarian cancer in first degree relatives of women with PCOS.

2. Assess the degree of morphological abnormalities in the ovarian epithelium of women with PCOS

3. Assess cell cycle and apoptotic protein expression in the ovarian surface epithelium of women with PCOS and healthy controls.

4. Assess gene expression in the ovaries of women with PCOS, healthy controls and women diagnosed with serous epithelial ovarian cancer

1.4 Background

PCOS affects 5–10% of women worldwide (Knochenhauer *et al*, 1998 Diamanti-Kandarakis *et al*, 1999, Asunción *et al*, 2000, Aziz *et al*, 2004& Broekmans *et al*, 2006). In view of this, PCOS is one of the most common human disorders and the single most common endocrinopathy in women of reproductive age. Although hetrogeneous in presentation the syndrome is characterised by signs of hyperandrogenism (hirsutism, acne or alopecia) and oligo ovulation (menstrual dysfunction or anovulatory infertility).

Additionally, ovarian cancer is the second most common gynaecological cancer in UK. In 2008, 6,537cases of ovarian cancer were diagnosed in the UK (Table1). The incidence of ovarian cancer in British women has increased over the last 30 years from around 15 per 100,000 women in 1975 to around 17 per 100,000 women in 2005; an increase of 15-17%. Surface epithelial tumours account for approximately 80% of ovarian cancers and occur predominantly in the older

reproductive and postmenopausal age groups (Capo-chichi *et al,* 2002, Yang *et al,* 2002 and Sheng *et al,* 2000). The main reason for the high mortality rate in women with ovarian cancer is that it usually remains clinically silent until it has metastasized (stage III or IV). There is therefore an urgent need for a non invasive screening test for early detection of ovarian cancer. In 2008, there were around 4,400 deaths from ovarian cancer in the UK, accounting for 6% of all female deaths from cancer (Office for National Statistics).

	Cases	AS rate
Cervix	2,938	8.7
Ovary	6,537	16.2
Uterus	7,703	19.4
Vagina	258	0.6
Vulva	1,157	2.5

Table1: Gynaecological Cancers in the UK 2008. Numbers of New Cases and Age-Standardised Incidence (AS) Rates per 100,000
Office for National Statistics. Cancer Statistics. http://www.statistics.gov.uk

1.4.1 Justification

There is some evidence to suggest a possible link between PCOS and ovarian cancer. This study was designed to explore whether there is any correlation between both conditions. Based on the high prevalence of PCOS, even a weak association with ovarian cancer could have an important effect on mortality.

1.4. 2 Ethical Considerations

Ethical approval was obtained from the Research Ethics Committee of Enfield and Haringey Health Authority.

1.5 Anatomy of the Ovary

The ovary is an important organ in the female reproductive system responsible for ovulation and production of steroid hormones.

1.5.1 Gross anatomy (Figure 1)

Outside the peritoneum, forming the floor of the ovarian fossa, are the obturator nerve and vessels. The tubal extremity of the ovary lies marginally below the level of the external iliac vessels, while the uterine extremity is immediately above the peritoneum of the pelvic floor.

The medial surface of each ovary is largely covered by the adjacent fallopian tube which, passing upwards near the mesovarian border, arches over the tubal extremity and then turns downwards in relation to the free border and posterior part of the medial surface before joining the uterus. The position of the ovaries is considerably more variable in women who have had children, because they are displaced during the first pregnancy and normally fail to return to their location within the ovarian fossae.

Anteriorly, the ovary is connected to the broad ligament by the mesovarium along the length of its mesovarian border.

A small peritoneal fold - the suspensory ligament - also passes upwards from the tubal end of the ovary to the peritoneum over the external iliac vessels and the psoas major muscles. This ligament is continuous below the ovary with the mesovarium and in front with the broad ligament. Between its two layers it contains the ovarian vessels and nerves as they pass down into the lesser pelvis to reach the hilum of the ovary via the mesovarium.

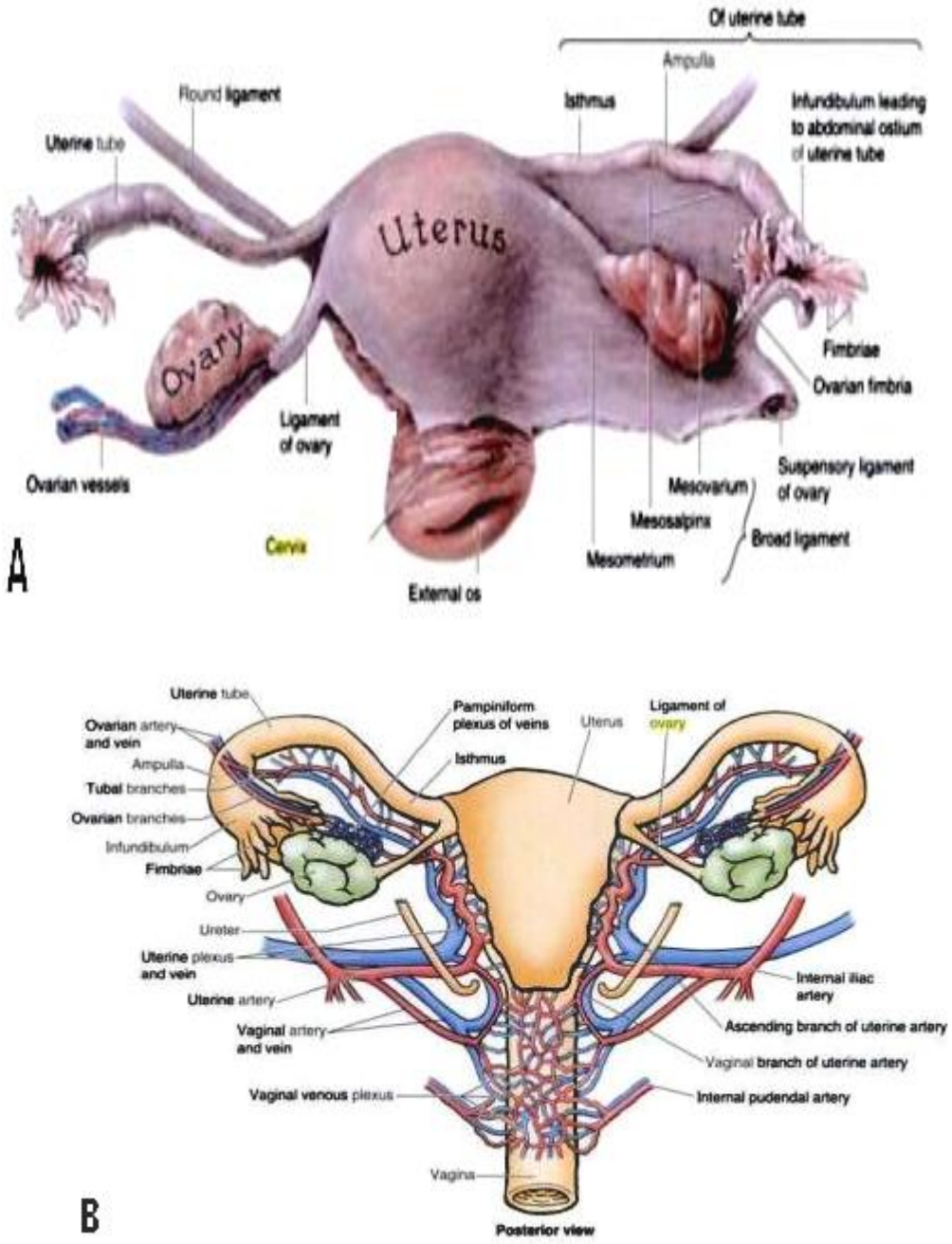


Figure 1: Gross anatomy of the Ovary: A. Female pelvic viscera B. Blood supply and venous drainage of ovaries, uterus and vagina

The uterine extremity is also connected with the uterus by the proper ligament of the ovary, which lies in the free border of the medial part of the mesovarium and is attached to the lateral angle of the uterus posterosuperior to the point of entrance of the fallopian tube. The tubal end of the ovary is directly connected with the ovarian fimbria, one of the largest of the fimbriae of the fallopian tube.

The ovarian arteries arise from the front of the aorta below the origin of the renal vessels. Each artery descends to the suspensory ligament and runs through it to enter the ovary at its mesovarian border. Close to the ovary the ovarian artery anastomoses with branches of the uterine artery. The smaller arteries within the adult ovary are spiral. Blood exits the ovary via a series of communicating veins, which eventually form one vein draining into the inferior vena cava on the right side and the renal vein on the left. The lymph vessels of the ovary follow the blood vessels and run with those from the fallopian tube and upper part of the uterus to end in the lymph nodes beside the aorta and inferior vena cava.

1.5. 2 Micro anatomy (Figure 2)

The ovary consists of three parts, the cortex which is the outermost layer, the central medulla and the hilum. The hilum is the point of attachment of the ovary to the mesovarium. It contains nerves, blood vessels, and hilus cells which have the potential to produce steroid hormones or to form tumours, these cells are very similar to the testosterone – producing Leydig cells of the testes. The outermost portion of the cortex is called the tunica albuginea; its surface is covered by a single layer of cuboidal epithelium, referred to as the ovarian surface epithelium or the ovarian methothelium. The oocytes, enclosed in complexes called follicles, are in the inner part of the cortex embedded in stromal tissue. The stromal tissue is composed of connective tissue and interstitial cells which are derived from mesenchymal cells, and have the ability to respond to LH or GnRH with androgen production. The central medullary area of the ovary is derived largely from mesonephric cells (Kumar *et al,* 2008).

Ovarian surface epithelium cells (OSE) are more complicated and physiologically adaptable than would be predicted from their ordinary appearance. The OSE has the capacity to modify the ovarian cortex through synthetic and physical functions which may influence ovulation and repair of ovulatory defects, as well as the ovarian shrinkage and cyst formation which occur with age (Kruk *et al,* (1994).
OSE cells secrete biologically active cytokines and growth factors, and that they have oestrogen receptors suggest that they play an integral part in the network of hormones and paracrine acting factors which regulate normal ovarian physiology.

Surface epithelial inclusion cysts are usually multiple, scattered singly or in small clusters throughout the superficial cortex. They are formed when the cortical invaginations of the ovarian surface epithelium lose their connection with the surface (Figure 2). There is an increased frequency of inclusion cysts (Figure 3) in apparently normal ovaries contralateral to ovarian cancer compared to ovaries from age matched women without ovarian cancer (Mittal *et al,* (1993). The surface epithelium of ovaries adjacent to epithelial ovarian cancer has a higher incidence of metaplastic and hyperplasic change than ovaries from healthy women (Resta *et al,* (1993).

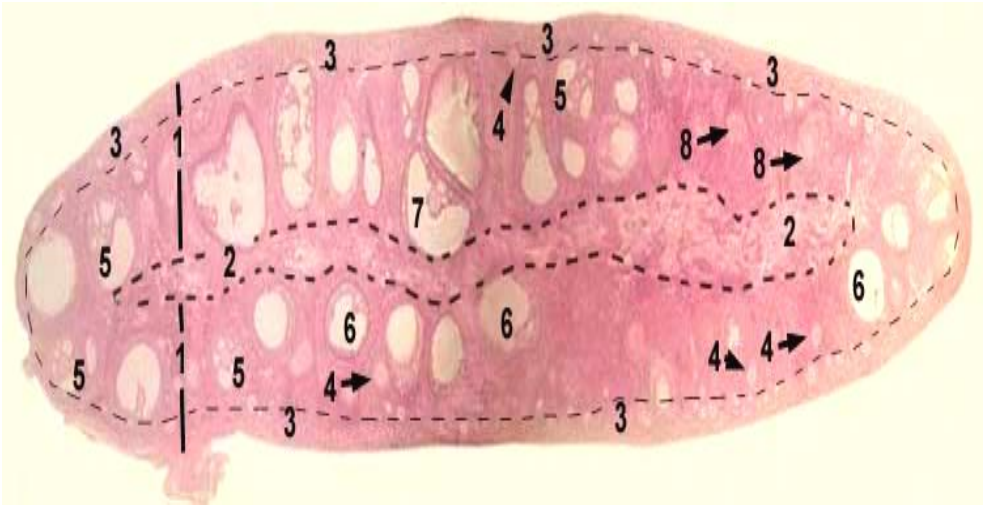


Figure 2: Ovary micro anatomy: 1 cortex, 2 medulla, 3 area where primordial follicles are located, 4 primordial follicles, 5 secondary follicles, 6 tertiary follicles, 7 mature follicles, 8 atretic follicles.

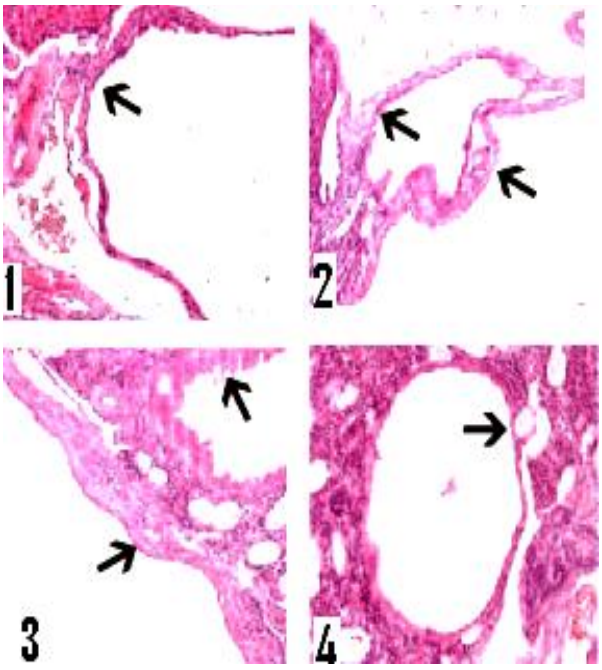


Figure 3: Inclusion Cyst (H&E stained): 1-4 show the invagination of the surface epithelium to form the inclusion cyst

1.6 Overview of Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is one of the most common hormonal disorders affects approximately 5 - 10 % of women in reproductive age (Aziz *et al*, 2004).

1.6.1 Clinical presentation

The characteristic features are menstrual changes and androgen excess (hirsutism and acne). It is a heterogeneous condition in which symptoms vary from woman to woman.

1.6.2 Historical aspects and diagnosis

The association of oligo/amenorrhoea, obesity, hirsutism with enlarged ovaries with multiple cysts and thickened tunica was first described in 1935 by Stein and Leventhal (Stein *et al*, (1935). In 1990, a consensus workshop sponsored by NIH suggested the following criteria:

- Menstrual irregularity due to oligo- or anovulation.
- Hyperandrogenism, whether clinical (hirsutism, acne, or male pattern balding) or biochemical (high serum androgen concentrations).
- Exclusion of other causes of hyperandrogenism and menstrual irregularity, such as congenital adrenal hyperplasia, androgen-secreting tumours, and hyperprolactinemia.

Notably, this NIH definition of PCOS did not include any criteria related to morphology of the ovary. However, in 2003, following a meeting of members of the European society of Human Reproduction and Embryology and the American Society of Reproductive Medicine (ESHRE /ASRM) new criteria were developed

to diagnose PCOS. According to this consensus definition the Syndrome could be diagnosed when two or three of the following criteria were met:

1. Oligo or anovulation.
2. Clinical and or biochemical signs of hyperandrogenism.
3. Polycystic ovaries as evidenced on ultrasound (Figure 4).

1.6.3 Ultrasound appearance of the ovaries

The criteria for the diagnosis of polycystic ovary morphology, using ultrasound were also agreed (Balen *et al*, 2003):

1. The PCO should have at least one of the following: either 12 or more follicles measuring 2-9 mm in diameter or increased ovarian volume (>10 cm^3). If there is evidence of a dominant follicle (>10 mm) or a corpus luteum (Figure 4), the scan should be repeated during the next cycle.

2. The subjective appearance of PCOS should not be substituted for this definition. The follicle distribution should be omitted as well as the increase in stromal echogenicity and/or volume. Although the latter is specific to polycystic ovary, it has been shown that measurement of the ovarian volume is a good surrogate for the quantification of the stroma in clinical practice.

3. Only one ovary fitting this definition or a single occurrence of one of the above criteria is sufficient to define the PCO. If there is evidence of a dominant follicle (>10 mm) or corpus luteum, the scan should be repeated next cycle. The presence of an abnormal cyst or ovarian asymmetry, which may suggest a homogeneous cyst, necessitates further investigation.

4. This definition does not apply to women taking the oral contraceptive pill, as ovarian size is reduced, even though the `polycystic' appearance may persist.

5. A woman having PCO in the absence of an ovulation disorder or hyperandrogenism (`asymptomatic PCO) should not be considered as having PCOS, until more is known about the situation.

6. In addition to its role in the definition of PCO, ultrasound is helpful to predict fertility outcome in patients with PCOS (response to clomiphene citrate, risk for ovarian hyperstimulation syndrome (OHSS), decision for in-vitro maturation of oocytes). It is recognized that the appearance of PCOs may be seen in women undergoing ovarian stimulation for IVF in the absence of overt signs of PCOS. Ultrasound also provides the opportunity to screen for endometrial hyperplasia.

7. The following technical recommendations are specified:

a) State-of-the-art equipment is required and should be operated by appropriately trained personnel.

b) Whenever possible, the transvaginal approach should be preferred, particularly in obese patients.

c) Regularly menstruating women should be scanned in the early follicular phase (days 3-5). Oligo-/amenorrhoeic women should be scanned either at random or between days 3-5 after a progestogen-induced bleed.

d) If there is evidence of a dominant follicle (>10mm) or a corpus luteum, the scan should be repeated the next cycle.

e) Follicle number should be estimated both in longitudinal, transverse and antero-posterior cross-sections of the ovaries. Follicle size should be expressed as the mean of the diameters measured in the three sections.

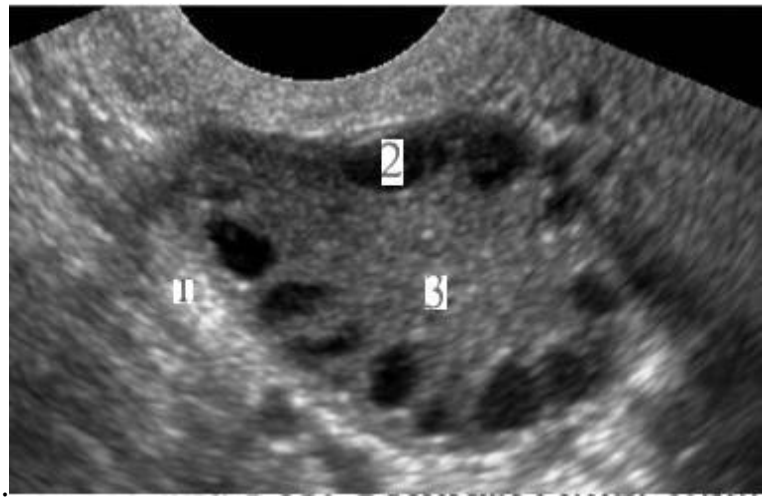


Figure 4: Ultrasound characteristic features of PCOS: (1) Thickened capsule, (2) cysts (12 in total) and (3) Increase in stromal echogenicity
www.gynaeonline.com/images/pcos.jpg

1.6.4 Histopathology of Polycystic Ovary

Typically the polycystic ovary is enlarged, two to five times the normal size with a white thickened cortex with multiple cysts that are typically less than 1 cm in diameter.

Microscopically, the superficial cortex is fibrotic and hypocellular and may contain prominent blood vessels. In addition to smaller atretic follicles, there are an increased number of follicles with luteinized theca interna. The stroma may contain luteinized stroma cells (Clement *et al,* (1987).

1. 6.5 Ovarian surface epithelial changes in PCOS

A small number of studies have demonstrated a possible correlation between PCOS and ovarian cancer but the results are conflicting (Spritzer *et al,* 2005). Resta *et al,* (1989) in a quantitative study examined ovarian surface epithelium in 50 women with PCOS having inclusion cysts and they were compared with 50 women with endometrial carcinoma and 50 women without any hyperplastic or

neoplastic genital tract pathology. In 34 women (68%) surface papillomatosis, hyperplastic and metaplastic changes were present on the ovarian surface and/or in the inclusion cysts. These findings were similar to those observed in the group of women with endometrial adenocarcinoma, while the surface epithelium was often normal in the control group. Their observations are compatible with the hypothesis of a hormonal influence in the hyper plastic and metaplastic modifications of the ovarian epithelium and in the related common epithelial tumours of the ovary.

In another study by the same author (Resta *et al,* 1993) sections of 200 specimens from hysterectomy and bilateral salpingo-oophorectomy were subjected to detailed histological examination. The cases were divided into four groups: those without hyperplastic or neoplastic disease in the tube, uterus, or vagina; those with contra-lateral epithelial ovarian tumours; those with endometrial adenocarcinomas; and those with polycystic ovary disease. A high frequency of hyperplastic and metaplastic changes in the surface epithelium or in the inclusion cysts in ovaries with contralateral epithelial ovarian tumours (92%), endometrial adenocarcinomas (76%), and polycystic ovary disease (68%) compared to ovaries without hyper-plastic or neoplastic disease (22%) were observed. These changes were frequently associated with intracystic papillae and psammoma bodies similar to those observed in epithelial tumours (Psammoma bodies are microscopic, laminated, calcified, extracellular bodies).

Resta *et al,* (1993) concluded that the hyperplastic and metaplastic changes of the surface epithelium and related inclusion cysts can be considered morphologic precursors of common epithelial tumours. Similar changes are found as a response

to a hormonal ovarian or extraovarian stimulus, which may play an important role in ovarian carcinogenesis.

Schildkraut *et al*, in 1996 reported similar findings in case control studies designed to investigate the relationship between PCOS and ovarian cancer. 476 subjects with histologically confirmed epithelial ovarian cancer were identified from 8 tumour registries of the Surveillance Epidemiology and End Results program. The study included 4081 controls ascertained via random-digit telephone dialling. All the candidates were aged 20-54 years. Seven subjects with ovarian cancer and 24 controls reported that they had been diagnosed with PCOS. The risk of ovarian cancer was increased 2.5-fold (95% CI 1.1-5.9) among women with PCOS. This risk was higher in women who had never used oral contraceptives (odds ratio [OR] 10.5, 95% CI 2.5-44.2) and women who were in the first quartile of body mass index (13.3-18.5) at age 18 (OR 15.6, 95% CI 3.4-71.0).

An Australia-wide, population-based case-control study (Olsen *et al*, 2008) was conducted to test a hypothesis relating to the role of androgens in stimulating ovarian epithelial proliferation. In this study 1276 cases aged 18-79 years with a new diagnosis of invasive epithelial ovarian cancer or borderline malignant tumour. 315 were identified through a network of clinics and cancer registries throughout Australia. Controls (n=1508) were selected from the National Electoral Roll. Women self-reported a history of PCOS, acne, hirsutism or the androgenic medication Danazol. There was no evidence that a history of PCOS, acne or hirsutism was associated with ovarian cancer overall, or with specific subtypes, with the exception of serous borderline tumours that were associated with a history of PCOS. However, it is not statistically significant (OR 2.6; 95% CI 1.0-6.1).

1.6. 6 Management

The effects of PCOS can be devastating for many patients who tend to suffer many psychological and physiological symptoms (hyperandrogenism or fertility problems or menstrual irregularity) as previously mentioned. There is no cure for PCOS. However, treatment is given according to symptoms.

1.6. 6. 1 Weight control

Between 38% and 88% of women diagnosed with PCOS are overweight or obese (Legro et al, 2000 and Balen et al, 1995). It is known that weight gain is associated with increasing insulin resistance. Hyperinsulinemia have adverse effects in women with PCOS through its action at non ovarian sites. This includes increase the pituitary LH pulse frequency and suppression of sex hormone binding globulin thereby increasing the free androgen index.

Weight control plays an important role in the improvement of all PCOS symptoms. Kiddy *et al*, (1992) reported improved menstrual function in 9 of 11 patients (82%) with oligomenorrhoea who lost >5% initial body weight (Range 5.9 to 22%) on a 1000 kcal/day, low-fat diet over 6 to 7 months. Conversely, only 1 of 11 patients (9%) losing <5% body weight demonstrated such improvement. In the same study, about 40% of obese women with PCOS (mean BMI ~34 kg/m^2) who lost >5% of initial body weight with caloric restriction achieved spontaneous pregnancy.

Standtmauer *et al*, (2005) in a review of the management of PCOS, which included a Medline search between 1986 and 2005 and the Cochrane Database, concluded that weight loss, exercise and metformin will induce ovulation in many insulin-resistant women and obese women with PCOS.

1.6. 6.2 Oral contraceptives

Oral contraceptives which have been one of the mainstays of treatment for decades are usually effective in normalizing menstrual cycles. Modern formulations are generally safer than those of years past, although their use in PCOS is under greater scrutiny with regards to their potentially detrimental effect on insulin sensitivity. Dianette also has a higher risk of inducing venous thromboembolism than 'third generation' preparations (Diamanti-Kandarakis *et al*, 2003).

1.6. 6.3 Infertility management

PCOS accounts for 75% of anovulatory infertility. It is also postulated that obese PCOS women are at increased risk of miscarriage (Homburg *et al*, 1988). Therefore, weight loss should be encouraged (Balen *et al*, 2006).

1.6. 6.3.1 Ovulation induction

Ovulation induction with clomiphene citrate (CC) is the first-line treatment. CC is structurally similar to oestrogen. This similarity allows it to inhibit the action of oestrogen on the pituitary by binding to oestrogen receptors (ER). Since oestrogen can no longer effectively exert negative feedback on the hypothalamus, *GnRH* secretion becomes more pulsatile, which results in increased pituitary gonadotropin release. Increased FSH level causes growth of more ovarian follicles resulting in ovulation.

Low-dose protocols of FSH are the second line of treatment, effective in inducing monofollicular development. Laparoscopic ovarian drilling can be an alternative but not as a first choice treatment in clomiphene-resistant patients.

1.6. 6.3. 2 Wedge resection and laparoscopic ovarian drilling

Surgical treatments for PCOS are aimed to reduce the androgen level and consequently restore the menstrual cycle. Although these procedures remove or destroy a portion of the ovaries, they generally do not impair a woman's fertility. Ovarian wedge resection has been used for a long time but because of the significant risk of adhesion formation, it is not currently used. Since the development of minimally invasive surgical techniques, laparoscopic ovarian surgery has become feasible. The potential advantages of laparoscopic ovarian surgery include repeated single ovulations and less adhesion formation.

1.6. 6.3. 3 Ultrasound-guided transvaginal ovarian interstitial laser

Ultrasound-guided transvaginal ovarian interstitial laser treatment is considered an effective new method to manage ovulation in PCOS patients. It could be conducted as an outpatient office procedure. The ease of scheduling, reduced costs, and rapid recovery would suggest it as a first-line treatment for PCOS cases resistant to CC (Zhu *et al*, 2006, Badawy *et al*, 2009 and Zhu *et al*, 2009).

1.7 Ovarian Cancer

1.7.1 Clinical presentation

Ovarian carcinoma does not cause early symptoms. As the tumour enlarges it may cause urinary urgency or frequency, constipation and a sense of heaviness in the pelvis, or dyspareunia. Malignancies of diameter >12 - 15 cm, or which initiate the development of ascites, cause abdominal enlargement. Other frequent symptoms are abdominal pain or discomfort, vaginal bleeding, bloating, dyspepsia, nausea, vomiting, cramps and diarrhoea. In one study (Goff *et al*, 2004), 22% cases were

referred to the surgeons because of gastrointestinal complaints. 28% of patients were referred with a diagnosis of pelvic mass and only 66% were referred to gynaecologists while the rest were referred to gastrointestinal specialists (9%), surgeons (9%) and occasionally to psychiatry. Ovarian cancer may present as an emergency because of torsion, rupture or intra-abdominal haemorrhage, though these are thought to be uncommon. Stages of ovarian cancer are illustrated in figure 5 and table 2.

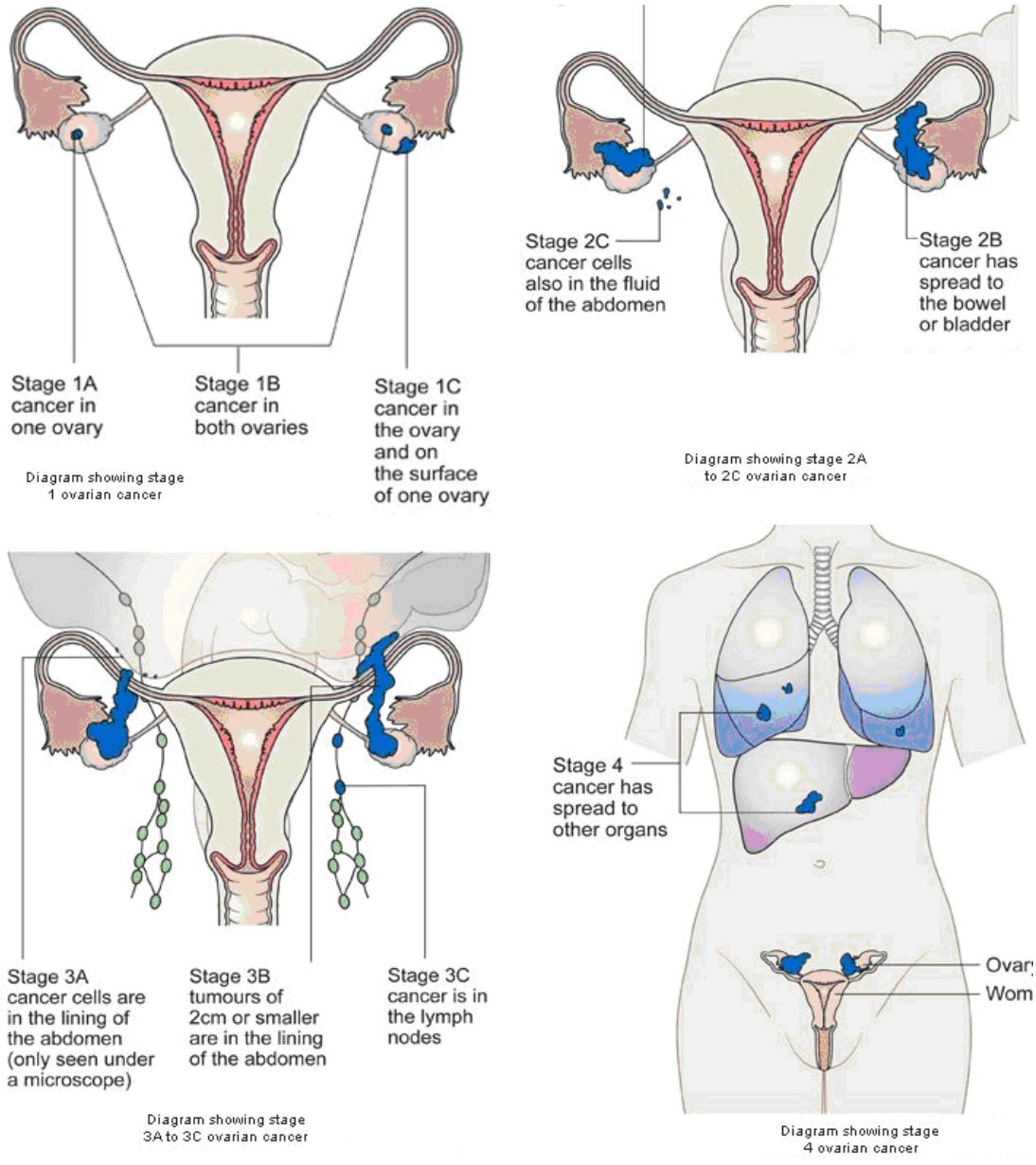


Figure 5: FIGO stage classification for ovarian cancer (www.cancerhelp.org.uk)

STAGE 1 Tumour is confined to the ovary / ovaries	
1A	• Only one ovary is affected by the tumour, the ovary capsule is intact • No tumour is detected on the surface of the ovary • Malignant cells are not detected in ascites or peritoneal washings
1B	• Both ovaries are affected by the tumour, the ovary capsule is intact • No tumour is detected on the surface of the ovaries Malignant cells are not detected in ascites or peritoneal washings
1C	The tumour is limited to one or both ovaries, with any of the following: • The ovary capsule is ruptured. • The tumour is detected on the ovary surface. • Positive malignant cells are detected in the ascites or peritoneal washings.
STAGE 2 Tumour involves one or both ovaries and has extended into the pelvis	
2A	• The tumour has extended and/or implanted into the uterus and/or the fallopian tubes. • Malignant cells are not detected in ascites or peritoneal washings
2B	• The tumour has extended to another organ in the pelvis • Malignant cells are not detected in ascites or peritoneal washings
2C	• Tumours are as defined in 2A/B, and malignant cells are detected in the ascites or peritoneal washings
STAGE 3 The tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis includes liver capsule metastasis.	
3A	Microscopic peritoneal metastasis beyond the pelvis
3B	Microscopic peritoneal metastasis beyond the pelvis 2 cm or less in greatest dimension
3C	Microscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension and/or regional lymph nodes metastasis
STAGE: 4 Distant metastasis beyond the peritoneal cavity. And, liver parenchyma metastasis.	

Table 2: FIGO staging of ovarian cancer. (www.targetovarian.org.uk)

Physical examination shows that 40 - 75% of cases have a palpable mass and 20 - 30% detectable ascites. Only 1 - 2% will have a negative examination. The bimanual rectovaginal palpation of the pelvic organs is essential since it provides the most accurate assessment of the posterior uterine surface, the pouch of Douglas and the parametria. The character of the adnexal or pelvic mass is an important indicator of its nature. Benign ovarian tumours tend to be mobile, smooth, cystic, small (<10cm) and unilateral. Solid, fixed, irregular, nodular, bilateral and large tumours are more likely to be malignant. Paradoxically, the extremely large ovarian tumours are usually benign mucinous cyst adenomas. Firm diagnosis ultimately depends upon operative intervention. Preoperative evaluation is directed towards excluding other causes of a pelvic mass.

1.7.2 Ovarian Cancer Screening

Numerous studies have demonstrated the importance of preoperative serum CA125 measurements in identifying patients with ovarian malignancy. Falsely elevated CA125 levels can be associated with pregnancy, endometriosis, adenomyosis, benign ovarian tumours and inflammatory conditions (Roett *et al,* 2009). Currently, the U.K Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) is monitoring 200,000 women between the ages of 50 and 74 based on two detection methods, serum CA125 and ultrasound scanning:

- Control group ~100,000 women - no screening
- Ultrasound scan group ~ 50,000 women to have annual transvaginal scanning

- Multimodal group ~ 50,000 women, where annual screening using serum CA125 as a primary test then serum CA125 and ultrasound scanning as the secondary test.

Preliminary results demonstrate the basic outcomes: 42 ovarian and tubal cancers were detected in the multimodal group; eight of which were borderline. 45 ovarian and tubal cancers were detected in the ultrasound scan group; 20 of which were borderline. Whether the detection of these tumours will have an effect on mortality rates will not be known until 2014, when the final results will be published (Menon *et al,* 2009).

1.7.3 Classification of Ovarian Tumours

The ovary is composed of a number of different cell types (surface epithelial, sex-cord, stromal, and germ cells), and ovarian tumours are classified on the basis of their origin from one of these cell types as shown in the following classification (Table 3) (Young *et al,* 2005 , Ulbright *et al,* 2005).

1.7.4 Classification of Surface Epithelial tumours

These tumours arise from the surface epithelium covering the ovary, which although not of Mullerian origin, has the potential to differentiate into a variety of Mullerian cell types, i.e. fallopian-tube-like epithelium (serous tumours), endocervical-like epithelium (mucinous tumours), endometrial-like epithelium (endometrioid tumours), in addition to tumours composed of clear cells (clear cell tumours) and transitional-type cells (most of which are Brenner tumours). In some tumours, the epithelial component is so poorly differentiated that it cannot be placed in any of the other categories and is simply diagnosed as an undifferentiated carcinoma. Surface epithelial tumours are further classified by the degree of

malignancy of the epithelial element, which is based on the degree of epithelial atypicality (cellular stratification, nuclear atypicality, mitotic activity) and the presence or absence of invasion of the fibrous stromal component by the epithelial component. These tumours are thus divided into benign tumours (absence of epithelial atypicality and invasion), tumours of low malignant potential or borderline malignancy (epithelial atypicality but no invasion), and carcinomas (epithelial atypicality and invasion) (Table 3).

1.7.5 Gross Pathology of Surface Epithelial tumours

Surface epithelial tumours are usually unilocular or multilocular cystic tumours, hence the diagnostic terms cystadenomas or cystadenocarcinomas. Papillary projections into the cystic spaces are most common in the serous tumours. The serous and undifferentiated carcinomas are frequently bilateral (1/3 to 1/2 of cases). Some of the tumours, especially the benign and borderline mucinous tumours, may reach enormous sizes, filling the entire pelvis or abdomen. The frankly carcinomatous tumours are usually focally solid, necrotic, and haemorrhagic. Brenner tumours differ in their gross appearance in that they are typically solid, tan, fibrous tumours.

1.7.5.1 Serous tumours

Serous tumours (Figure 6&7) are the most commonly found histological type, comprising 40 - 50% of all primary ovarian cancers (Silverberg *et al*, 2000). They tend to be large tumours and the majority exhibit mixed papillary and cystic characteristics, with multiple friable papillary masses and solid nodules. The fluid within the cyst tends to be watery, thin and straw-coloured; however in up to 28%

Cancer type	Percentage of all ovarian cancers	Characteristics
Epithelial cell	85 to 95	Most common in patients older than 50 years; 15 percent of epithelial cell ovarian cancers are borderline or have low malignant potential, with a 10-year survival rate up to 99 percent for stage I
Serous		40 percent of all ovarian cancers; most common ovarian cancer
Endometrioid		20 percent of all ovarian cancers; 15 percent of endometrioid carcinomas coexist with endometriosis; 40 percent bilateral
Mucinous		25 percent of all ovarian cancers; origin unclear; may occur in association with endometriosis; associated with pseudomyxoma peritonei
Stromal cell	5 to 8	Derived from the sex cord of embryonic gonads
Granulosa-theca		Wide age range; may produce precocious sexual development in prepubertal girls; may be associated with endometrial hyperplasia, cystic disease of the breast, and endometrial carcinoma in adults; ascites in 40 percent of fibroma and thecoma tumours; may be associated with ascites and hydrothorax (Meigs syndrome)
Sertoli-Leydig (androblastomas)		Common in adolescence; may be masculinising; may block normal female sexual development
Germ cell	3 to 5	Found mostly in children and young adults 20 to 30 years of age; highly malignant; usually unilateral
Endodermal sinus tumour		Most common germ cell ovarian cancer in children; usually larger than 15 cm; median age of patients is 18 years, one third of patients are premenarchal
Embryonal (multipotential)		Extra embryonic: yolk sac carcinoma
		embryonal carcinoma (highly malignant), precocious puberty
		Somatic: immature teratoma
		Trophoblast: choriocarcinomas
		Undifferentiated: dysgerminomas (most common malignant germ cell ovarian cancer), 10 to 20 percent bilateral, radiosensitive
Mature		Mostly benign, dermoid cysts
Metastasis to ovaries (Krukenberg tumour)	5 percent	Typically from breast or gastrointestinal

Table 3: Classification of Ovarian Tumours (Roett *et al*, 2009)

of cases it is viscous and mucoid. About 8% of serous tumours are solid adenocarcinomas (Malpica *et al*, 2004).

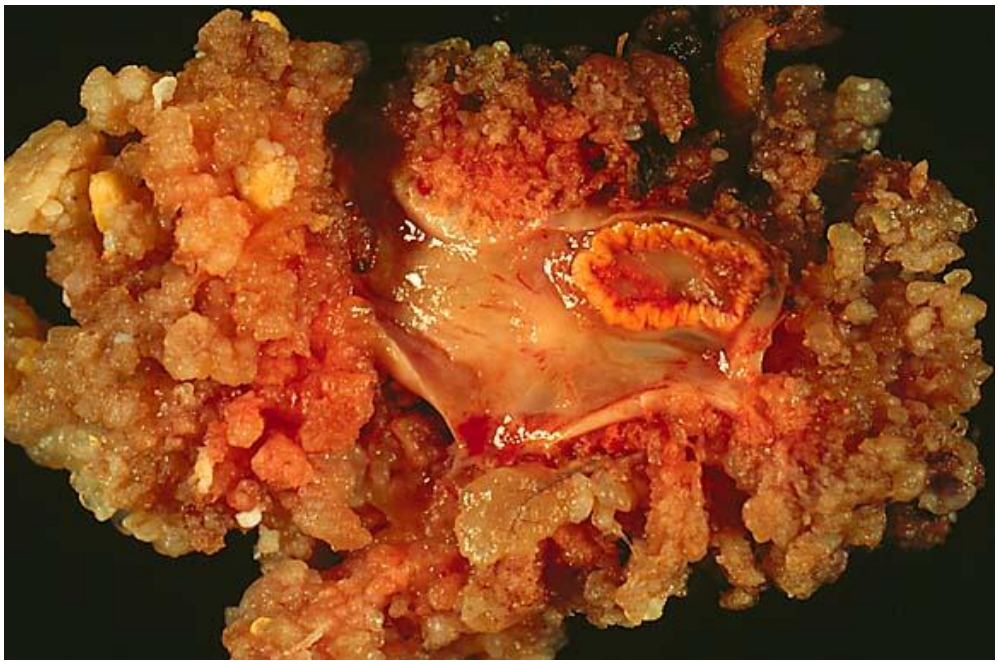


Figure 6: Ovarian serous papillary tumour

Ovarian serous tumours are bilateral in 35-50% of cases and are associated with rapid spread, leading to widespread tumour metastases within the peritoneal cavity, and marked ascites formation. Consequently, they normally have a poor prognosis (Silverberg *et al*, 2000).

Serous tumours are composed of cells resembling the surface epithelium of the ovary or fallopian tubal mucosa. Nuclei are large, vesicular and have prominent nucleoli. Size and number of nucleoli increases with histological grade. Rounded hyaline globules similar to those seen in endodermal sinus tumours are often present. Small, laminated psammoma bodies are frequently found in papillary serous tumours.

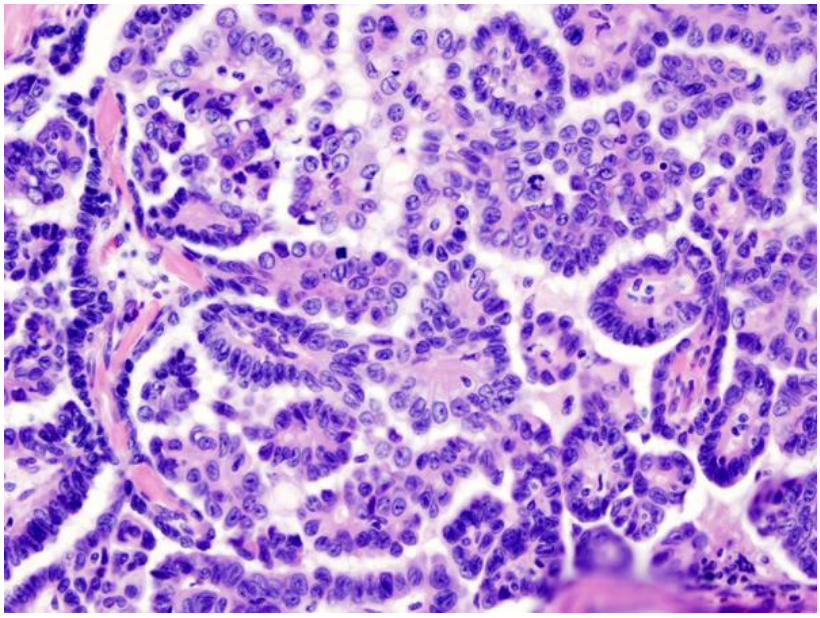


Figure 7: Ovarian papillary serous cystadenocarcinoma (high magnification); the ovary is lined by tall columnar, ciliated epithelial cells and are filled with clear serous fluid. http://en.wikipedia.org/wiki/File:Ovarian_serous_adenocarcinoma_(3).jpg

1.7.5.2 Mucinous tumours

Mucinous carcinomas account for 15% of primary ovarian malignancies and are bilateral in only 10% of cases; Mucinous tumours (Figures 8 & 9) histologically resemble endocervical epithelium. They tend to be the largest epithelial ovarian neoplasm, with a median diameter of 18 to 20 cm, but tend to remain confined to the ovaries. Pseudomyxoma peritonei, a clinical syndrome characterized by accumulation of a gelatinous ascites, may be present. Furthermore, primary ovarian mucinous tumours can be difficult to distinguish from metastatic mucinous tumours from the appendix, colon/rectum, cervix, or pancreas. Primary tumours tend to be large and unilateral (Danijela *et al,* 2011 and Khunamornpong *et al,* 2006).

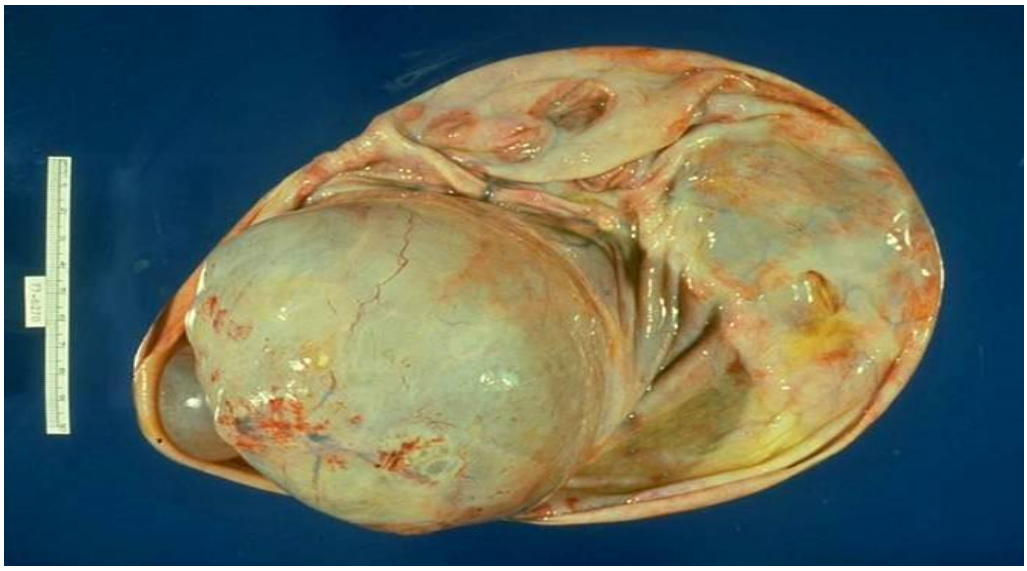


Figure 8: Ovarian mucinous cystoadeno carcinoma (Macroscopic view)

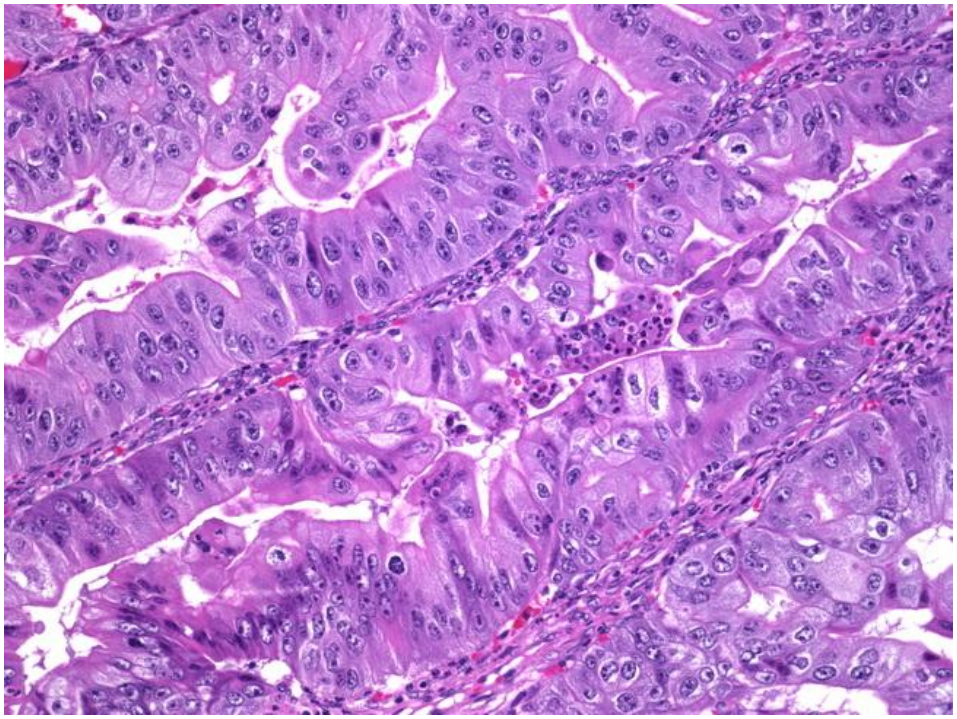


Figure 9: Ovarian mucinous cystoadeno carcinoma (Microscopic view)

http://www.webpathology.com/image.asp?n=14&Case=526

1.7.5.3 Endometrioid tumours

Endometrioid ovarian tumours (Figures 10 & 11) have features of one or more of the typical forms of endometrial neoplasia. Histogenesis is possibly from the epithelium of antecedent endometriosis or from the surface epithelium of the ovary. They account for 16-30% of all ovarian malignancies and vary in size from 2 to 35 cm. Endometrioid tumours tend to be less cystic than serous or mucinous tumours, but they often exhibit solid nodular areas of yellowish to dark red tissue alternating with cysts. Intracystic papillary projections are infrequent but, when present, papillae are blunt and broad rather than finely branched as in serous tumours.

Dark haemorrhagic fluid may indicate its origin from an endometriotic cyst. While psammoma bodies are rare, squamous metaplasia is characteristic of endometrioid tumours. Ovarian endometrioid carcinoma has ultra structural features similar to those of well-differentiated adenocarcinoma of the endometrium. Indeed, ovarian endometrioid carcinoma is commonly associated with carcinoma or hyperplasia of the endometrium; up to 20% of women with endometrioid ovarian tumours also have a uterine endometrial malignancy. Up to 33% of endometrioid ovarian malignancies are bilateral and 5-year survival for patients with tumours is around 50%. Ovarian endometriosis is present in up to 17% of cases while pelvic endometriosis has been reported in up 28% of patients (Storey *et al*, 2008).

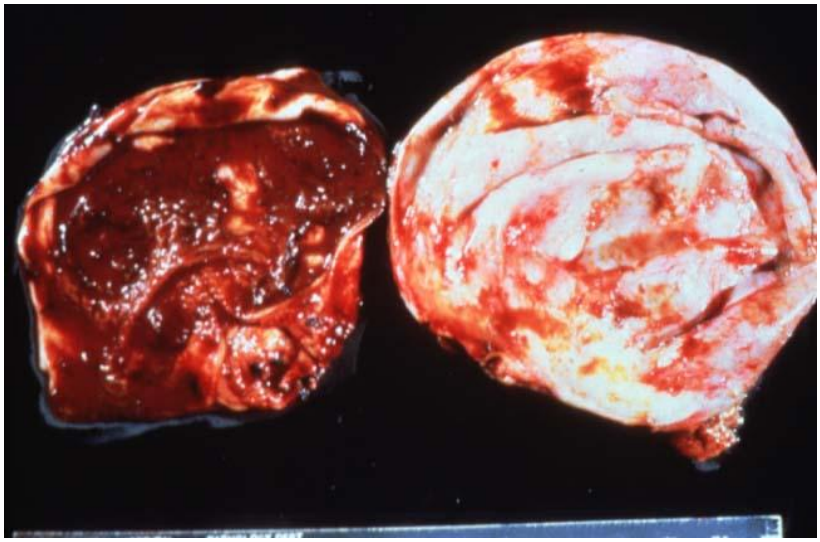


Figure 10: Ovarian endometrioid tumour (Macroscopic view)

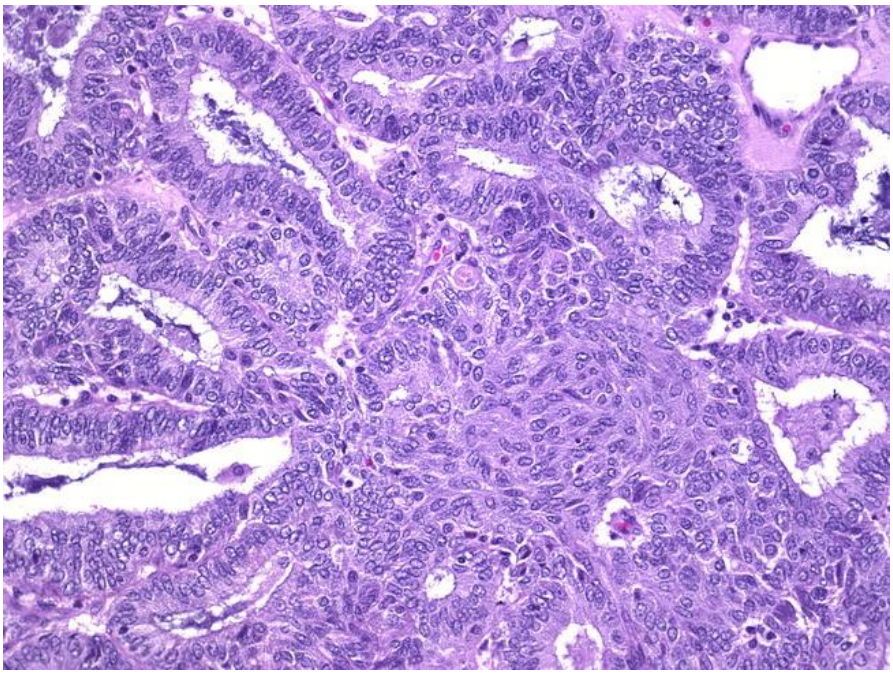


Figure 11: Ovarian endometrioid tumour (Microscopic view)
http://www.webpathology.com/image.asp?case=527&n=13

1.7.5. 4 Clear cell tumours

These account for 5 - 10% of all epithelial ovarian malignancies. The histogenesis is generally accepted as Müllerian in nature, derived from remnants of the embryological paramesonephric duct system. Histologically identical clear cell tumours arise in the cervix, vagina and endometrium. These tumours normally

exceed 15cm in size and consist of a mixture of cystic and solid areas. Bilaterality is rare. Ovarian or pelvic endometriosis has been demonstrated in up to 25% of patients with clear cell ovarian tumours.

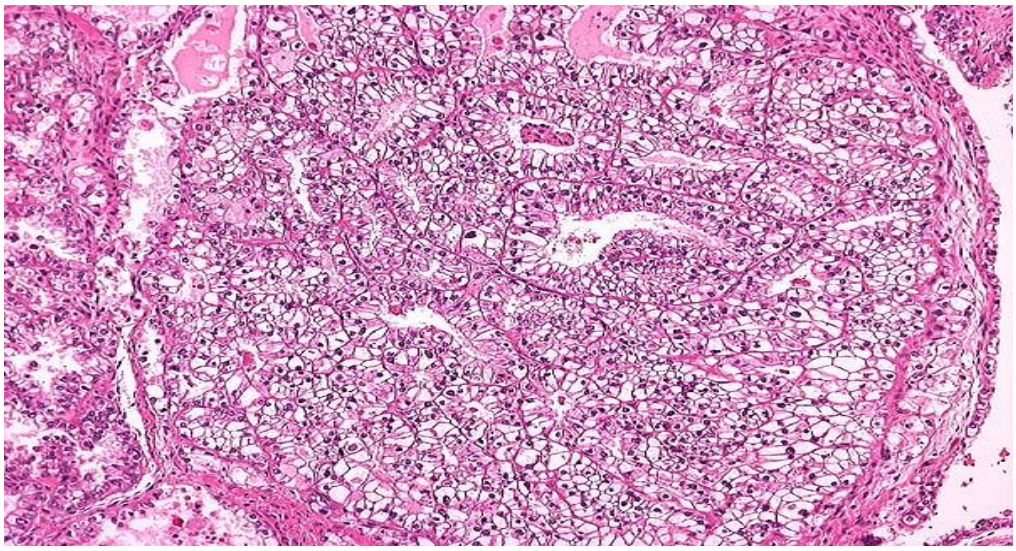


Figure 12: Ovarian clear cell carcinoma (Microscopic view)

Microscopically (Figure 12), clear cell carcinoma exhibits abundant clear or vacuolated cell cytoplasm containing glycogen. Clear cells are usually mixed with other cell types. They have abundant hyaline material in papillary areas. These cells have marked nuclear pleomorphism and scattered large hyperchromatic, bizarre cells. They also have a marked infiltrate of inflammatory lymphoid cells. Clear cell carcinomas tend to have a higher percentage of stage I disease than advanced stage disease. This suggests that clear cell carcinomas may have more symptoms when confined to the ovary and are therefore more easily diagnosed at an early stage, or that they have a lower propensity for spread. In support of the former theory, clear cell carcinomas may be associated with a large pelvic mass. Recurrences are more frequent than with other histologies (Danijela *et al,* 2011, Pectasides *et al*, 2006, Behbakht *et al*, 1989).

1.7.6 Epidemiology of Surface Epithelial tumours

1.7.6.1 Nulliparity

Nulliparity appears to be associated with an increased risk of ovarian cancer. McGowan reported a 2.5-fold increased risk of ovarian cancer in women without children (McGowan *et al*, 1988, McGowan *et al*, 1979 and Fathalla *et al*, 1971).

Hildreth *et al*,(1981) in a case-control study examining reproductive factors and ovarian cancer, studied 62 cases of ovarian cancer and 1068 controls between the ages of 45 and 74 years. Women with history of previous cancer of the breast or female reproductive tract and controls who had had their ovaries removed were excluded from the study. The findings revealed that women who remained voluntarily nulliparous had a 1.7-fold increased risk of ovarian cancer (95% CI 0.9 - 3.1), whilst if nulliparity was involuntary (i.e. in infertile patients) there was a 2-fold increase in risk (95% CI 1.0 - 4.1). Furthermore, the greater the number of pregnancies experienced by a woman, the lower was her risk of ovarian malignancy.

It has also been reported that late age of menopause increases the risk of ovarian cancer. This effect is directly related to the years of ovulation experienced; the odds ratio (OR) for ovarian cancer being 1 in those who ovulate for less than 25 years versus 4.5 in those who ovulate for more than 40 years. Conversely, the risk of ovarian cancer is unaffected by the age of menarche (OR 0.5, 95% CI 0.2 - 1.5). The use of the oral contraceptive pill (OCP) is associated with a reduced risk of ovarian cancer (Booth *et al.,* 1989)

The findings further revealed that nulliparity was associated with an 8-fold increased risk of ovarian cancer (95% CI, 1.5 - 73.4). The large 95% confidence interval reflects the small sample sets analysed in this study which, to a degree, reduces confidence in its risk estimates. Further limitations include its retrospective design and the low response rates. Additionally, the authors suggested that pregnancy at an early age is protective, but this apparent effect could not be separated from the protective effect of increasing number of pregnancies, due to sample size limitations.

Nevertheless, similar findings were reported in Mosgaard *et al*, (1997) case-control study. That study assessed the risk of invasive ovarian cancer among 684 infertile Danish women (below the age of 60 years) with ovarian cancer during the period from 1989 to 1994 and 1,721 age-matched controls. Nulliparous women had an increased risk of ovarian cancer compared with parous women: OR 1.5 to 2.0. Infertile, untreated nulliparous women had an OR of 2.7 (1.3 to 5.5) compared with non infertile nulliparous women. The OR of ovarian cancer among treated nulliparous women was 0.8 (0.4 to 2.0) and among treated parous 0.6 (0.2 to 1.3), compared with untreated nulliparous and parous infertile women, respectively.

Mosgaard *et al.* (1997) concluded that nulliparity confers a 1.5- to 2-fold increased risk of ovarian cancer. It was further stated that infertility without medical treatment among these women increased the risk. Among parous as well as nulliparous women, treatment with fertility drugs did not increase the ovarian cancer risk compared with untreated infertile women.

1.7.6.2 Ovulation Induction

The first report of invasive ovarian epithelial cancer associated with ovulation induction was presented by Bamford *et al,* (1982). A 32 years old woman with primary infertility had a history of eight cycles of ovulation induction using hCG in one year and another three cycles using FSH and LH later on. Ovulation was achieved in all these attempts. During the last cycle the left ovary was found to be slightly enlarged. Pelvic ultrasonography demonstrated bilateral ovarian cysts. At the end of that cycle she suffered heavy vaginal bleeding. At that stage a palpable left ovarian cyst was noted and arrangement was made to review the patient after one month. Three weeks later, she presented with sudden onset lower abdominal pain and tender mass arising to the umbilicus. Emergency laparotomy was performed to reveal a left ovarian mass (25 cm in diameter). The right ovary was approximately 3 cm in size. Total hysterectomy and right salpingecto-oophrectomy was performed one week later. The histology confirmed endometrioid tumour with encroachment of the capsule. The uterus showed endometrial hyperplasia which was adenomatous and atypical in areas in conjunction with a well differentiated adenocarcinoma which extended to the superior parts of the endocervical canal. The right tube and ovary were free of tumour but there was evidence of stromal hyperplasia and cystic follicle formation. These findings suggest neoplastic changes. Similar findings were reported in three other cases of ovarian carcinoma associated with fertility drugs. Two patients were hyperstimulated by clomiphene citrate. The third had hMG + CC. Two of these patients had a borderline ovarian carcinoma and the third had an invasive ovarian carcinoma associated with endometrial carcinoma (Abboud *et al,* 1997).

Another case was reported by Bandera *et al,* (1995) with similar history but with poorer outcome. That woman was diagnosed with stage 1C grade 1 mucinous epithelial ovarian cancer and died of recurrent disease shortly after receiving gonadotrophin therapy for ovulation induction. Over three years she underwent two cycles of ovulation induction with exogenous gonadotrophins five months after the second cycle. The patient presented with bowel obstruction and extensive recurrence of the disease. Two months later, she died despite extensive surgical debulking and chemotherapy. Hull *et al,* (1996) presented a 40-year-old woman who complained of secondary infertility. She conceived after five cycles of human menopausal gonadotropins with intrauterine insemination. Eight months after delivery, she presented with right lower quadrant pain and a right adnexal mass. At exploratory laparotomy, the patient was found to have a poorly differentiated papillary serous carcinoma of the ovary.

In a retrospective cohort study by Brinton *et al,* (2004) to assess the risk of cancer among infertile patients, 12,193 women with infertility problems were recruited for the study during the period of 1965-1988 at five clinical sites, 45 women were diagnosed with ovarian cancers during follow-up appointments. Standardized incidence ratios (SIR) were used to report the risk of cancer among the infertile patients to the general population, whereas analyses within the cohort allowed the derivation of rate ratios for fertility drug usage compared with no usage after adjustment for other ovarian cancer risk factors. The infertility patients had a significantly elevated ovarian cancer risk compared with the general population (standardized incidence ratio 1.98, 95% CI 1.4, 2.6). However, the rate was not significantly increased after adjusting for patient demographics and known risk factors within the infertile women, (0.82 (95% CI 0.4, 1.5) for CC and 1.09 (95%

CI 0.4, 2.8) for gonadotrophins. There were higher, albeit non significant, risks with follow-up time, with the rate ratios after 15 or more years being 1.48 (95% CI 0.7, 3.2) for exposure to CC (5 exposed Cancer patients) and 2.46 (95% CI 0.7, 8.3) for gonadotrophins (3 exposed Cancer patients). Although drug effects did not vary by causes of infertility, there was a slightly higher risk associated with CC use among women who remained nulligravid, based on 6 exposed patients (rate ratio 1.75; 95% CI 0.5, 5.7).

Another study was conducted with women who had undergone in-vitro-fertilisation (IVF) to investigate the incidence of invasive cancer of the breast, ovary, and uterus in relation to the cause of infertility or exposure to fertility drugs to induce super ovulation was associated with an increased risk (Venn *et al,* 1999). The cohort consisted of 29,700 women: 20,656 were exposed to fertility drugs and 9044 were not. 143 breast cancers, 13 ovarian cancers, and 12 cancers of the uterus were detected among these women. Women who had been exposed to fertility drugs seem to have a transient increase in the risk of having breast or uterine cancer diagnosed in the first year after treatment, although the incidence overall was not significant. Unexplained infertility was associated with an increased risk of ovarian or endometrial cancer.

In another population based cohort, Jensen *et al,* (2009) examined the effects of fertility drugs and risks of ovarian cancer. 54,362 women with infertility problems, 156 women with invasive epithelial ovarian cancer (cases) and 1241 sub cohort members identified in the cohort during follow-up in 2006. Analyses within cohort showed no overall increased risk of ovarian cancer after any use of gonadotrophins (rate ratio 0.83, 95% CI 0.50 to 1.37), clomifene (1.14, 0.79 to 1.64), human

chorionic gonadotrophin (0.89, 0.62 to 1.29), or gonadotrophin releasing hormone (0.80, 0.42 to 1.51).

Similarly, Silva Idos *et al.* (2009) examined the long-term health effects of ovarian stimulation drugs for over 20 years in a British cohort of 7355 women with ovulatory disorders. 43% of were prescribed ovarian-stimulation drugs. Total of 274 deaths and 367 incident cancers. Relative to the general population, the cohort experienced lower mortality from most causes, including from all neoplasms combined, and lower incidence of cervical cancer, but higher incidence of cancers of the breast (relative risk: 1.13; 95% CI 0.97, 1.30) and corpus uteri (2.02; 1.37, 2.87). There were no significant differences in the risk of cancers of the breast, corpus uteri, ovary, or of any other site, between women who had been prescribed ovarian-stimulation drugs and those who had not.

In summary, a great many studies have been performed to assess a possible association between fertility drugs and ovarian cancer risk (Table 4), infertility and ovarian cancer risk (Table 5), case–control studies exploring the association between infertility and ovarian cancer risk (Table 6), case–control studies investigating the association between fertility drugs and ovarian cancer risk (Table 7).

Although the results of some of these studies are to some degree contradictory, the balance of evidence suggests that infertility appears to increase the risk of ovarian cancer but this is true only for those women who do not eventually conceive. This increased risk is eliminated if pregnancy is achieved irrespective of the duration of the preceding subfertility. Nulliparous women are at an increase risk of developing ovarian cancer; where this is by choice the risk is half of that compared to those

with infertility. The cause of infertility does not seem to have a particular effect on the risk of ovarian cancer.

In summary, overall the literature suggests that fertility drugs do not increase the risk of ovarian malignancy.

Authors	Population	No. of ovarian cancers	Standardized incidence ratios (95% CIs) vs. general population	
Rossing et al. 1994	**3837**	**11**	No drug	1.4 (0.2–5.0)
			Clomiphene	**3.1 (1.4–5.9)**
			hMG/FSH	5.6 (0.1–31.0)
Modan et al. 1998	2496	12	No drug	1.6 (0.6–3.5)
			All treatments	1.7 (0.6–3.8)
			Clomiphene	2.7 (0.9–5.8)
Doyle et al. 2002	5556	6	No treatment	1.7 (0.2–6.0)
			Treatment	0.6 (0.2–2.2)
Brinton et al. 2004	**12,193**	**45**	**No clomiphene**	**2.1 (1.4–3.0)**
			Clomiphene	1.8 (1.0–3.0)
Klip et al. 2002	23,592	15	No IVF	1.4 (0.4–3.2)
			IVF	1.4 (0.7–2.6)

Table 4: Selected cohort studies studying the association between fertility drugs and ovarian cancer risk

Authors	**Population**	**No. of ovarian cancer**	**Standardized incidence ratios (95% CIs) vs. general population**	
Brinton et al. 1989	2335	11	Evaluated for infertility	1.28 (not given)
Modan et al. 1998	2496	12	Infertility patients treated	1.6 (0.8–2.9)
Venn et al. 1999	29,700	13	IVF patients evaluated	0.99 (0.57–1.70)
Potashnik et al. 1999	1197	2	Evaluated for infertility	0.91 (0.1–3.27)
Rossing et al. 1994	**3837**	**11**	**Evaluated for infertility**	**2.5 (1.3–4.5)**
Brinton et al. 2004	**12,193**	**45**	**Evaluated for infertility**	**1.98 (1.4–2.6)**

Table 5: Selected cohort studies studying the association between infertility and ovarian cancer risk

Authors	No. of cases	No. of controls	Comparison	OR (95% CI)
Whittemore et al. 1992	2197	8893	12 US case–control studies (1956–1986)	Nulligravid: 1.4 (0.86–0.23)
				Gravid: 0.87 (0.67–1.1)
Ness et al. 2002	**5207**	**7705**	**Eight case–control studies (1989–1999)**	Nulligravid: 1.1 (0.91–1.55)
				Gravid: 1.1 (1.02–1.31)

Table 6: Case control studies studying the association between infertility and ovarian cancer risk

Authors	No. of cases	No. of controls	Comparison	OR (95% CI)
Shu et al. 1989	229	229	Drugs vs no drugs use	2.1 (0.2–22.7)
Whittemore et al. 1992	**718**	**1236**	**Fertility drugs use vs no infertility**	**2.8 (1.3–6.1)**
Franceschi et al. 1994	195	1339	Drugs vs no drugs use	0.7 (0.2–3.3)
Ness et al. 2002	149	911	Drugs vs no drugs use	0.93 (0.7–1.2)
Parazzini et al. 1997	971	2758	Drugs vs no drugs use	1.1 (0.4–3.3)
			≥6 cycles	1.0 (0.2–3.8)

Table 7: Case control studies studying the association between fertility drugs and ovarian cancer risk

1.7.7 Ovarian cancer pathogenesis

The cell of origin of epithelial ovarian cancer has been long debated. The traditional view of ovarian tumours was that it derived from the ovarian surface epithelium and subsequent metaplastic changes leading to the development of the different cell types namely the serous, endometroid, clear cell, mucinous and transitional cell which morphologically resemble the epithelia of the fallopian tube, endometrium, gastrointestinal tract or endocervix and urinary bladder.

Fathalla *et al,* (1971) postulated that ovulation causes a minor trauma on the epithelial surface, and the recurrent surface epithelial trauma contributes to the pathogenesis of ovarian cancer. To explore Fathalla`s theory Kosec *et al,* (1999) studied transvaginal colour Doppler ultrasonography screening for early stage ovarian cancer in 110 asymptomatic women who received an ovulation induction therapy for infertility. Seven subjects had abnormal ovarian findings. One

underwent surgery for a benign cyst and one was diagnosed with early stage ovarian malignancy.

However, recent morphologic, immunohistochemical, and molecular genetic studies have led to the development of a new theory for the pathogenesis and origin of epithelial ovarian cancer based on a dualistic model of carcinogenesis that divides epithelial ovarian cancer into two broad categories designated types I and II. Type I tumours comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas, and Brenner tumours. They are generally present in stage I (tumour confined to the ovary), and are characterized by specific mutations, including *KRAS, BRAF, ERBB2, CTNNB1, PTEN, PIK3CA, ARID1A, and PPP2R1A*, which target specific cell signalling pathways. Type I tumours rarely harbour TP53 mutations and are relatively stable genetically. Type II tumours comprise high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumours (carcinosarcomas), and undifferentiated carcinomas. They are aggressive, present in advanced stage, and have a very high frequency of *TP53* mutations but rarely harbour the mutations detected in type I tumours. In addition, type II tumours have molecular alterations that perturb expression of *BRCA* either by mutation of the gene or by promoter methylation. A hallmark of these tumours is that they are genetically highly unstable (Kurman *et al*, 2011).

In the review by Crum *et al*, (2007) , they concluded that a significant percentage of tumours currently classified as ovarian and primary peritoneal carcinoma are associated with, and genetically related to, an early serous carcinoma in the fallopian tube.

In that respect, Kindelberger et al (2007) surveyed a consecutive series of pelvic serous carcinomas in which the entire tubal epithelium was analyzed. They found that after exclusion of primary fallopian tube carcinomas, approximately half of serous carcinomas co-existed with an intraepithelial carcinoma of the fimbria. They went on to demonstrate that in all cases, the early tubal cancer was genetically identical (based on p53 mutation status) to the ovarian or peritoneal tumour component – compelling evidence that the two are causally related.

Seidman *et al.,* (2010) highlighted the significance of fimbrial tubal epithelium in the origin of serous ovarian carcinomas. They studied 613 junctional foci in 228 fallopian tube specimens from 182 patients who underwent surgery for a variety of indications, including 27 risk-reducing salpingo-oophorectomy specimens. Transitional metaplasia was found at the junction (Figure 13) in 20% of patients and mesothelial hyperplasia in 17%. Inflammation at the junction was seen predominantly in patients with salpingitis, torsion, or tubal pregnancy. Ovarian-type stroma was found at the junction in 5% of patients, and was found elsewhere in the tubal lamina propria in an additional 27% of patients.

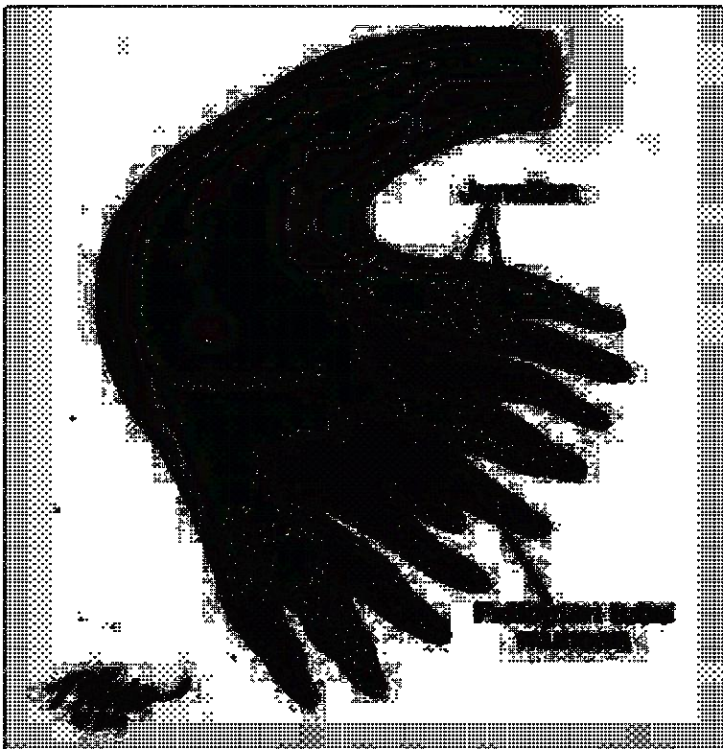


Figure 13: The Fallopian Tube-Peritoneal Junction.
The junction is tortuous with tongues of mesothelium extending for variable distances from the serosa at the outer edges of the fimbriae, onto the fimbrial plicae joining the tubal epithelium at various points along and between fimbrial plicae and at the plical tips .

A recent study by Kurman *et al.* (2010) strongly suggest that fallopian tube epithelium (benign or malignant) that implants on the ovary is the source of low-grade and high-grade serous carcinoma rather than the ovarian surface epithelium as initially postulated (Figure 14).

Similarly, it is widely accepted that endometriosis is the precursor of endometrioid and clear cell carcinomas and, as endometriosis is thought to develop from retrograde menstruation. These tumours can also be regarded as involving the ovary secondarily. The origin of mucinous and transitional cell (Brenner) tumours is still not well established, although a possible origin from transitional epithelial nests located in paraovarian locations at the tuboperitoneal junction.

Thus, it now appears that type I and type II ovarian tumours develop independently along different molecular pathways and that both types develop outside the ovary and involves it secondarily. If this concept is confirmed, it leads to the conclusion that the only true primary ovarian neoplasms are gonadal stromal and germ cell tumours analogous to testicular tumours. This new paradigm of ovarian carcinogenesis has important clinical implications. By shifting the early events of ovarian carcinogenesis to the fallopian tube and endometrium instead of the ovary, prevention approaches, for example, salpingectomy with ovarian conservation may play an important role in reducing the burden of ovarian cancer while preserving hormonal function and fertility.

Piek *et al*. (2001) in a Dutch study investigating the occurrence of preneoplastic lesions in overtly normal fallopian tubes from women predisposed to developing ovarian carcinoma, had similar conclusions. The presences of preneoplastic lesions were scored in histological specimens from 12 women with a genetically determined predisposition for ovarian cancer, of whom seven tested positive for BRCA1 mutation. When compared to a control group of 13 healthy women, all the results were normal. Immunohistochemistry was used to determine the expression of p21, p27, p53, Cyclin A, Cyclin D1, bcl-2, Ki67, HER-2/neu, and the oestrogen and progesterone receptors. Loss of heterozygosity analysis on the *BRCA1* locus was also assessed in dysplastic tissue by polymerase chain reaction (PCR) studies.

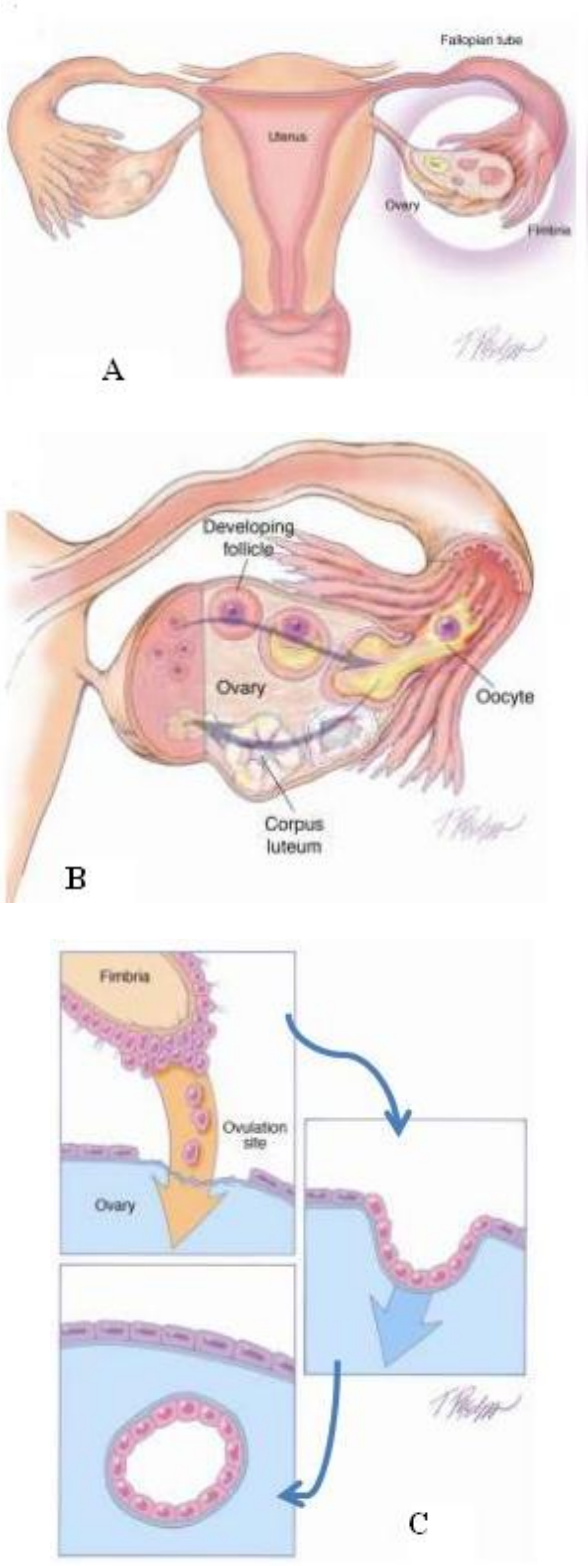


Figure 14: Transfer of normal tubal epithelium to the ovary. A. Anatomical relationship of fallopian tube to the ovary at the time of ovulation. The fimbria envelops the ovary. B. Ovulation. The ovarian surface ruptures with expulsion and transfer of the oocyte to the fimbria. The fimbria is in intimate contact with the ovary at the site of rupture. C. Tubal epithelial cells from the fimbria are dislodged and implant on the denuded surface of the ovary resulting in the formation of an inclusion cyst.

Of the 12 women with a predisposition for ovarian cancer, six showed dysplasia, including a severe case. Five harboured hyperplastic lesions and in one woman, no histological aberrations were found in the Fallopian tube. No hyperplastic, dysplastic or neoplastic lesions were detected in the Fallopian tubes of control subjects. In the cases studied, morphologically normal tubal epithelium contained a higher proportion of Ki67-expressing cells ($p=0.005$) and lower fractions of cells expressing p21 ($p<0.0001$) and p27 ($p=0.006$) than in the control group. Even higher fractions of proliferating cells were found in dysplastic areas ($p=0.07$) and accumulation of p53 was observed in the severely dysplastic lesion. Expression patterns of other proteins studied, including the hormone receptors, were similar in both groups. However, one subject, *BRCA1* mutation carrier, showed loss of the wild-type *BRCA1* allele in the severely dysplastic lesion.

The conclusion was that the Fallopian tubes of women predisposed to developing ovarian cancer frequently harbour dysplastic changes, accompanied by changes in cell-cycle and apoptosis-related proteins, indicating an increased risk of developing tubal cancer.

Approximately 10% of EOCs are associated with inheritance of an autosomal dominant genetic aberration which leads to cancer predisposition with a high penetrance (Boyd *et al*, 2003). Inheritance of a deleterious mutation in one of BRCA genes is associated with a 45% to 80% life time risk of breast cancer and 27% to 44% life time risk of ovarian cancer (Jelovac *et al*, 2011).

The exact cells of origin for *BRCA* associated ovarian cancer is yet to be identified. However, the most common premalignant pathologic findings in risk reducing

salpingo-oophorectomy specimens from *BRCA* mutation carriers are in the distal, fimbriated end of the fallopian tube (Crum *et al,* 2009).

Deligdisch *et al,* (1999) examined slides of ovarian tissue of 54 women. All had a family history of ovarian carcinoma; 44 women were BRCA positive, 31 women underwent prophylactic oophorectomy and 23 underwent oophorectomy for ovarian carcinoma. Normal, dysplastic, and ovarian carcinoma epithelial cells were analysed morphometrically combining nuclear area measurements with chromatin texture assessment using a novel method based on the computation of autocorrelation coefficients and a derived parameter. Discriminate analysis between classificatory algorithms was used to obtain results. Ovarian dysplasia was identified in 77.6% of the prophylactic oophorectomy specimens. Another study by Diniz *et al,* (2011) where thirty-four cases of serous pelvic carcinoma with clinical presentations suggesting an ovarian origin was analyzed. Histological samples of fallopian tube tissues were analyzed. Probable primary sites, types of tubal involvement, tissues involved in the neoplasia and vascular involvement were evaluated. Fallopian tube involvement was observed in 24 (70.6%) of 34 cases. In 4 (11.8%) of these cases, an intraepithelial neoplasia was present, and therefore these cases were hypothesized to be primary from fallopian tubes. For an additional 7 (20.6%) cases, a fallopian tube origin was considered a possible primary. It was concluded that fallopian tubes can be the primary site for a subset of pelvic high-grade serous carcinomas.

Ovarian dysplasia has been established as a histological entity based on microscopic and morphometric criteria. These changes have been considered important in pathogenesis of EOC but this theory is now controversial. Chêne *et*

al, (2009) conducted a retrospective cohort study using specimens from 90 patients who had undergone bilateral oophorectomy or ovarian cystectomy; 28 prophylactic oophorectomies for genetic predisposition to ovarian cancer and 62 controls- fertile with no malignancy between 1992 and 2005 and whose ovaries were reported as normal. The ovaries were analysed and reviewed by four pathologists blinded to the clinical data. An ovarian dysplasia score based on eleven epithelial cytological and architectural features was devised to quantify extent of ovarian epithelial abnormalities. The results showed a higher mean dysplasia score in the prophylactic oophorectomies than in the controls (9.67 vs 4.19, $P < 0.001$). There was a gradation in the severity of the dysplastic lesions between proven BRCA mutations and prophylactic oophorectomies without mutations (11.26 vs. 8.1) and according to age (10.27 > 50 years vs. 8.6 before age 50 years, $P = 0.2962$).

Corakci *et al*, (2005) evaluated the effects of ovulation induction on Ki67 expression and dysplasia scores of female rat ovaries. Twenty female rats were randomized either to receive 150 iu human menopausal gonadotropin on oestrous day 2 and 75 iu hMG on the day of preoestrous (induction group, n= 10) or saline as placebo on the corresponding days (control group, n= 10). After five oestrous cycles bilateral oophorectomy was performed to compare the Ki67 expression and dysplasia score of the ovarian epithelium. The mean number Ki67 positive cells was 159.6 +/- 101.92 in the follicles, 283.4 +/- 42 in the corpus luteum , and 151 +/- 75.1 in the stroma of the study group compared to 41.8 +/- 35.6 (p 0.03), 43.2 +/- 28.3 (p 0.007), and 55.6 +/- 18.6 (p 0.01), respectively, in the control group. The mean number and rate of cells that stained positive for Ki67 in the epithelium was significantly higher in the ovulation induction group (758 +/- 71 and 63 +/-

1.6%, respectively) compared to the control group (386 +/- 23, $p < 0.001$; and 60 +/- 1.1%, $p< 0.001$; respectively). The mean dysplasia score was significantly higher (9.6 +/- 1.3) in the study group compared to the control group (5.08 +/- 0.9, $p< 0.001$). The results showed that ovulation induction in rats results in increased Ki67 expression and dysplastic features in the ovarian epithelial cells.

Brewer *et al*, (2004) investigated putatitive preneoplastic changes in the ovary. Ovaries were collected from 10 low-risk women, from seven high-risk women and from three women with ovarian cancer. Five micron sections were cut and haematoxylin and eosin stained. High-resolution images were recorded from the epithelium lining inclusion cysts and from the underlying stroma of ovaries from these 20 subjects. A total of 2860 epithelial nuclei and 3610 stromal nuclei were recorded. Karyometric features and nuclear abnormality were computed. Discriminate analyses and unsupervised learning algorithms defined deviations from normal that were designated "above threshold" and used to compute average nuclear abnormality of a second nuclear phenotype. Histologically normal epithelium from inclusion cysts of ovaries harbouring a malignant lesion was shown to exhibit changes in the nuclear chromatin pattern that were statistically significant using quantitative image analysis procedures. Similar changes were seen in the inclusion cyst epithelia of high-risk ovaries. A subpopulation of cells representing a new phenotype was detected in the underlying stroma of women harbouring a malignant ovarian lesion and in women at high risk of ovarian cancer. Brewer *et al*, (2004) concluded that the karyometric changes observed in the epithelium lining inclusion cysts and in the underlying stroma of ovaries, either with ovarian cancer or at high risk of ovarian cancer, suggest the presence of preneoplastic changes in histologically normal tissue.

Okamura *et al*, (2001) assessed specific changes in the architecture and in the cytological characteristics of human ovarian epithelial cells on the surface and within inclusion cysts. Normal ovaries were retrieved from 215 patients undergoing incidental oophorectomy and 33 patients with contralateral ovarian tumours. In addition, 75 patients diagnosed with epithelial ovarian cancer and 26 ovarian endometriosis patients undergoing oophorectomy were selected. The incidence of cortical invasion, epithelial papillomatosis and pseudo stratification was no different in normal ovaries of pre- and post-menopausal women, whereas inclusion cysts were more frequently observed in post-menopausal women ($p<0.05$). The prevalence of invagination and inclusion cysts were significantly higher in normal ovaries examined after incidental oophorectomy ($p<0.05$) and in those with contralateral ovarian tumours ($p<0.05$), respectively.

Although serous metaplastic change with cilia was most common and did not differ before and after menopause, the frequency of mucinous, endometrioid and transitional cell changes were low in the two groups of pre- and post-menopausal women. Mucinous metaplasia was more frequently observed in patients with contralateral ovarian tumours than those without ovarian tumours (12.1% vs. 1.9%; $p<0.05$). Four out of 75 ovarian cancer patients revealed malignant transformed cells from a single layer of normal epithelium covering the ovarian surface or inclusion cyst. Ovarian or extra ovarian endometriosis was identified in 16 out of 75 epithelial ovarian cancer patients analyzed. In situ carcinogenesis in an endometriotic cyst was seen in three epithelial ovarian cancers. In seven out of 26 ovarian endometriosis cases, epithelial cells on the ovarian surface or within the inclusion cyst were histologically changed to endometriotic gland cells. Careful

and extensive observations of surgical specimens with ovarian carcinoma and ovarian endometriosis showed that they originated from the ovarian epithelium lining the surface and cortical small cysts.

Feeley *et al,* (2001) suggested that the exposure of the mesothelial lining of an inclusion cyst to the ovarian stromal microenvironment may be responsible for the phenotypic change to Mullerian epithelium commonly seen in these cysts. Mullerian metaplasia is usually serous phenotype, and it is possible that undefined molecular events occurring in an inclusion cyst that has undergone Mullerian metaplasia may initiate neoplastic change in these cysts. This may be the developmental pathway of most invasive serous carcinomas. Occasional rare cases of ovarian intraepithelial neoplasia, manifested by epithelial atypia in an inclusion cyst or on the surface epithelium without invasive carcinoma, are identified histologically.

1.8 PCOS and ovarian Cancer

Chittenden *et al* (2009) conducted a systematic review to determine whether there is an association between PCOS and gynaecological malignancy. Medline and Embase databases (1968-2008) were searched. A total of 19 studies exploring the association between PCOS and breast, endometrial and ovarian cancer were identified. They concluded a possible link between PCOS and ovarian cancer and the results are conflicting but suggest that this association is unlikely.

1.8.1Gene expression in PCOS ovaries

To date, the results of genetic studies have failed to identify specific gene/s with clear clinical significance (Diamanti-Kandarakis *et al,* 2005) (Table 8). The

comparison of gene expression profiles between PCOS and normal ovaries may reveal novel information regarding genes associated with early and later stages of antral follicle development, ovulation, and corpus luteum formation under normal conditions.

Pathophysiological mechanisms	Candidate genes	Comments	References
Genes involved in biosynthesis and metabolism of androgens	LH and its receptor	Multicentre study, mutation of LH receptor, no linkage or association	Tapanainen et al. (1999)
	CYP11	Randomized clinical study, partial association	Gharani et al. (1996), Urbanek et al. (1999), Kahsar-Miller et al. (2000)
	-cytochrome P450 side chain cleavage enzyme		
	CYP17-cytochrome P450 17	No association or linkage	Carey et al. (1994), Witchel et al. (1998), Diamanti-Kandarakis et al. (1999b)
	CYP21-cytochrome P450 21-hydroxylase	Mutation, no association	Escobar-Moreale et al. (1999), Witchel and Aston (2000)
	Androgen receptor	Family studies, no association	Urbanek et al. (1999), Mifsud et al. (2000)
	Sex hormone binding globulin (SHBG)	Polymorphism, no association	Hoogeveen et al. (2002), Xita et al. (2003)
	Other steroidogenic genes	Family study, no association	Urbanek et al. (1999)
Genes involved in the secretion and action of insulin	Insulin gene VNTR	One study showed linkage and association of VNTR with PCOS. Further family studies showed no association.	Bennett et al. (1995), Waterworth et al. (1997), Urbanek et al. (1999), Calvo et al. (2002), Vankova et al. (2002)

	Insulin receptor gene	Polymorphism in the tyrosine kinase domain of INSR showed association with PCOS. Caucausian family studies, D19S884 marker near insulin receptor gene, chromosome 19p13.3 showed linkage and association	Dunaif et al. (1995), Moran et al. (2001), Tucci et al. (2001), Siegel et al. (2002), Ukkola et al. (2002)
	Insulin receptor substrate (IRS) proteins	Polymorphisms in IRS1 and IRS2, no association	Ehrmann et al. (2002a), Ibanez et al. (2002)
	Insulin-like growth factors (IGF)	Association with IGF2 and PCOS in Spain, no linkage	Conover et al. (1992), Buyalos et al. (1995), Urbanek et al. (1999), Cataldo et al. (2003), San Millan et al. (2004)
	Calpain-10	Contradictory data, association in Spanish population	Ehrmann et al. (2002b); Gonzalez et al (2002, 2003), Haddad et al. (2002)
Genes involved in gonadotrophin action and regulation	Dopamine receptor genes	Polymorphisms, no association	Kahsar-Miller et al. (1999)
	Follistatin gene	Family studies, no clear association or linkage	Urbanek et al. (1999, 2000)
Genes involved in obesity and insulin resistance	Peroxisome proliferator-activated receptor-	Polymorphism, prevalence in finish population, no association in US and Spain	Witchel et al. (2001), Hara et al. (2002), San Millan et al. (2004)
	Human sorbin and SH3 domain-containing 1 gene (SORBS1)	Multicentric European study, no association	Lin et al. (2001), Nieters et al. (2002), Witchel et al. (2003)

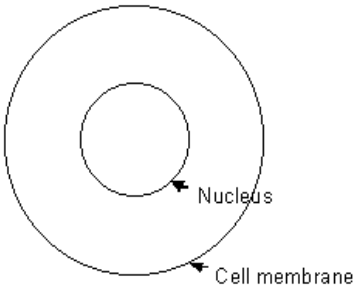
	Paraoxonase (PON1)	Polymorphism, no association	Leviev et al. (2001), San Millan et al. (2004)
	Genes encoding other molecules related to insulin resistance	No association or linkage	Rajkhowa et al. (1996), Oksanen et al. (2000), Urbanek et al. (2003), San Millan et al. (2004)
Genes involved in chronic inflammation	Plasminogen activator inhibitor-1 (PAI-1)	Association of 4G5G polymorphism in Greek population of PCOS women	Diamanti-Kandarakis et al. (2004b)
	Tumour Necrosis Factor-	No association	Hotamisligil et al. (1996, 1999), Milner et al. (1999), Escobar-Morreale et al. (2001)
	Type 2 TNF receptor gene	No association	Fernandez-Real et al. (1998), Peralet al. (2002)
	Interleukin-6 gene (IL-6)	No linkage, or association	Villuendas et al. (2002), Escobar-Morreale et al. (2003a)
	IL-6 signal transducer gp130 (IL-6ST)	No association	Escobar-Morreale et al. (2003b)

Table 8: Systematic review of candidate genes investigated for their possible association with PCOS

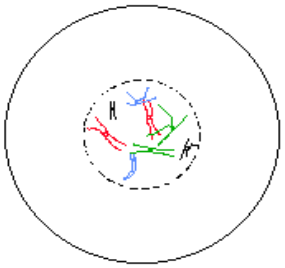
1.9 Overview of the cell cycle

This section will review the physiology and regulation of the cell cycle and its role in cancer process when it is altered. In particular, I will focus on the control of the cell cycle by a subfamily of Cyclin-dependent kinases, their activators (Cyclins), and their inhibitors (CKIs), and how deregulation of their activity in cancer cells is associated with tumourigenesis.

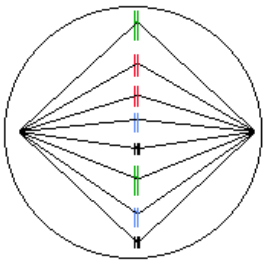
To start with, the cell cycle is divided into two stages: (a) Mitosis (M) which is a process of nuclear division which includes prophase, metaphase, anaphase and telophase and (b) interphase which includes G1, S and G2 phases (Norbury *et al*, (1992). Replication of DNA occurs in S phase which is preceded by a gap called G1 during which the cell is preparing for DNA synthesis, followed by another gap called G2 during which the cell prepare for mitosis (Figure 15).



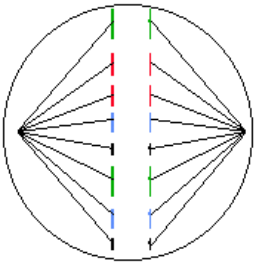
Interphase (G_1 and G_2)
Chromosomes are not visible
because they are uncoiled



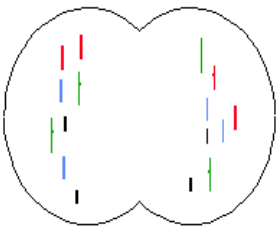
Prophase
The chromosomes coil,
The nuclear membrane disintegrates
The spindle apparatus forms.



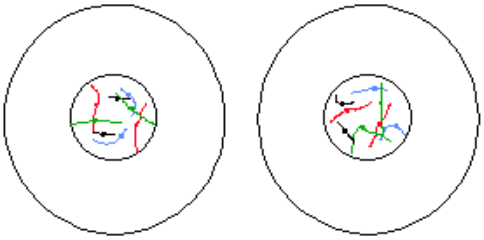
Metaphase
The chromosomes become aligned.



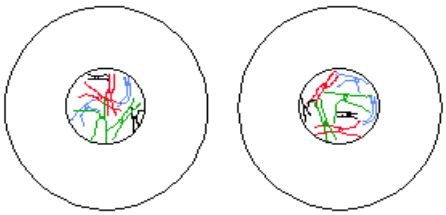
Anaphase
The chromatids separate
The number of chromosomes doubles



Telophase
The nuclear membrane reappears
The chromosomes uncoil.
The spindle apparatus breaks down.
The cell divides into two.



G_1 Interphase
The chromosomes have one
chromatid



G_2 Interphase
The chromosomes have two chromatids

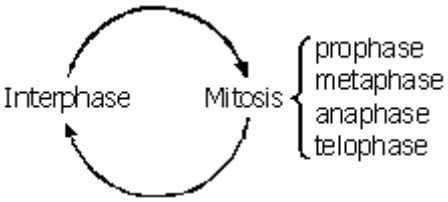


Figure 15: overview of the cell cycle

http://faculty.clintoncc.suny.edu/faculty/michael.gregory/files/bio%20101/bio%20101%20lectures/mitosis/mitosis.htm

1. 9.1 Control of the cell cycle

The regulation of the cell cycle is precise and evolutionarily highly conserved. It involves complex and dynamic structural interactions between proteins such as p21, p27, p53, the retinoblastoma gene protein product (pRb), the Cyclins, the Cyclin-dependent protein kinases (CDK) and proliferating cell nuclear antigen or Ki67, which impose logic and temporal order on the cell cycle (Clurman *et al*, (1995).

1. 9.1.1 Cyclin D

Cyclin D gene is located at chromosome 11q13 and codes for the first Cyclin protein acting in G1. Three forms of Cyclin D have been characterized, Cyclin D1, D2 and D3. They are rate limiting for cell cycle progression. Cyclin D complexes with Cyclin dependent kinase (CDK) CDK4 and/or CDK6 and the CDK activity can then be positively or negatively regulated through phosphorylation/ dephosphorylation by CAK and Cyclin kinase inhibitors (CKI).

1. 9.1.2 Cyclin dependent kinase (CDK)

CDK are proteins that become activated at specific points of the cell cycle (Morgan *et al*, 1995 and Pines *et al*, (1995).

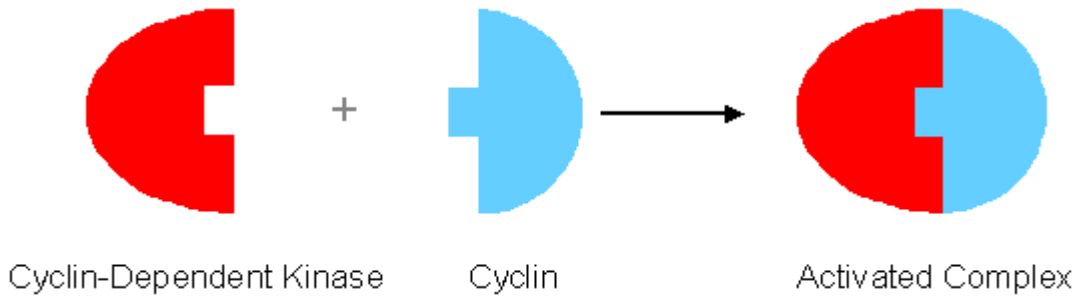


Figure 16: The activated Cyclin kinase complex
http://faculty.clintoncc.suny.edu/faculty/michael.gregory/files/bio%20101/bio%20101%20lectures/mitosis/mitosis.htm

Transition through the different phases of the cell cycle is regulated by the activities of Cyclins, CDK and their inhibitors (CKI). CDK activity is regulated by phosphorylation and dephosphorylation through Cyclin-activated kinase (CAK), which only activates CDKs when the specific Cyclin bound reaches a critical concentration (Figure 16&17). Once CDKs have carried out their role they become deactivated and decline in concentration. Eight CDKs have been identified (CDK1 to CDK8) which exhibit considerable structural similarity and 75% sequence homology (Assoian *et al,* 1997). Cyclins are not all involved directly in the cell cycle, neither are they present all the time during the cell cycle but only at particular phases. Before the onset of the active cell cycle, while cells lie in G0, p27 (a Kip/Cip family CDK inhibitor) is present in high concentrations which prevent CDK4 and CDK6 from being activated by Cyclin D.

Activated Cyclin D/CDK4 and Cyclin D/CDK6 complexes phosphorylate pRb causing release of the E2F transcription factor to which it was bound (Witzel *et al,* 2010). The release of activated E2F transcription factor is required to prepare the nucleus for DNA replication and initiate this process. At the onset of S phase, Cyclin D concentrations decline while Cyclin E-CDK2 complex concentrations rise in late G1 and drive the cell cycle transition from G1 into S phase. Once the cell enters the S phase, this complex declines and the Cyclin A-CDK2 complex takes over as the main driver of the cell cycle through the S phase, promoting DNA replication for example, and on into G2. There is another restriction point in G2, at which the cell cycle is held to allow complete and accurate DNA replication before progression to the M phase. This is stimulated by mitosis promoting factor (MPF), a complex of CDK1 (also known as cdc2) and Cyclin A or B. The CDK1-Cyclin, a complex considered more important in promoting the G1-M phase

transition, while the CDK1-Cyclin B complex is more important during the M phase since it controls the onset, sequence and completion of mitosis.

CDK inhibitors also control the transition between cell cycle phases and increases in their activity can block CDK activation and cell proliferation. Two types of CDK inhibitors exist, the Kip/Cip family (p21, p27 and p57) which inhibit CDK2, CDK4 and CDK6, and the INK4 family (p15, p16, p18 and p19) which also inhibit CDK4 and CDK6 but, in addition, they inactivate murine double minute 2 (MDM2) protein thus increasing p53 stability. This effect of INK4 proteins further induces cell cycle arrest because p53 induces expression of p21, a Kip/Cip mediator of cell cycle arrest.

Over expression of Cyclin D1 has been found in 28% of epithelial ovarian cancers (Worsley *et al*, 1997).

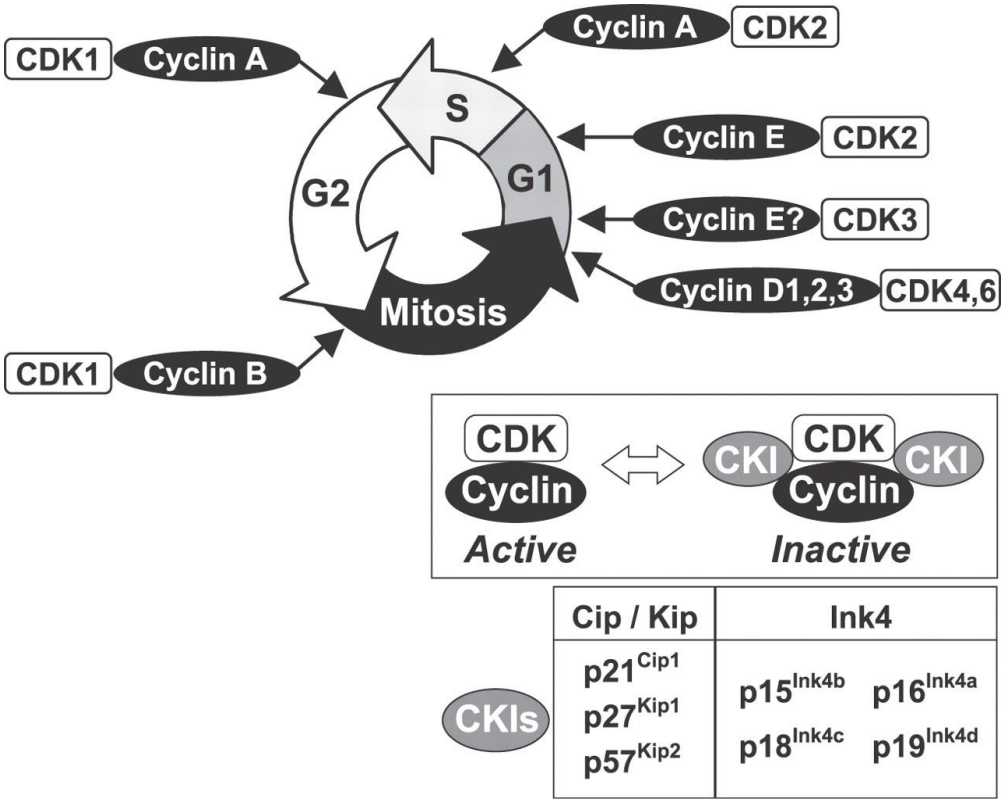


Figure 17: Cell cycle control. Activation of specific CDK/cyclin complexes drives progression through the cell cycle (CDK1=CDC2). CKIs interact with and inactivate CDK/cyclin holoenzymes. (Andres 2004)

1. 9.1.3 Check point control

The restriction point ‘R’ is a point of no return in G1, following which the cell is committed to enter the cell cycle. Experiments demonstrate that cells starved of serum before the restriction point enter a G0 like state, while cells starved after R are unaffected and continue through mitosis (Pardee *et al,* 1974).

At the G1/S checkpoint, cell cycle arrest induced by DNA damage is p53 dependent. Usually, cellular p53 level is low but DNA damage can lead to rapid induction of p53 activity (Levine *et al,* 1997).

Check point control is an essential part of the cycle progression. Cell cycle check points sense the flows in critical events such as DNA replication and chromosomal segregation (Elledge *et al,* 1996). These signals cause delay in cycle progression, until the danger of mutation has been averted.

1. 9.2 Cell cycle abnormalities

The time required to complete a cycle is called generation time (GT). Tumour cells usually have shorter GT than normal cells and subsequently, a smaller percentage of cells in the resting phase. Initially, there is an increase in the growth followed by a plateau phase when the cell death nearly equals to the rate of formation of new cells. This is due to exhaustion of the nutrients and oxygen for the rapidly growing tumour. Generation of cancer cells is mainly due to mutations of oncogenes and tumour suppressor genes. These mutations affect the function and the amount of protein products that regulate the cell growth, division and DNA repair.

1. 9.2.1 Oncogenes

Oncogenes are abnormal forms of normal genes that regulate various aspects of

cell growth. Theses mutations affect the intracellular signal transduction, transcription factors, secretion of the growth factors which control the cell growth and division. Tumour suppressor genes, for example p53, play a role in cell division and DNA repair and detecting inappropriate growth signals in the cells. If these genes become unable to function, genetic mutation in other genes can precede unchecked leading to neoplastic transformation.

1. p53

p53 was first discovered and identified as a tumour suppressor gene (Lane *et al,* 1970). Later, it was found to participate in almost all cell activities. p53 tumour suppressor gene is located on the short arm of chromosome 17. It controls the expression of the gene encoding the p21 regulatory protein of Cyclin dependent kinase. During p53 mediated suppression of cell proliferation, p21 and proliferating cell nuclear antigen (PCNA) are involved in coordinating the repair of damaged DNA (Levine *et al,* 1991) (Figure 18).

p53 plays a crucial role in the cell cycle since its activity is responsive to the integrity of the replicated genome. It can prevent genetically damaged cells from progressing through the cell cycle. This occurs because p53 inhibits phosphorylation of pRb, which causes the cell cycle to stall allowing time for DNA repair. In some cell lines, this has been attributed to it blocking S phase entry while in others it functions at a second restriction point in G2.

The activity of p53 within a cell is normally very low since MDM2 protein directly inhibits p53 transcription and also binds p53, decreasing its activity and labelling it for nuclear export and proteasomal degradation. Expression and activation of p53 is rapidly increased in the presence of hypoxia, radiation, DNA

damage and chemotherapeutic drugs. This is partly because p53 promotes its own transcription and activation by phosphorylation, but also because MDM2 activity is decreased under these conditions, due to increased activity of its negative transcriptional regulator, the INK4 protein p19. The consequent increase in p53 activity promotes the transcription of the Kip/Cip protein p21, which inhibits CDK 4, 6 and 2 activities, reducing pRb phosphorylation and causing cell cycle arrest in G1. This gives time for the activation of DNA repair mechanisms and the cell cycle can resume after repair. However, if damage is too severe, the continued elevated p53 activity can induce programmed cell death (i.e. apoptosis) through the bax protein pathway. Therefore, p53 acts as a 'gatekeeper' in the co-ordination of repairs to cellular, notably genomic, damage.

2. Ki-67

Ki-67 was identified by Gerdes et al in 1991 as a nuclear nonhistone protein. The *Ki-67* gene is on the long arm of human chromosome 10 (10q25) (Fonatcsch *et al*, 1991) (Figure 9). The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G 1, S, G2, and mitosis), but is absent from resting cells (G0), makes it an excellent marker for determining the so-called growth fraction of a given cell population. Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle (Scholzen *et al*, 2000).

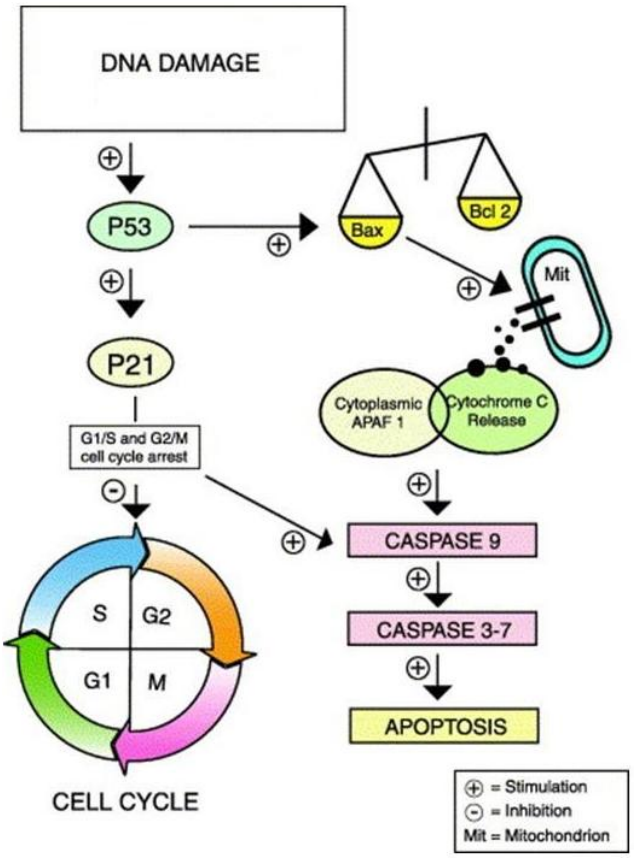


Figure 18: Flow diagram of the P53/apoptosis pathway. (Gillham *et al.* 2007)

3. *Bcl-2*

Bcl-2, an antiapoptotic protein located on chromosome 18 (Figure 19). It has been reported as being critical for development, tissue homeostasis, and protection against pathogens.

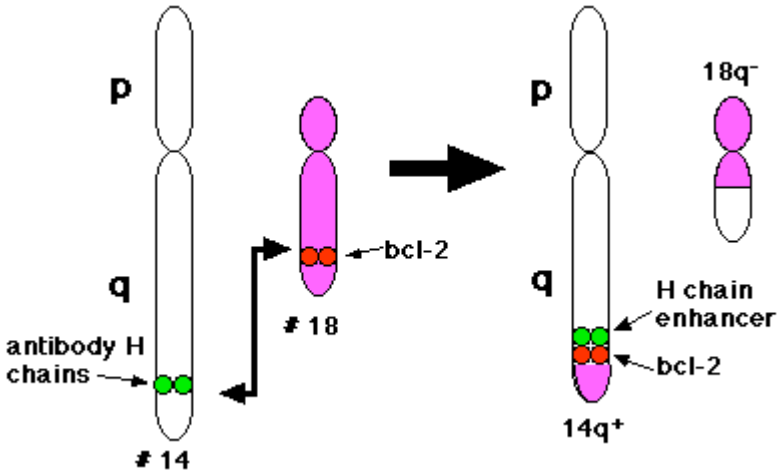


Figure 19: Chromosome 18 containing the *BCL-2*
http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/B/BCL-2.html

Bcl-2 promotes cell survival by inhibiting adapters needed for activation of the proteases caspases that dismantle the cell through mechanisms that include displacing the adapters from the pro-survival proteins. Thus, for many but not all apoptotic signals, the balance between these competing activities determines cell fate. In a healthy cell the outer membrane displays the Bcl-2 protein on the surface which inhibits apoptosis. Internal cell damage protein called Bax migrate to the surface of the cell where it inhibits the protective effect of Bcl-2 and inserted into the outer membrane causing cytochrome c to leak out. The release of cytochrome c binds to a protein called apoptotic protease activating factor 1 (Apaf-1). By the use of ATP these complexes aggregate to form apoptosomes which bind to and activate capase 9 - a protease. Caspase 9 cleaves and in so doing, activates other caspases (caspase-3 and -7). This leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell (Figure 20). Bcl-2 family members are essential for maintenance of major organ systems, and mutations affecting them are implicated in cancer (Jerry *et al*, 1998). Bcl-2 is inversely correlated with baseline Ki-67 (Bottini *et al*, 2001).

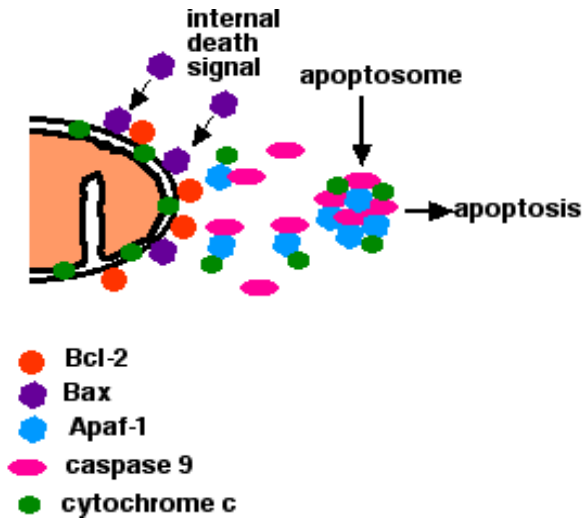


Figure 20: Apoptosis process
http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/Apoptosis.html#Bcl-2

Chapter Two

Materials and Methods

In this chapter, I will discuss the materials and methodologies used for the studies conducted. Four areas were explored namely:

- Family history in patients with PCOS
- Ovarian surface epithelial morphology
- Cell cycle and apoptotic protein expression and
- Gene expression.

2.1 Family history in patients with PCOS

2.1.1 Sampling frame

The design of the study was a cross sectional survey targeting 211 women aged 20-54 years and from different ethnic backgrounds, with or without PCOS, previously seen at the Gynaecology outpatient clinics at the North Middlesex Hospital, London in the period between 2001 - 2005. An additional 10 women were seen at The Royal Free Hospital Gynaecology outpatient clinic. The diagnosis of PCOS was made according to Rotterdam criteria (2003) as mentioned in chapter one. The clinical notes of the subjects recruited into the study were reviewed and relevant data extracted.

2.1.2 Design of the Questionnaire

The questionnaire was designed in simple English. All the questions were placed on one page with adequate spacing in between each question (Stone *et al,* 1993, Streiner *et al,* 1989, McDowell *et al,* 1987 and Bennett *et al,* 1975). Demographic data included age, race and occupation. Clinical data which included past medical history of PCOS, fertility treatment, diabetes, cardiovascular disease, deep vein

thrombosis, ovarian, breast cancer and whether or not they had children were obtained. The questions were designed in 'Yes or No' format and where the answer was 'Yes', the subject was asked to provide further details (Figure 21). By so doing, it was hoped that the recruits would have openly discussed their experiences (Stone *et al,* 1993 & Streiner *et al,* 1989).

The questionnaire was sent to the candidates' home address with a covering letter (Appendix2), inviting them to participate in the study and explaining the importance of their contribution. It was made clear that the decision of whether or not to complete the questionnaire would not affect the candidates' medical care. A prepaid envelope was provided for their response. For those whose first language was not English, an appropriate interpreter was made available to help.

Eighty responses were received in the first two weeks and seven letters returned as 'not known at this address'. A reminder was sent to the remaining patients with a different colour questionnaire (white changed to green) and further assistance was offered in completing the form (Appendix 3). One week later a further 33 responses were received. Another reminder to the remaining patients was sent and the following week an additional 11 responses were received. Another 10 patients from the gynaecology outpatient clinic also replied. The total number of responses received was 134 (61%) (Table 9).

	Patients (n=221)	Response (%)
1st Request	211	38
2nd Request	131	23
3rd Request	101	11
GOPD	10	100

Table 9: Percentage of responses in relation to questionnaire request

Confidential

Infertility and general health questionnaire

Please write your answers on the dotted lines or circle the correct answer

Do you have any children? Yes / No
If yes how many? .....

Have you had fertility treatment in the past? Yes / No
If yes what treatment? .....

Are you diabetic? Yes / No
If yes what type of treatment .....
Do you have a family history of diabetes? Yes / No
If yes who? .....

Do you have a family history of breast cancer? Yes / No
If yes who? .....
Have you been diagnosed with breast cancer? Yes / No
If yes when? .....

Have you ever been diagnosed with Poly Cystic Ovary Syndrome (PCOS)?
Yes / No
If yes when? .....
Have you been on medication for PCOS? Yes / No
If yes what treatment? & for how long? .....

Do you have a family history of cancer of the ovary? Yes / No
If yes who .....

Have you ever been diagnosed with cancer of the ovary? Yes / No

Has any member of your family had a heart attack? Yes / No
If yes who? .....

Have you had a heart attack? Yes / No
Has any member of your family been diagnosed with blood clots in their veins?
(DVT) deep vein thrombosis. Yes / No
If yes who? .....

Have you ever been diagnosed with having blood clots in your veins?
Yes / No

Thank you very much for your co-operation**.**

Figure 21: Infertility and general health questionnaire

2.2 Ovarian surface epithelial morphology

This was a retrospective cohort study designed to compare the degree of dysplasia in the ovarian surface epithelium of women who suffered from subfertility as a consequence of polycystic ovary syndrome. Available ovarian sections from 128 patients who had undergone wedge resection or ovarian biopsy at the North Middlesex and the Royal Free Hospitals between January 1991 and December 1995 were examined. Wedge resection was performed as part of fertility management at that time.

The clinical notes of the recruits were examined and the diagnosis of PCOS made based on the hormonal analysis, patients' presentation, and ovarian sonography and also by histological features. Women who had pre-existing cancer were excluded from the study. Thirty PCOS patients and 72 controls (without PCOS) were identified. The remaining 26 patients were excluded from the study because there was insufficient cortical tissue to allow adequate examination of the surface epithelium. Approval from the local Ethical Research Committee was obtained.

2.2.1 Histological features selected for assessment

The histological features selected for dysplasia assessment and scoring were selected based on Chene *et al*., (2011), Nieto *et al*, (2001) and Deligdisch 1997 studies. They comprised tufting, papillomatosis, psammoma bodies, epithelial stratification, mitoses, nuclear pleomorphism, chromatin pattern irregularity and nuclear cytoplasmic ratio.

3.2.2 Tissue Preparation

Haematoxylin and Eosin (H&E) staining was performed by the histopathology department at the Royal Free Hospital where the subjects had their operations performed. The H&E procedure stains tissue components on the basis of their pH. Haematoxylin stains acidic tissue components, such as nucleic acids, while eosin stains more alkaline tissue components. In general therefore, nuclei appear blue-black, the cell cytoplasm varying shades of pink, while fibrous tissue and muscle fibres appear a deep pinky red.

Gill *et al's* (1974) haematoxylin protocol was used. Five µm paraffin wax embedded ovarian sections were dewaxed in three changes of xylene for 5 minutes each and rehydrated in 100% v/v ethanol for 3 x 3 min, 95 % v/v ethanol for 1 x 3 min, 80 % v/v ethanol for 1 x 3 min then in deionised water for 1 x 5 min. Excess water was blotted off and the sections were stained for 5 min in Mayer's haematoxylin, prepared by mixing 1.0 g haematoxylin (Cl 75290), 50 g aluminium ammonium sulphate and 0.2 g sodium iodate in 1L deionized water, after which 1.0 g citric acid and 50 g chloral hydrate were added and the solution was boiled for 5 min, cooled and filter before use. Sections were rinsed in a trough filled with running tap water for 20 min or until the water was clear, washed in acid-alcohol (1% v/v concentrated HCl in 70% v/v ethanol) for a few seconds then in deionised water for 5 min. After blotting excess water from the sections, they were placed in eosin (1.0 g Eosin Yellowish dissolved in 100 ml distilled Water) for 5 min and washed for 3 x 1 min in deionised water. Sections were then dehydrated sequentially in 95 % v/v ethanol for 2 x 1 mins, 100% v/v ethanol for 2 x 1 mins and Xylene for 2 x 5 min, dried and mounted in DPX under cover slips.

2.3 Cell cycle and apoptotic protein expression

2.3.1 Principles of immunohistochemistry

Immunohistochemistry (IHC) is a combination of anatomical, immunological and biochemical techniques for the detection of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue (Ramos-Vara *et al*, 2011).

Immunohistochemistry is the study of the expression of antigens within tissues and cells using antibodies to detect them. Antigens within cells or on cell surfaces are detected through their high affinity interaction with applied target-specific antibodies, with subsequent development of a visual signal at the site of antigen-antibody interaction, using fluorophores or one of several enzymatic systems which generate chromogenes. Examination of the antibody-stained tissue section with a fluorescent microscope (for fluorophores) or standard light microscope (for enzymatically generated chromogens) allowed for assessment of antigen distribution and variability of expression within the tissue specimen.

2.3.2 Tissue preparation and Antibody labelling

Immunohistochemistry for all antigens was performed on sections cut from formalin fixed paraffin wax embedded ovarian biopsies, therefore antigen retrieval was required to increase target antigenicity.

5µm sections were cut, placed onto coated slides and dried for at least one hour at 60ºC. De-paraffinisation, antigen retrieval, immunostaining and counterstaining were carried out on a Bond maX automated immunostainer (Vision Bio Systems, city) at UCL Advanced Diagnostics laboratory (Figure 21&22). The protocol used

for the study is enclosed in Appendix 5. Antibodies used for this study, supplier, dilution and antigen retrieval are summarised in table 10.

Antibody	Supplier	Dilution	Antigen Retrieval
p53	Novocastra NCL-p53-DO7	1/100	ER1 30’
MIB-1	Dako M7240	1/100	ER2 20’
bcl-2	Novocastra NCL-L-bcl2	1/50	ER2 30’
Cyclin D1	Lab Vision RM-9104-S	1/40	ER2 30’

Table 10: Antibodies, supplier, dilution and antigen retrieval.

Figure 21: Vision Automated Immunohistochemistry Staining System
http://www.leica-microsystems.com/products/total-histology/ihc-ish-advanced-staining/details/product/leica-bond-max/

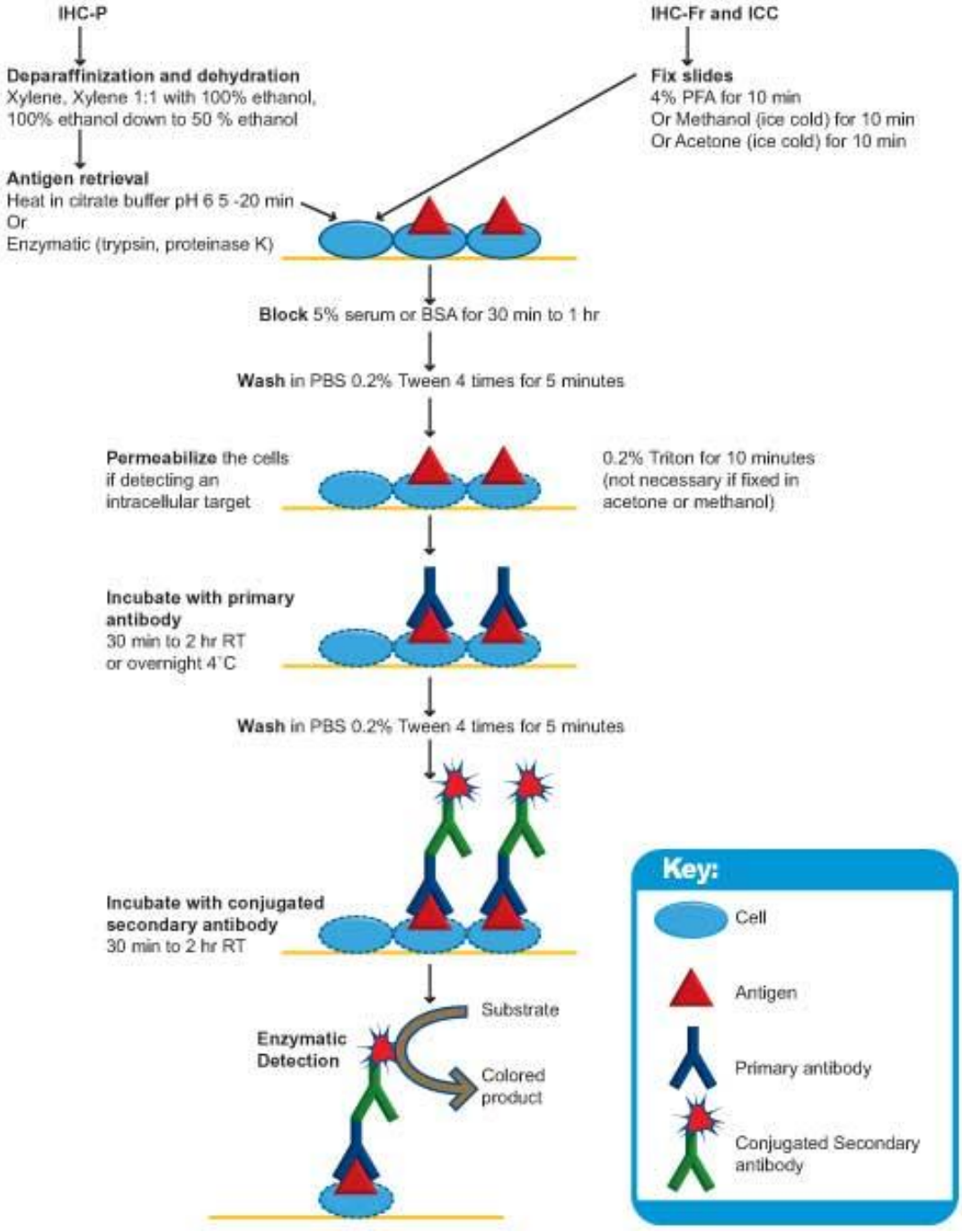


Figure 21: Immunostaining flow diagram
www.abcam.com/ps/CMS/Images/immuno_staining2.jpg

2.3.3. Ovarian Epithelial Dysplasia scoring

Assessment of the extent and intensity of specific staining in the dysplastic areas of the ovarian biopsies was performed independently by Dr. Soha El Sheikh, Consultant Histopathologist at the Royal Free Hospital and me. Scoring was carried out on standard histopathology grade light microscopes, at a total magnification of x100 with standard light field optics, on 5 randomly selected fields per section. At this magnification, visual fields contained approximately 100 cells. Sections were examined to check for the proportion of cells staining and the intensity of staining.

- *Score 0*: A scoring of Zero indicated that epithelium architecture was normal. This score was also given when focal nuclear stratification, the nuclear-to-cytoplasmic ratio was normal, and nuclear pleomorphism was minimal.
- *Score 1*: This score was given when there was the presence of cytological or architectural abnormalities in 15% of the cells.
- *Score 2*: This score was given where significant dysplasia was present in more than 30% of the cells, The cytological features include nuclear pleomorphism, hyperchromatism, increased N: C ratio, markedly enlarged nucleoli, cytoplasmic basophilia, excessive nuclear stratification and increased number of abnormal mitotic figures. The architectural features include irregularly shaped, crowded, papillary extensions; and a villiform configuration of the surface.

Some samples were not appropriate for examination where there was not any epithelium to comment. Three sections were reviewed for each case. The mean was calculated to establish the level of significance.

2.3.4 Imuunohistochemistry scoring

Imuunostained sections were examined at high power fields (× 400) under a standard light microscope (Zeiss Axioskop2 MAT microscope) (Figure 23).

In some cases more than one slide were used for the same specimen. Thirty to one hundred and fifty (30-150) cells were examined on each slide. Three pictures were taken from different angles of each slide. They were examined to check for the proportion of cells staining and the intensity of staining. For p53 and Ki-67, a positive score was recorded when at least 10% of ovarian epithelial cell nuclear stained. For Cyclin D1 and bcl2, nuclear and/or cytoplasmic staining of at least 10% of cells was regarded as positive.

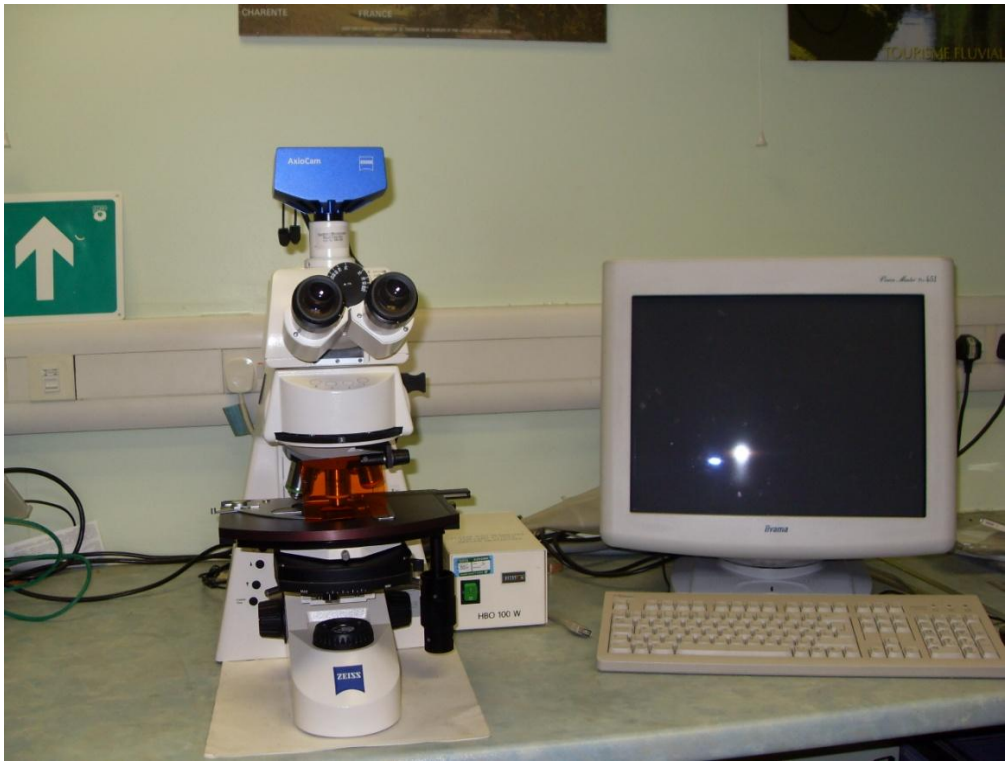


Figure 23: Zeiss Axioskop2 MAT microscope

2.4. Gene expression

2.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method developed by Kary Mullis in the 1980s for amplifying segments of DNA, by generating multiple copies using DNA polymerase enzymes under controlled conditions. The process of PCR has made it possible to perform DNA sequencing and identify the order of nucleotides in individual genes. Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of three discrete temperature steps (Figure 24):

Denaturation: This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing: The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically, the annealing temperature is about 3-5 degrees Celsius below the temperature of the primers used. Hydrogen bonds are only formed when the primer sequence very closely matches the template sequence eg. C to G, A to T. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used. Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3'

direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. Under optimum conditions at each extension step, the amount of DNA target is doubled, leading to geometric amplification of the specific DNA fragment.

Final elongation: This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final step: This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

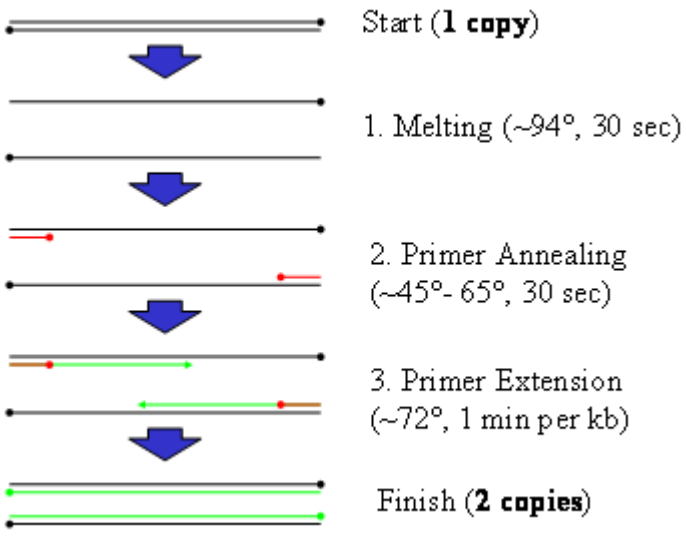


Figure 24: The Polymerase Chain Reaction steps.
https://medschool.mc.vanderbilt.edu/student/igp_material/20050919081419_298_3504.ppt

2.4.2 Differential Display

One of the most effective methods to investigate differential gene expression is the so-called 'differential display (DD)' method which was originally introduced by Liang and Padre (1994). Different variations of the original method have been developed which can be basically classified as 'arbitrarily primed' and 'systematic' differential display approaches (Liang *et al,* 2002, Matz & Lukyanov, 1998 and Sturtevant *et al,* 2000). Microarrays are another method used in gene expression**.**

2.4.3 Fundamental Differences between DD and DNA Microarrays

Both differential display (DD) and microarrays are conceptually simple to use. However, the two methods have key differences. The fundamental difference is that differential display visualizes the mRNAs in subsets directly without any data normalization after their amplification and labelling by either isotopes or fluorescent dyes. In contrast, DNA microarrays visualize the mRNAs indirectly after the hybridization of the extremely complex mixture of fluorescence labelled cDNA species derived from an RNA sample, to a set of cDNA templates spotted on a glass surface. In fact, a cDNA probe mixture used for microarray can be so complex that it consists of as many as 10,000 different species, representing mRNAs present from only a few copies to thousands of copies per cell. Further compounding the problem in signal specificity has been the fact that eukaryotic genes often come in families with many conserved sequences among the family members.

Therefore, lack of sensitivity, nonspecific- and cross-hybridization are problems with microarrays. Microarrays have limited detection of whatever genes that are

spotted on a slide, making it a "closed' system for gene discovery. In contrast, DD is capable of detecting both known and novel genes with much higher gene coverage.

2.4.4 The advantages of differential display method (DD) over Microarray analysis

The differential display method (DD) was chosen above the microarrays analysis method for the reasons listed below. Additionally, at the time of conducting this experiment, the DDRT-PCR protocol was the established protocol in use in the Department of Molecular Endocrinology UCL, Middlesex Hospital. London. The DD method was less expensive when compared to the microarray method and this was a key factor in choosing that protocol.

1. Simplicity: Technically, DD is based on well established methodologies used in RT-PCR and DNA sequencing gel electrophoresis.

2. Sensitivity: Five to ten micrograms (µg) of total RNA is enough to cover the majority of mRNAs. Also, DD is sequence-dependent. Therefore, if the sequences of the arbitrary primer match the target RNA, whether abundant or rare, it will be amplified and detected.

3. Reproducibility: Up to 99% of the bands on an mRNA display are reproducible.

4. Versatility: More than two RNA samples can be compared simultaneously allowing complex comparisons in the same experiment. Also, both up- and down-regulated genes can be detected at the same time.

5. Detecting Novel Genes: Unlike microarray, DD does not require prior knowledge of the mRNA sequence to be detected. Therefore, DD is considered an

"open system", as opposed to a "closed system" as exemplified by microarray analysis (http://www.genhunter.com/products/differential/).

2.4. 5 Materials

The ovarian samples were archived at the tissue bank at the Royal Free Hospital at -80ºC. I started the RNA extraction using 23 normal ovaries, 19 ovarian papillary serous adenocarcinomas and seven ovaries from subjects with PCOS. All subjects were 35-45 years at the time of biopsy.

The normal and PCOS ovaries were obtained after total hysterectomy for prophylactic reasons related to a family history of breast cancer or ovarian cancer. The clinical notes for these patients had been reviewed and data extracted on the clinical history, biochemical investigations, ultrasound scan report and history report confirming either normal or PCOS. The notes of these patients have been microfilmed and I had to access these files through the medical records department at the Royal Free Hospital, after approval from the Local Research Ethics Committee was obtained.

The cancer tissue was obtained, with informed consent, from patients who were diagnosed with papillary serous adenocarcinoma.

Figure 22 demonstrates the schematic flowchart of the reverse transcriptase-polymerase chain reaction differential display procedure performed for the gene expression.

For clarity, the main steps (Figure 25) used in DD PCR protocol were:

1. Collecting RNA
2. Treating RNA with DNase

3. Splitting RNA into aliquots and performing reverse transcription reaction on each using a different oligo-dT (anchored) primer
4. Performing PCR using cDNA subsets as template with specific anchored primer together with an arbitrary primer, include 33P-dATP in reaction
5. Loading PCR reactions on sequencing gel
6. Identifying induced or inhibited genes
7. Repeating experiment to confirm results
8. Excising band from gel
9. Reamplifying cDNA using same PCR conditions
10. Cloning cDNA's
11. Screening clones to identify unique species
12. Sequencing clone and/or using it to obtain full-length cDNA.

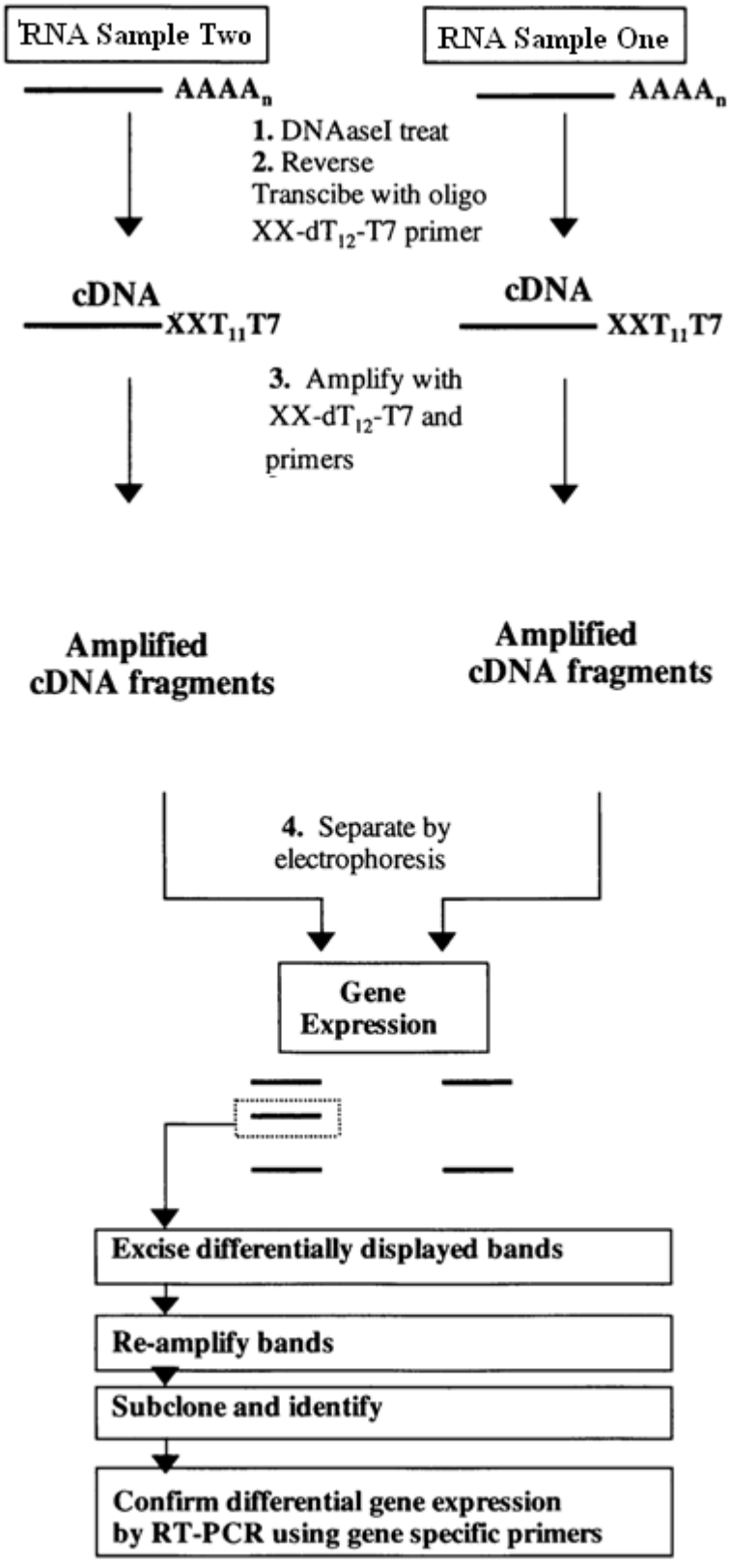


Figure 25: Schematic flowchart of the reverse transcriptase-polymerase chain reaction differential display procedure performed

2.4. 6 Isolation of total RNA

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (Chomzynski *et al,* 2006). Ovarian tissue was homogenised in 9 vol. denaturing solution (solution D, 4M guanidinium thiocyanate, 0.5 %(w/v), sarcosyl, 0.1 M 2-mercaptoethanol in 25 mM sodium citrate: pH 7.0). Homogenates were mixed with 0.1 vol. 2M sodium acetate (pH 4.0), 1 vol. 0.1 M citrate buffer- saturated phenol pH 4.5 (ICN Biomedical, Inc) and 0.2 vol. chloroform: isoamyl alcohol (49:1 mixture). The tubes were mixed vigorously for 60 seconds then placed on ice for 15 min, before being centrifuged (10.000 x g, 20 min at 4ºC). The upper aqueous phase was collected into fresh tubes taking care to avoid contamination with DNA interface layer, and an equal volume of isopropanol was added. The tubes were then mixed and incubated ≥ 1 hour or overnight at -20ºC to allow RNA precipitation.

Tubes were recentrifuged (10.000 x g, 20 min at 4ºC), then the RNA pellet was resuspended in 0.5 ml solution D, transferred into a fresh tube containing an equal volume of isopropanol, re-precipitated for ≥ 1 hour at -20ºC, and re-centrifuged at 10.000x g, 10 min at 4ºC. Pellets were then washed twice with 75% ethanol (v/v), air dried, dissolved in nuclease free water (0.25 µl/mg wet weight tissues) at 65ºC, and stored at - 20ºC.

2.4. 7 Spectrophotometeric determination of RNA concentration

Samples were diluted with nuclease free water and the absorbance read in a Hitachi Spectrophotometer (Figure 26) at 230, 260 and 280 nm against a blank consisting of nuclease free water. RNA concentration was estimated based on the assumption that a solution of 40 µg/ml RNA has an absorbance of 1 at 260 nm

(Figure 27). An A_{260} /A_{280} ratio of 1.6-2.0 indicated reasonably pure RNA, relatively free of protein contamination.

Figure 26: Spectrophotometer (model U-1500; HITACHI. WOKINGHAM, UK).

2.4. 8 Analysis of RNA integrity by agarose gel electrophoresis

2 µg RNA in 4 µl nuclease free water was mixed with 16 µl RNA sample buffer, heated at 55ºC for 15 min and chilled on ice. Samples were mixed with 2.0 µl RNA loading buffer, before loading onto gels (1% (w/v) agarose (Sigma), 18% (v/v) formaldehyde in 1X MAE). Gels were run at 100-120 V for 0.5-1 hour. Bands were visualised by UV Transillumination and photographed using a digital camera and shroud. A 28/18S ribosomal RNA intensity ratio of approximately 2:1 is indicative of reasonably intact RNA (Figure 27).

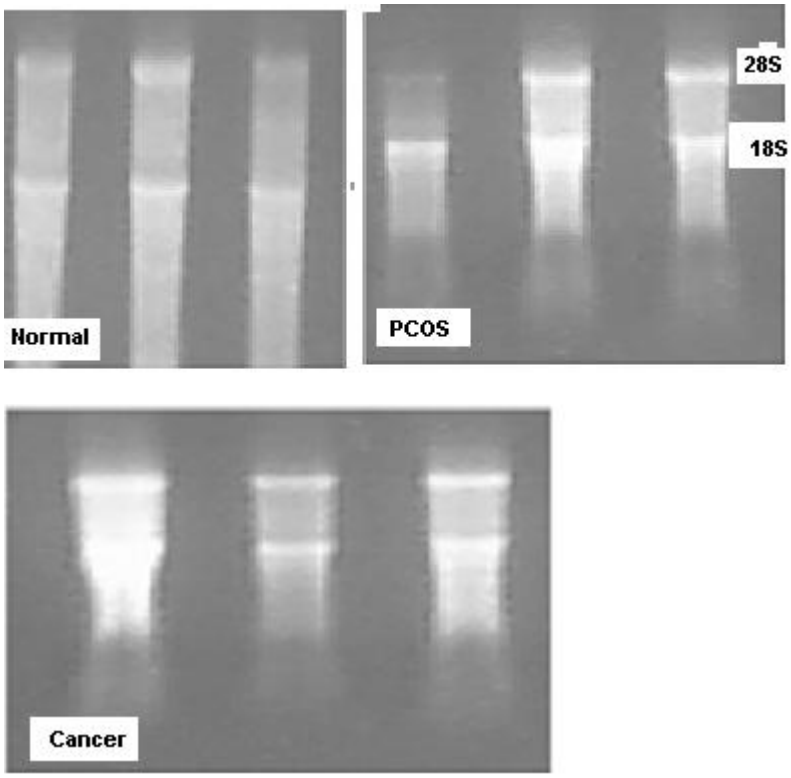


Figure 27: Total isolated RNA from normal, PCOS and cancerous ovaries

2.4.9 DNase treatment of RNA samples

Samples were treated with RNase free DNase (Invitogen) in order to remove any traces of contaminating genomic DNA. Reactions consisted of 2.5 µg RNA, 20 U RNasin, 1X DNase buffer and 0.5 U DNase I in a final vol. of 25 µl.

Samples were incubated for 15 min at room temperature. Reactions were terminated by addition of 2.5 µl 25 mM EDTA, heated for 10 min at 70ºC then chilled on ice. 25µl water was added just before reverse transcription (total volume 52.5 µl).

2.4.10 Reverse transcription

A. for general PCR

Reverse transcription (RT) of RNA samples was carried out using random hexamer primers (1.15 µg/ µl.) Added in 1 µl to 10 µl DNase digested RNA,

produced using method 2.4.7 Each sample was then reverse transcribed in a reaction volume of 20 µl containing 11 µl RNA/primer mix, 1X first strand buffer, 0.01 M dithiothreitol (DTT), 2 nM deoxynucleotide triphosphates (dNTP) and 200 U Superscript II reverse transcriptase (RT^+ reactions). Duplicate reactions containing no enzyme were set up for each sample as a negative control (RT^- reactions). The reactions were incubated at 25ºC for 10 min, then at 42ºC for 60 min to allow elongation. The enzyme was then deactivated by incubation at 70ºC for 15 min and samples were stored at - 20ºC.

B. For Differential display

RT of RNA samples was carried out in three reactions using one of the three $ET_{12}X$ downstream primers (where E is *Xho*I restriction enzyme site and X is A, C, or G) in each reaction. Thus RNA was reverse transcribed into 3 cDNA populations, differing at the last nucleotide before the poly (A) tail. To each of three aliquots of DNase digested RNA was added $ET_{12}A$, $ET_{12}C$ or $ET_{12}G$ primer (4.76 µM) to a final vol. of 10.5 µl. Samples were mixed, denatured at 80ºC for 5 min, and then annealed at 4ºC for 2 min. The reaction temperature was raised to 42ºC for 2 min before addition of reagents to: 1X first strand buffer, 10 mM DTT, 20 µM dNTP, 10U RNasin and 200 U Superscript II in a final vol. of 20 µl. Reverse transcription was allowed to proceed at 42ºC for 70 min, before enzyme denaturation at 80ºC for 10 min.

2.4.11 PCR amplification with arbitrary primers

To display the cDNAs present in the 3 populations, PCR was performed using the relevant $ET_{12}X$ downstream primer and one of 7 different EAP arbitrary upstream

primers (EAP1-7) in the presence of [α-^{33}P] dATP. EAP primers consisted of an *Eco*RI site (E), and an arbitrary primer (AP) consisting of 10 bases of 3` random sequence with 50% GC content.

The PCR reaction (10 µl total volume) contained 1 µl reverse transcription reaction containing cDNA , 0.75 U Taq DNA polymerase, 1X PCR buffer, 3 mM $MgCl_2$, 20 µM dNTP , 15 nM [α-^{33}P] dATP , 1 µM relevant $ET_{12}X$ primer and 1 µM EAP primer. The thermal profile used for amplification of cDNA consisted of 94ºC for 2 min, then 2 cycles (denaturation 94ºC for 30s, annealing 42ºC for 1 min; elongation 72 ºC for 2 min), followed by 38 cycles (denaturation 94ºC for 30s, annealing 55ºC for 1 min; elongation 72 ºC for 2 min) and a final elongation step (72 ºC for 5 min). Samples were stored at -20ºC, but were electrophoresed the next day to minimise loss of activity in ^{33}P-labelled PCR products.

2.4.12 Analysis of DDRT-PCR products

DDRT-PCR products were analysed using polyacrylamide gel electrophoresis on a SequiGen GT sequencing cell (Bio-Rad Laboratories Ltd, Hemel Hempstedf, UK) (Figure 28).

Figure 28: Sequi-Gen GT System (http://www.bio-rad.com)

A 6% non-denaturing gel was prepared by degassing a mixture containing 6% (w/v) acrylamide (acrylamide;bis-acrylamid 1:19), 0.5X Tris/Borate/EDTA buffer TBE, 0.001% (v/v) TEMED, before addition of 0.05% (w/v) ammonium persulphate. A 0.4 mm thick gel was cast in the sequencing cell according to the manufacturer`s protocol using a 60 place comb; the setting time was 1 hour, or sometimes overnight. Gel running buffer for lower and upper reservoirs was 0.5X TBE. PCR products (10 µl) were mixed with 2 µl of Blue/Orange loading dye and loaded at 3 µl/well. Electrophoresis was performed at 80 W for 2.5 hours; the maximum temperature was 40ºC.

Following electrophoresis, gels were mounted on chromatography paper, sealed with a layer of Saran Wrap and dried under vacuum at 60ºC for 1.5 hr. Dried gels were exposed to Kodak Biomax Maximum Sensitivity film which was developed following the manufacturer's recommended methods. Auto radiograms were also scanned using a Typhoon FLA 7000 ™ to create digital images for easy storage (Figure 29).

2.4. 13 Isolation and re-precipitation of differentially expressed fragments

Auto radiograms were aligned onto gels to allow the marking and excision of differentially expressed bands (Appendix 6) into sterile tubes using a sterile scalpel. Following the excision of bands, gels were re-exposed to film to compare with the original autoradiogram to confirm the accuracy of band excision.

Figure 29: Typhoon FLA 7000™
(http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/55DEA1D48F26687EC125771900828CAD/$file/28961073AB.pdf)

DNA was eluted by boiling each gel fragment in 250 µl nuclease free water for 10 min in a water bath followed by centrifugation (16,000x g for 5 min). 180 µl supernatant was mixed with 2 µl Pellet Paint (Novogen, Madison, USA) and 0.5 M ammonium acetate in a final volume of 200 µl. 630 µl of absolute ethanol was then added and the DNA precipitated overnight at -20 ºC. Samples were re-centrifuged

(16,000x g for 5 min) and pellets were washed in 75% ethanol twice, vacuum dried for 30 min, then dissolved in 50 µl nuclease free water and stored at -20 ºC.

2.4.14 Amplification of recovered DNA

DNA recovered from differentially expressed bands was subjected to PCR using the same $ET_{12}X$ and EAP primer pair used to generate it. Reactions consisted of 25 µl fragment DNA (generated using method 2.4.9) , I U taq DNA polymerase, 1X PCR buffer , 3 mM $MgCl_2$, 50 µM dNTP, 0.5 µM ET_{12} X primer and 0.5 µM EAP primer in a total volume of 100 µl. Thermal conditions were 94ºC for 10 min, followed by 38 cycles (denaturation 94ºC for 30s, annealing 55ºC for 1 min; elongation 72 ºC for 2 min) and a final elongation step (72 ºC for 5 min). Samples were stored at -20ºC.

In order to verify successful PCR, gel electrophoresis was carried out using 5 µl PCR product mixed with 2 µl DNA loading buffer on a 2% (w/v) agarose gel in 0.5X TBE, alongside a 100bp marker ladder. Remaining products were reprecipitated using pellet paint. Pellets were re-dissolved in 30 µl nuclease free water.

2.4.15 Re-*purification of samples using HYBAID columns*

The amplified DNA fragments were purified using the Hybaid Recovery DNA Purification Kit II, a silica gel based spin prep kit for purification of DNA (200bp-300kb) from agarose gels and solutions. The kits were used according to the manufacturer's instructions. In brief, 400 µl of binding buffer with silica gel suspension was added to a spin filter, and then a cDNA sample was added and mixed. This spin filter was then spun at maximum speed in a microfuge for 15-30 seconds to transfer buffer to the catch tube, leaving DNA bound on the silica gel.

This was then washed by adding 500 µl of washing solution to the filter, and spinning for 30 seconds before emptying the catch tube, then spinning for 1 minute to dry the gel. DNA was then eluted from the gel into a clean catch tube by adding 15 µl of elution solution and resuspending the binding matrix/DNA by flicking the tube. The spin filter was then centrifuged at maximum speed in a microfuge for 30 seconds to transfer eluted DNA into the catch tube. This elution step was repeated to maximise recovery giving a total DNA elute of 30 µl. In order to verify successful purification, gel electrophoresis was carried out using 5.25 µl DNA mixed with 2.33 µl DNA loading buffer and 6.42 µl water on 2% (w/v) agarose gel in 0.5X TBE, loading 6, 4 and 2 µl from each DNA sample (i.e. equivalent to 2.25, 1.12, 0.056 µl eluted DNA preparation) alongside a 2, 1, 0.5, 0.25 and 0.125 kb DNA marker ladder.

2.4.16 Cloning *of differentially expressed PCR fragments*

2.4.16.1 Restriction digestion of PCR products

The purified PCR products generated above were digested with *Eco*RI and *Xho*I in order to facilitate sticky-ended ligation into the pBluescript II SK^+ plasmid vector (Strategene) (Figure 30). Reactions contained re-purified PCR products (7 µl), acetylated BSA (5 µg), *Xho*I (24 U; Promega), *Eco*RI (10 U; Promega) and buffer H (X1; 10 mM $MgCl_2$, 50 mM NaCl, 90 mM Tris HCL, pH 7.5; Promega) in a final vol. of 50 µl. After incubation at 37 °C for 3 h, reactions were made up to 200 µl with nuclease free water, before addition of an equal volume of a mixture containing phenol (pH 7.9):chloroform:isoamyl alcohol (PCIAA; 25:24:1). The tubes were mixed vigorously then centrifuged (16,000*g*, 10 min; 25 °C). The aqueous layer was transferred into a fresh tube containing an equal volume of

PCIAA and re-centrifuged (as above). The non-aqueous layer was also mixed with an equal volume of water and re-centrifuged (as above). The resulting aqueous layers were combined and DNA re-precipitated using Pellet Paint and ammonium acetate as described previously. The size and concentration of re-purified restriction fragments was assessed by electrophoresis on a 2% (w/v) agarose gel in 0.5X TBE alongside a 1 kb ladder (Stratagene).

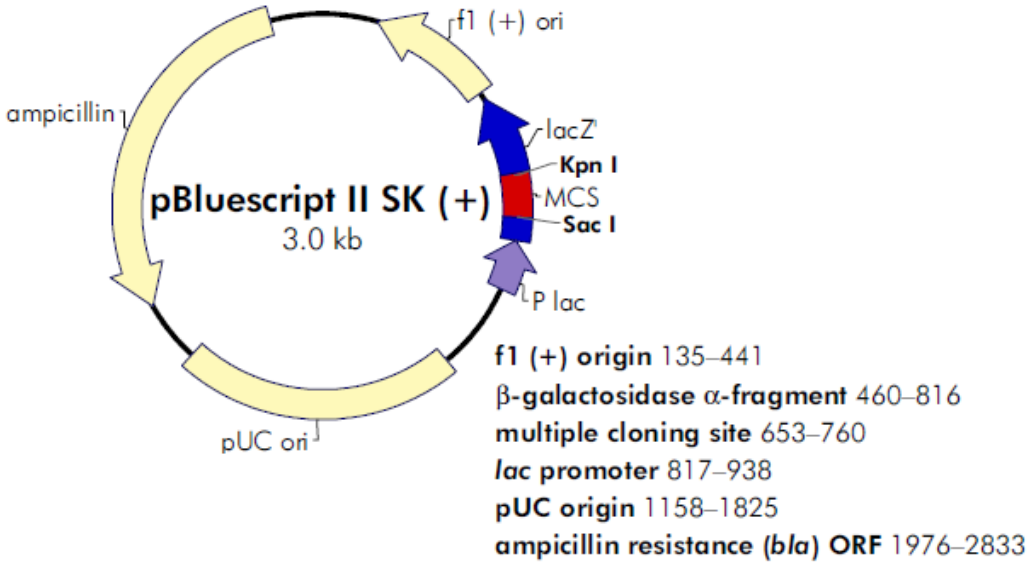


Figure 30: Blue script II SK (+) Multiple Cloning Site Regions

2.4.16.2 Ligation of PCR fragments into cloning vector

Digested PCR fragments were ligated into *Xho*I/*Eco*RI digested pBluescript II SK^{+} plasmid vector (Stratagene). Ligation reactions contained digested PCR fragment (*ca.* 5 ng), plasmid vector (100 ng), T4 DNA ligase (1U; Promega), ligase buffer (1X; 1 mM ATP, 10 mM DTT, 10 mM $MgCl_2$, Tris-HCL, pH 7.8; Promega) in 10 µl with water. Reactions were left to stand at 25 °C for 2 h then at 4 °C overnight.

2.4.16. 3 Transformation of competent E.coli

Aliquots (50 µl) of INVαF′ competent *E.coli* cells (Invitrogen Life Technologies) were mixed with β-mercaptoethanol (2 µl) and an aliquot (4 µl) of ligation mixture (Section 2.10.3.2). Cells were incubated on ice for 30 min, heat-shocked for 30 s at 42 °C, then incubated on ice for a further 2 min, before addition of SOC medium (250 µl) and horizontal shaking (225 rpm) at 37 °C for 1 h 20 min. Cells were then spread-plated on Petri dishes containing Lennox Luria Bertani (LB) agar supplemented with sodium ampicillin (75 µg/µl) and 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal; 40 mg/ml in formamide; applied as a top coat onto the gel). X-Gal allows blue-white colour selection of recombinant (white) clones. Plates were allowed to dry for 10 min, inverted then incubated at 37 °C for > 18 h.

2.4.16.4 Cloning

Four white colonies were picked off each plate and suspended in separate 5 ml aliquots of LB medium containing ampicillin (50 µg/ml) and 0.1 % (w/v) glucose. Colonies were grown overnight at 37 °C with shaking as before, then stored in 30% (v/v) glycerol at –20 °C.

2.4.16.5 Plasmid purification

Plasmid purification was carried out using the GenElute Plasmid Mini-Prep Kit (Sigma), according to the manufacturer's protocol. Briefly, overnight culture (2.3 ml) was pelleted by centrifugation (16,000 *g*, 1 min; 25 °C) and the supernatant discarded. Cells were resuspended by titration in resuspension solution (200 µl), followed by addition of lyses solution (200 µl). The solution was mixed by

inversion, then allowed to clear for 5 min. Addition of neutralization solution (350 µl) was followed by mixing, then centrifugation (16,000 *g*, 10 min; 25 °C). The cleared lysate was transferred to a Mini Spin Column, then centrifuged (16,000 *g*, 1 min; 25 °C). Columns were washed with wash solution (750 µl) by centrifugation (as above), then dried by re-centrifugation. Elution solution (100 µl) was added to columns and the purified plasmid DNA was collected by centrifugation (as above).

2.4.16.6 Sequencing and identification of plasmids

Recovered plasmids were linearised with *EcoR*1 and the DNA concentration in digests was estimated by agarose gel electrophoresis alongside a 1 kb ladder. Samples were adjusted to 150 ng DNA/6 µl with nuclease-free water before sequencing was carried out with T7 primer using a Beckman Coulter CEQ 2000XL DNA analysis system in a local facility. The Blast™ programme (www.ncbi.nlm.nih.gov) was used to compare insert sequences with entries in the GenBank database. Primers were designed to amplify regions within the identified genes and semi-quantitative RT-PCR was used to screen ovarian RNA samples to verify differential expression of target genes.

2.5 Statistical analysis

2.5.1 Family history in patients with PCOS

The programme G. Power 2 for Macintosh was used to perform a power analysis using 2 tailed T test. This provided the number of samples required for testing, with certain power, the null hypothesis, the means of two independent sets of normally distributed data of defined homogenous variance are equivalent at a defined level of significant (normally $P < 0.05$). An estimated sample size of 53 PCOS women and 53 controls, assuming a prevalence of x and y in the two groups respectively, with a power of 80% and significance of $P<0.05$. Categorical variables were summarized as proportions and differences among groups were assessed using the chi-square test and T test.

2.5.2 Ovarian surface epithelial morphology

All statistical analyses were performed using the SPSS programme version 10.0 for Windows. The main outcome measure for histological assessment of the ovarian epithelium study was the total dysplasia score for each subject or the highest score where more than one section was available from the same ovary. The score for each subject was based on the eight histological features. The *a priori* hypothesis tested in this thesis was that PCOS was associated with higher dysplasia scores. Student's t-test analysis was repeated using non-parametric tests (Kruskal-Wallis or Mann Whitney U test, as appropriate). In cases where there was departure from assumptions required for parametric testing, multiple regression analysis, using the general linear model (GLM in SPSS®) procedure was used to obtain adjusted mean dysplasia scores. The dysplasia scores were arbitrarily classified as 'zero' in cases where there was no abnormality detected, 'one' in cases with slight changes and 'two' when significant changes were seen.

2.5.3 Cell cycle and apoptotic protein expression

The Mann-Whitney U test was used to measure the associations between the groups with regards to their immunohistochemical expressions and assess their significance. A value of $P<0.05$ was considered statistically significant.

The proportion of subjects whose ovaries expressed each of the cell cycle proteins studied was compared to those with dysplasia scores, using the chi-square test.

Chapter Three

Results

3.1 Family history in patients with PCOS

The total number of responses after sending two reminders was 134 (response rate 60%). 52 (38.8%) women were diagnosed with PCOS and 82 (61.2%) were controls. From table 11 and figure 31, it is evident that with the controls, fewer women are presenting in the younger age groups and with increasing years, more women are presenting. There could be some possible reasons for this increasing trend. Younger women tend to have fewer problems with fertility compared to older women. Also, social trends are changing and some women in the pursuit of having a career defer starting their families and as a consequence, tend to present later with fertility problems. A similar trend can be seen in patients with PCOS, particularly in the 26 to 34 years age groups. A possible explanation for the reduced number in the 35 to 40 years age group could be that they have already had their fertility treatment or they have presented with different symptoms of PCOS.

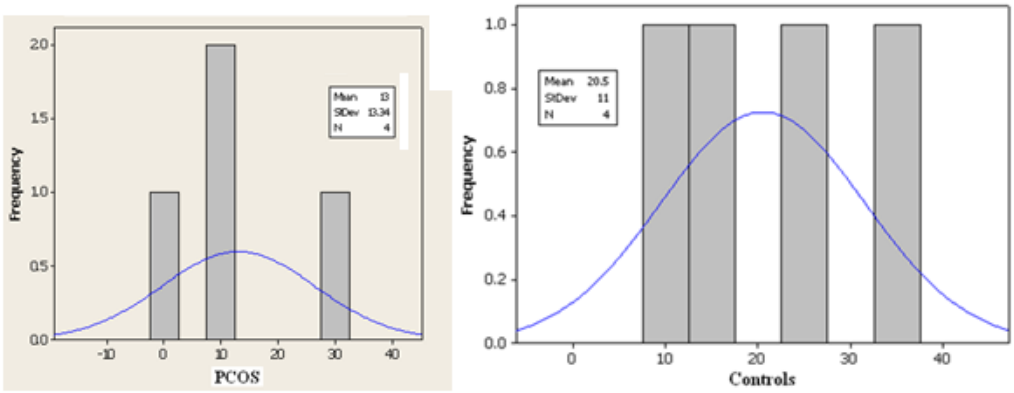
The difference and variation in the sample population mean between PCOS and the control group in respect of age or ethnicity (p value 0.419 and 0.0635) which is statistically insignificant (Table 12). Meanwhile at 95% confidence interval for the difference in age is (-28.66, 13.66) and (-0.33 to 8.90) in case of ethnicity.

Age (Years)	PCOS (%)	Controls (%)
19 and less	1.56	0.82
20-25	2.08	8.2
26-29	8.32	10.66
30-34	10.92	33.62
35-40	1.92	18.04
Mean	6.76	20.2
St Dev	13.34	11
Minimum	1	11
P value	0.419	

Table 11: Age in PCOS cases and controls

Ethnic group	Control (%)	PCOS (%)
Caucasian	28.7	16.64
Black	18.86	5.72
Asian	10.66	4.16
Didn't answer	9.02	5.52
Mean	11.71	7.43
P value	0.0635	

Table 12: Ethnicity in PCOS cases and controls



.Figure 31: Histogram with normal curve of PCOS and controls by age

From Tables 13&14 there was a significantly higher proportion of women in the PCOS group who had a first degree relative affected with breast cancer than in the control (non PCOS) women (P=.0.0066). The difference between the relatives in both groups is statistically insignificant (P=0.1563). However grandmother is the highest percentage in both groups (Figure 32) which could be explained by knowing that breast cancer tends to affect older women.

The proportion with a family history of myocardial infarction shows borderline significance in the PCOS women compared to the controls (P=0.0583). However there was no significant association between family history of thromboembolism (P> 0.9999) and ovarian cancer (P > 0.9999) was also not statistically significant (Table 13).

	PCOS (%)	Controls	P value
Family history of breast cancer	23	7	0.0066
Family history of ovarian cancer	4	4	> 0.9999
Family history of myocardial infarction	31	11.7	0.0583
Family history of DVT	15	15	> 0.9999
Family history of DM	131	40	0.3575

Table 13: Family history in PCOS and controls

	Controls (%)	PCOS (%)
Mother	0	1.56
Sister	0.82	0
Grandmother	1.64	2.6
Aunt	1.64	0.52
Grandmother & mother	0	0.52
Grandmother & sister	0	0.52
Aunt & grandmother	0	0.52
Mean	0.71	1.71
P value	0.1563	

Table 14: Family history of Breast Cancer in PCOS and controls

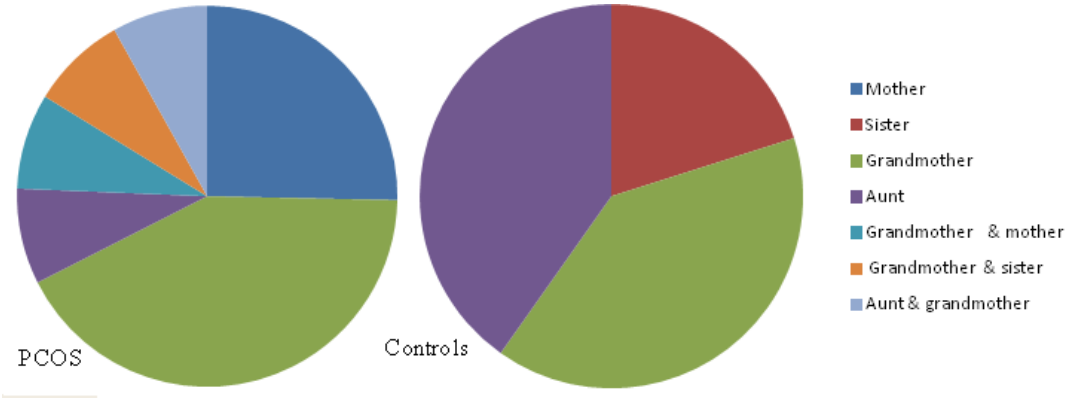


Figure 32: Family history of Breast Cancer in PCOS and controls with regards to family members.

Personal past medical history of DVT is statistically insignificant (P=0.2014) (Table 15). None of the candidates for the study experienced breast cancer, ovarian cancer or myocardial infarction.

	PCOS (%)	Controls (%)	P value
Live birth	52	55	0.8590
Fertility treatment	54	57	0.7237
History of DVT	4%	11	0.2014

Table 15: Past medical history in PCOS and Controls

3.2 Ovarian surface epithelial morphology

3.2.1 Subject characteristics

All subjects in both groups had been previously exposed to ovulation induction therapy .The most common drug used was clomiphene citrate (CC), followed by human menopausal gonadotropins (hMG) and gonadotropin -releasing hormone (GnRH). The subjects had different exposures to these drugs with regards to the combination and duration of use. The difference in duration of CC use was not statistically different between PCOS subjects and controls (P 0.9395). 95% confidence interval of this difference was -4.27 to 4.56 (Table 16).

	Control (n = 20)	PCOS (n = 21)
1 month	5	0
2 months	3	2
3 months	12	6
4 months	0	5
5 months	0	0
6 months	0	7
More than 6 months	0	1
Mean	2.86	3
P value	0.9395	

Table 16: Duration of exposure to clomiphene citrate in control and PCOS subjects

With regards to hMG and GnRH use alone or in combination again it was also statistically insignificant (P= 0.1478). 95% confidence interval of this difference from -4.73 to 21 (Table 17).

Drug	Cycle	Control	PCOS
GnRH	1	14	1
hMG	1	29	4
CC + GnRH	2	1	1
CC + hMG	2	2	3
GnRH + hMG	2	6	0
Mean		10.4	1.8
P value	0.1478		

Table 17: Duration of exposure to GnRH, hMG and CC in control and PCOS

3.2.2 Morphology of the ovarian surface epithelium

Psammoma bodies (Figure 33) and mitoses (Figures 38&39) were found to be statistically more common in PCOS (p value 0.0011 and 0.0065 respectively) (Table 18). The prevalence of other features assessed did not differ significantly between the PCOS and controls (Figures 34, 35, 36, 37, 38& 40). The results were also analysed according to whether the particular morphological abnormality was present or absent i.e. disregarding whether or not there was dysplasia, likewise the degree of dysplasia (Tables 18&19). Following the analysis of the data the presence of only two factors (as above) is statistically higher in PCOS.

	Control 72			PCOS 30			P
Score	0	1	2	0	1	2	
Surface tufting	53	8	11	27	1	2	0.1819
Psammoma bodies	68	0	4	22	5	3	0.0011
Epithelial stratification	68	3	1	30	0	0	0.4201
Mitoses	72	0	0	27	3	0	0.0065
Surface nucleoli	72	0	0	30	0	0	Na
Nuclear pleomorphism	66	6	0	30	0	0	0.1031
Chromatin pattern	68	4	0	26	4	0	0.1831
Nuclear cytoplasm ratio	50	14	8	19	8	3	0.7213

Table 18: Dysplasia scoring in PCOS and controls

	Control 72		PCOS 30		P
Score	-ve	+ ve	-ve	+ve	
Surface tufting	53	19	27	3	0.1108
Psammoma bodies	68	4	22	8	0.0058
Epithelial stratification	68	4	30	0	0.3173
Mitoses	72	0	27	3	0.0236
Surface nucleoli	72	0	30	0	Na
Nuclear pleomorphism	66	6	30	0	0.1761
Chromatin pattern	68	4	26	4	0.2295
Nuclear cytoplasm ratio	50	22	19	11	0.6434

Table 19: Dysplasia in PCOS and controls (Analysis in view of negative or positive presence of Dysplasia)

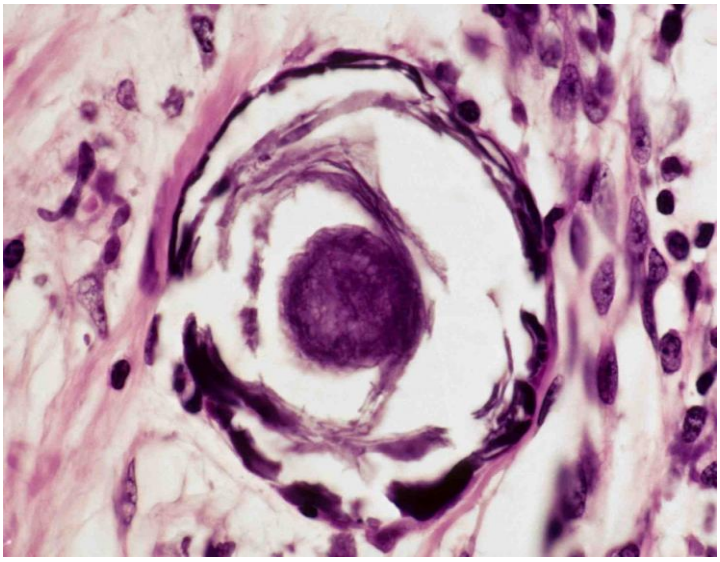


Figure 33: Psammoma Body

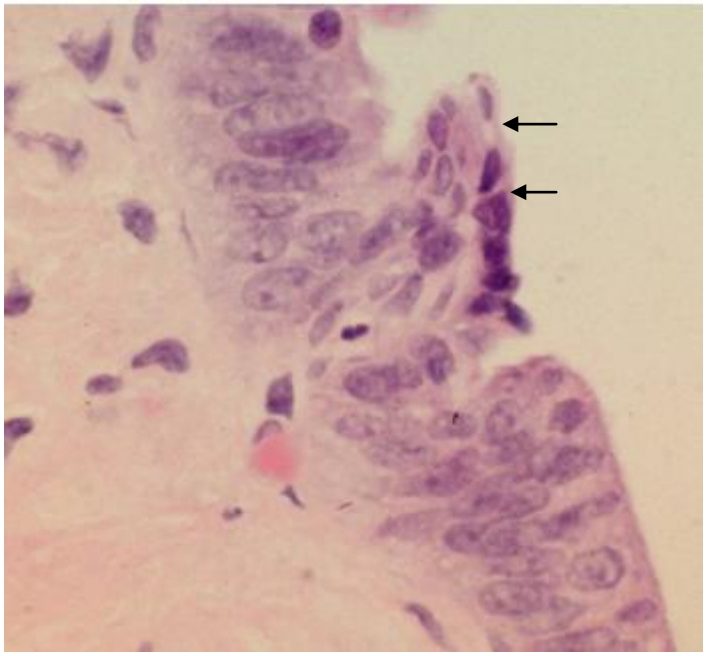


Figure 34: Presence of tufting on surface epithelium of ovary

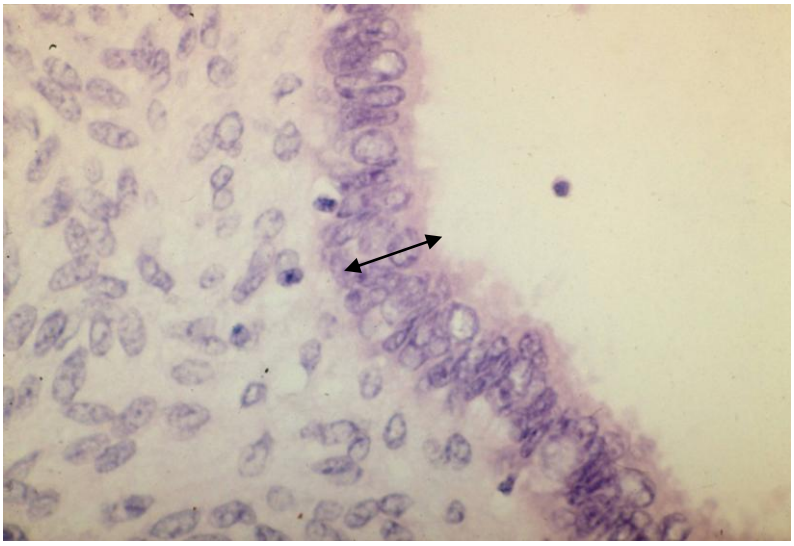


Figure 35: Cellular pleomorphism in epithelial surface of ovary

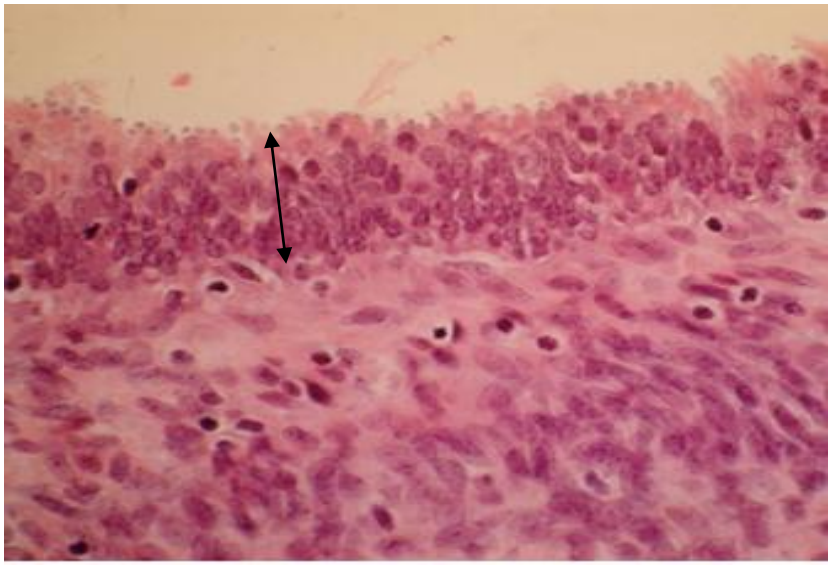


Figure 36: Multilayering in epithelial ovarian surface

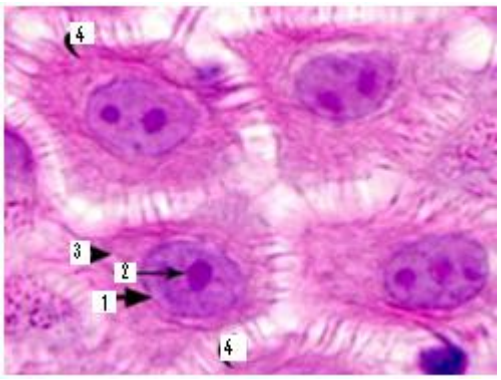


Figure 37: Normal nuclear cytoplasmic ratio. The nucleus (1) in the centre bounded by a cell membrane. The nucleus contains nucleolus (2). The cytoplasm (3).Note that adjacent cells are connected to each other by intercellular junctions (4) http://www.pathpedia.com/Education/eAtlas/Histology/cells_and_epithelia/Images.aspx

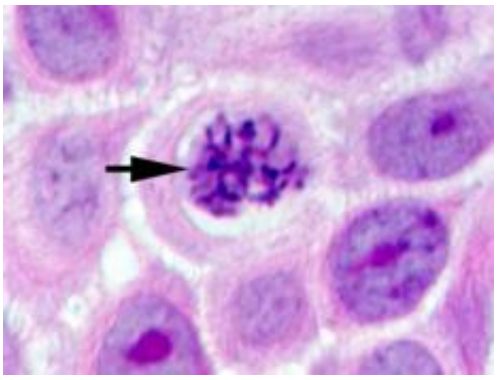


Figure 38: Normal mitosis .The arrow indicates a cell in late prophase. All other cells in the surrounding are in interphase.

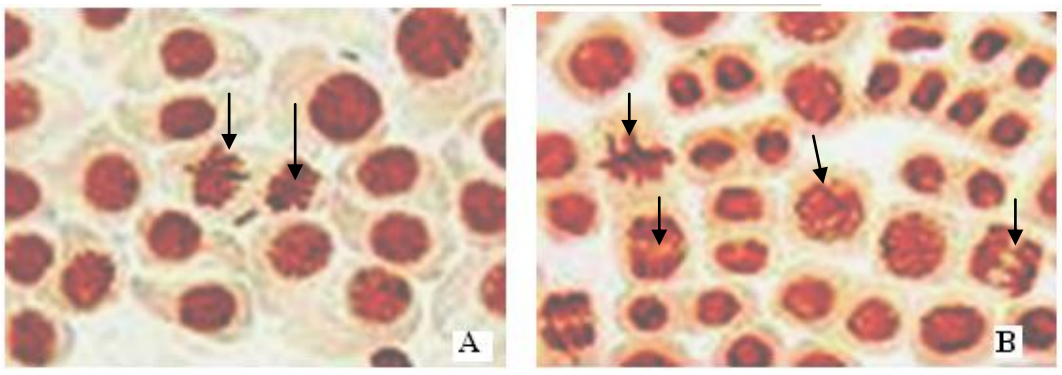


Figure 39: Mitosis: (A) score one, (B) score Two. Alkaline Phosphatase staining

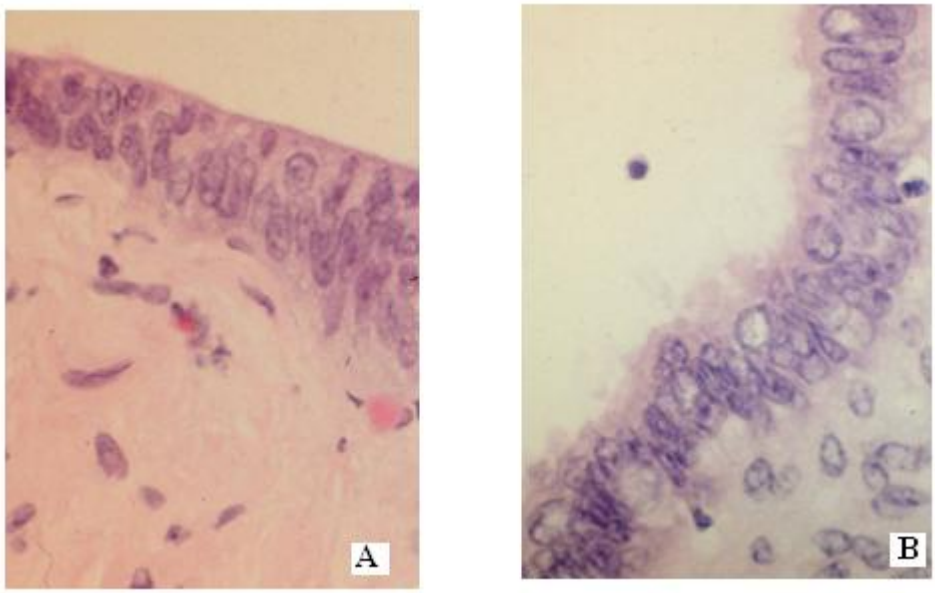


Figure 40: Nuclear cytoplasic ratio. (A) Score one, (B) Score Two

3.3 Cell cycle and apoptotic protein expression

I examined all the sections after being trained by Dr. El Sheikh, Consultant Histopathologist at the Royal Free Hospital. To test for consistency and efficacy in the results, 20% of the samples I examined were re-examined by Dr El Sheikh. In 87.5 % of these cases, the Kappa test was used and identified that the difference between the scores calculated by myself and Dr. El Sheikh was less than 10% (2.15) which was not significant and demonstrated a high level of consistency. (Table 20) (Figures 41 to 47).

No	Essam El Mahdi %	Dr El Sheikh %	Difference	% Difference
1	0	0	0	0
2	0	0	0	0
3	1.40	1.29	0.11	2.5
4	0.94	0.86	0.08	8.51
5	9.85	9.75	7.29	1.01
6	0	0	0	0
7	2.0	1.89	0.11	5.5
8	0	0	0	0
9	0	0	0	0
10	10.9	9.8	1.1	10
11	1.94	1.9	0.04	2.06
12	0	0	0	0
13	27.4	27.39	0.01	0.04
14	14.99	14.87	0.12	0.80
15	74.94	74.74	0.2	0.26
16	26.89	26.31	0.58	2.16
17	93.14	93.45	3.69	3.79
18	3.1	3.03	0.07	2.25
19	100	100	0	0
20	98.75	93.75	5	5.06
21	85.14	84.78	0.36	0.42
22	100	98.36	1.64	1.64
Mean	15.55	15.43	0.78	2.15

Table 20: Reproducibility immunohistochemistry scoring

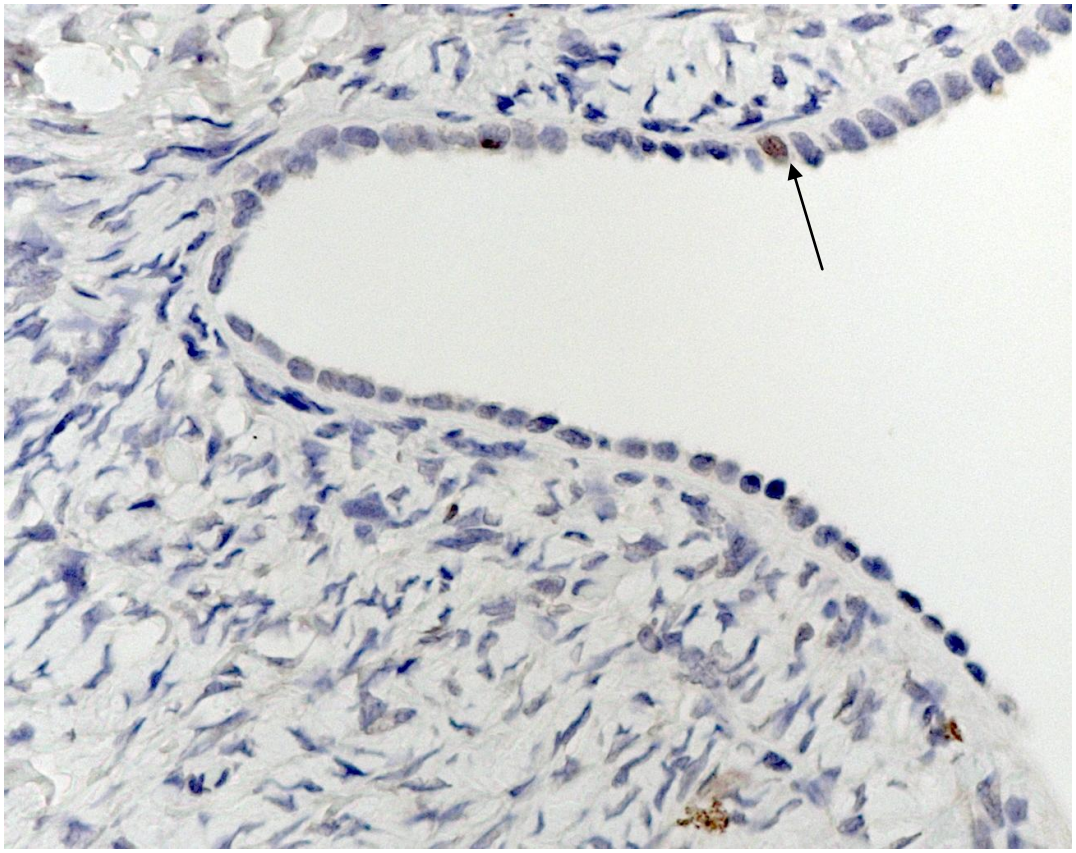


Figure 41**:** Cyclin D1 positively stained cell

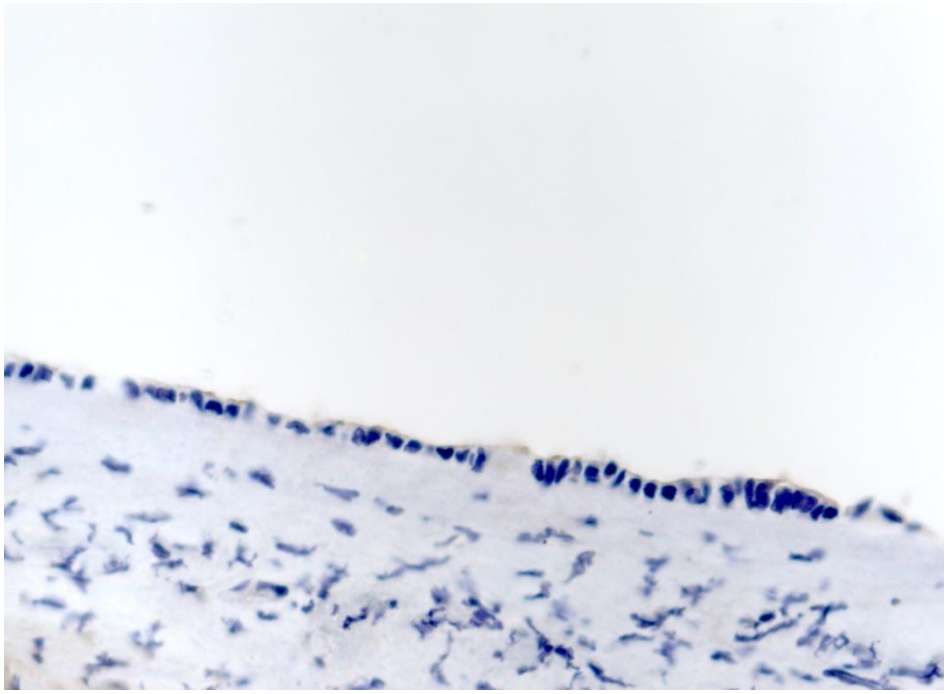


Figure 42: No Cyclin D1 staining is seen in surface epithelial cells

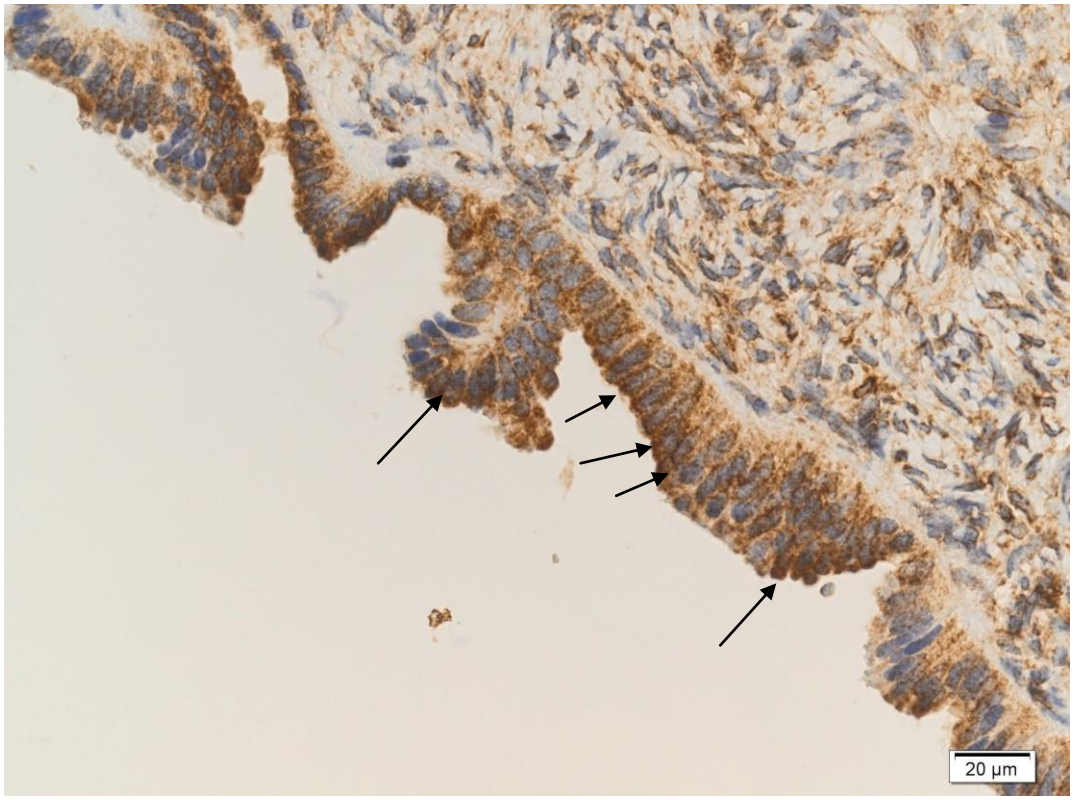


Figure 43: BCL-2 positively stains the cytoplasm of surface epithelial cells and some stromal cells

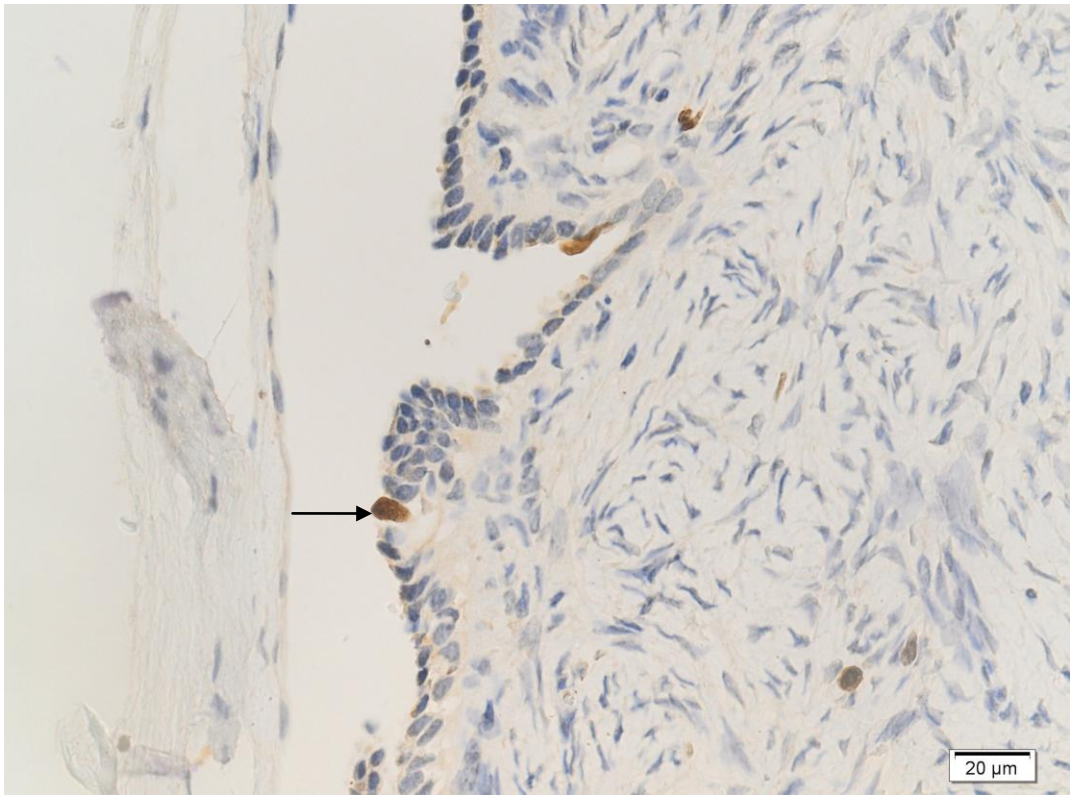


Figure 44: Ki-67 positively stained nuclei.

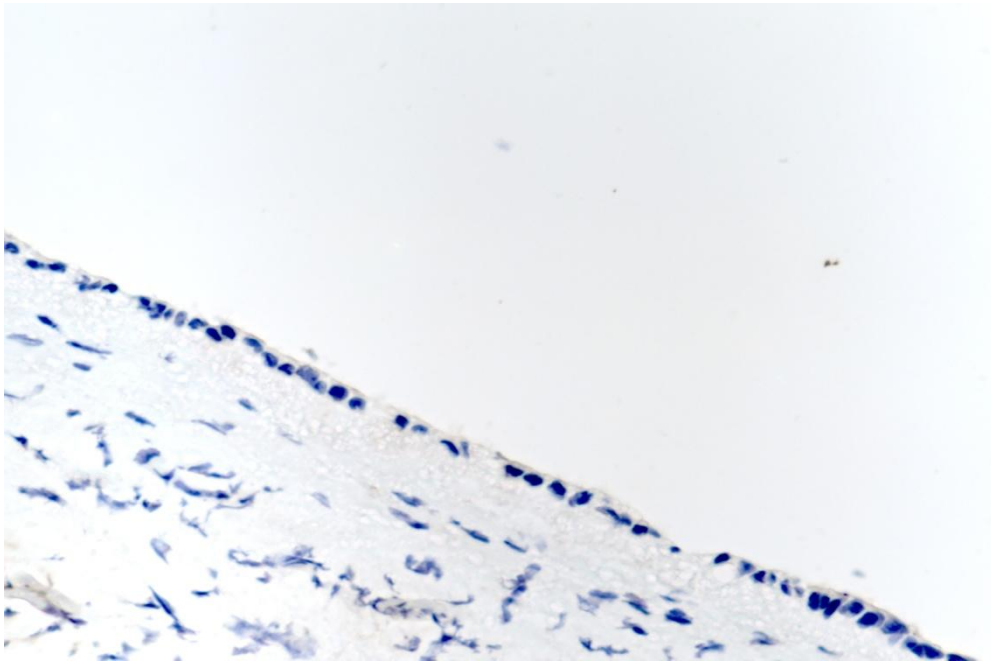


Figure 45: All surface epithelial cells lack ki-67 expression denoting quiescent G0 state.

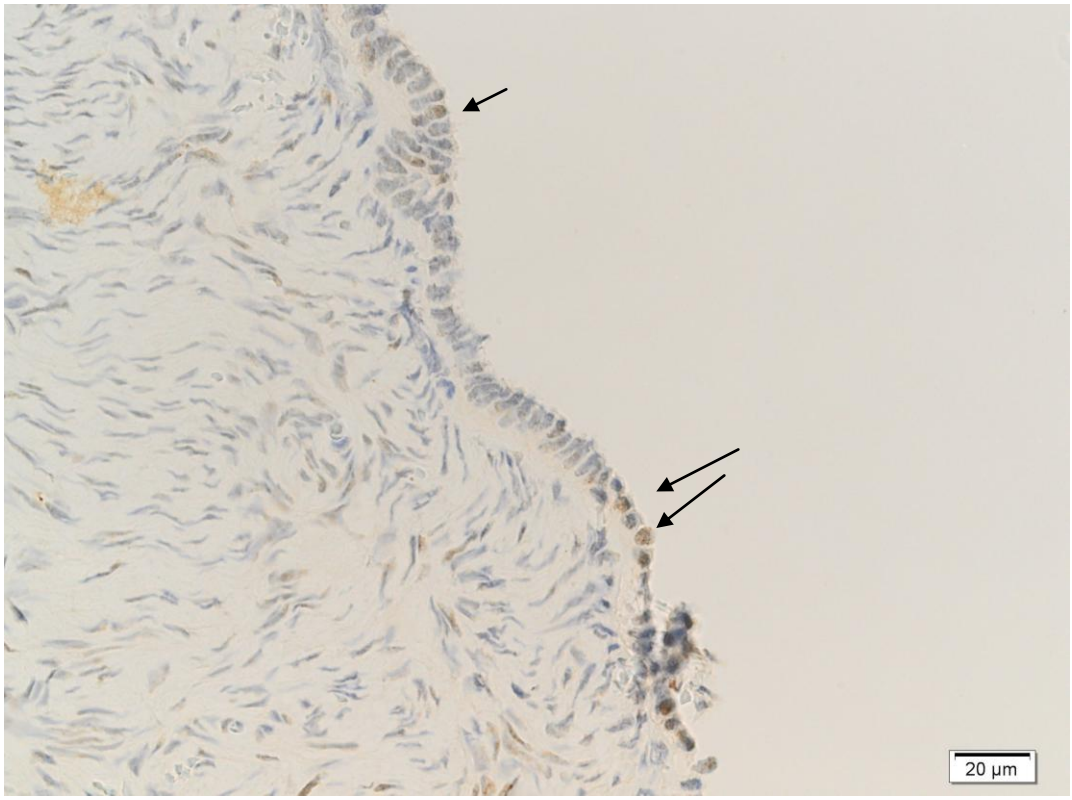


Figure 46: The brown nuclei (arrows) are p53 positively stained surface epithelial cells.

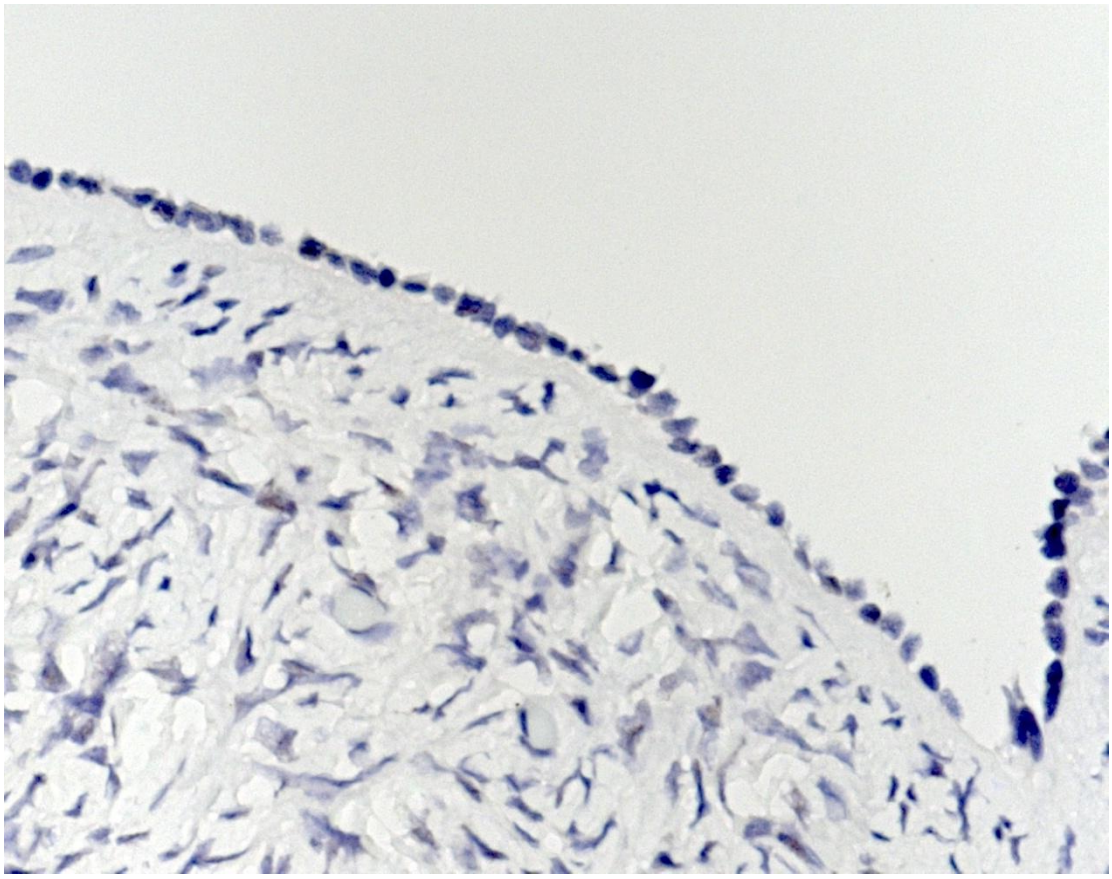


Figure 47: Surface epithelium showing no p53 staining.

The percentage cells staining positive for p53 expression in the ovarian surface epithelium of PCOS women and infertile controls is significant compared to mib and bcl; P value for p53 is 0.0025. However Cyclin D is marginally significant (P=0.0536) (Table 21).

		p53	Cyclin D	mib	bcl	TDS
Control	Mean	13.1	2.2	8.9	78.9	2.3
PCOS	Mean	39.8	7.3	9.9	85.1	1.5
	P Value	0.002545	0.053672	0.897063	0.579269	0.225025

Table 21: Mean percentage cells stained positive for Cyclin D1, mib, p53 and bcl in the ovarian surface epithelium of PCOS subjects and controls

The effect of clomiphene citrate ovulation induction therapy on p53, Cyclin D, mib and bcl expression (% positive cells) and total dysplasia score was not significant (Table 22).

			p53	Cyclin D	mib	bcl
Clomiphene exposed	Control	Mean	70.2	62.6	58.2	81.1
	PCOS	Mean	70.9	46.2	54.3	91.8
Clomiphene unexposed	Control	Mean	79.5	63.6	62.1	87.8
	PCOS	Mean	73.9	70.4	66.2	91.7
		P Value	0.329502	0.138404	0.290241	0.505528

Table 22: Cell cycle protein expression (% positive cells) in relation to exposure to clomiphene citrate

Similarly, the expression of cell cycle proteins was not significantly affected by prior exposure to human menopausal gonadotropins or gonadotropin-releasing hormone (Tables 23 and 24).

			p53	Cyclin D	mib	bcl
hMG exposed	Control	Mean	69.8	60.4	56.1	78.7
	PCOS	Mean	87.0	44.0	80.0	73.0
hMG unexposed	Control	Mean	63.8	64.7	53.2	82.7
	PCOS	Mean	74.5	69.1	68.2	91.2
		P Value	0.049974	0.781109	0.43099	0.508516

Table 23: Cell cycle protein expression (% positive cells) and total dysplasia score in exposed subjects to human menopausal gonadtrophins staining for cell cycle proteins.

			p53	Cyclin D	mib	bcl
GnRH exposed	Control	Mean	79.1	60.2	64.0	80.1
	PCOS	Mean	74.5	69.1	68.2	91.2
GnRH unexposed	Control	Mean	63.8	64.7	53.2	82.7
	PCOS	Mean	0	0	0	0
		P Value	0.327579	0.91277	0.524073	0.238233

Table 24: Cell cycle protein expression (% positive cells) and total dysplasia score in exposed subjects to gonadotrophin releasing hormone.

Previous ovulation induction exposure to any treatment did not significantly affect the total dysplasia score or percentage cells staining for cell cycle proteins (Table 25).

			p53	Cyclin D	mib	bcl
Any treatment exposed	Control	Mean	70.1	65.7	60.0	88.3
	PCOS	Mean	66.5	23.0	51.0	93.5
Any treatment unexposed	Control	Mean	87	66	51.5	85
	PCOS	Mean	73.9	70.4	66.2	91.7
		P Value	0.067483	0.147673	0.566064	0.853533

Table 25: Cell cycle protein expression (% positive cells) and total dysplasia score in exposed subjects to any ovulation induction treatment

The total dysplasia score was compared in relation to each of cell cycle elements (p53, Cyclin D, mib and bcl) in PCOS and control group. However, the results did not show any statistically significant difference (Figure 48 to 56).

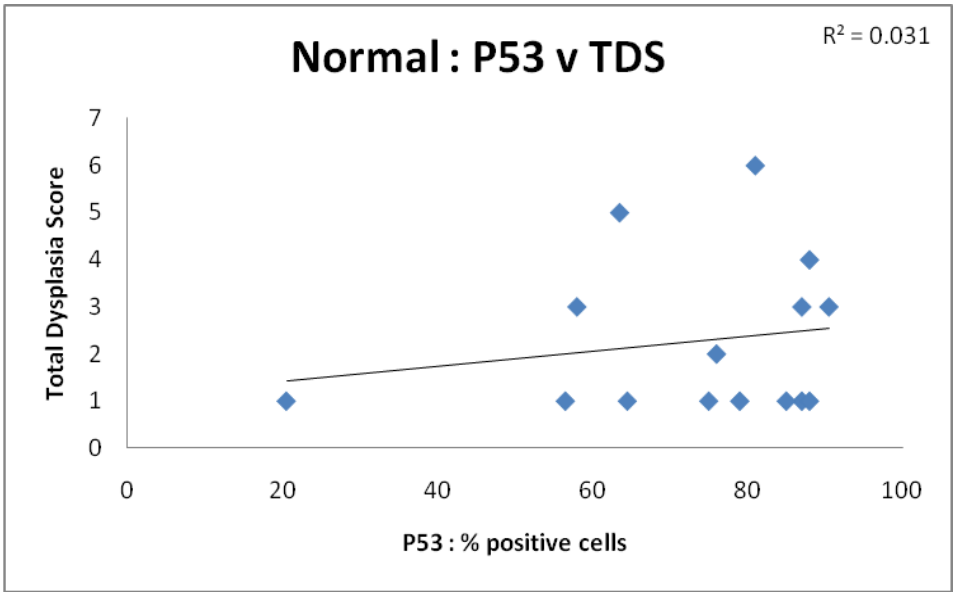


Figure 48: Mean of percentage of p53 positive stained cells in relation to total dysplasia score in control group

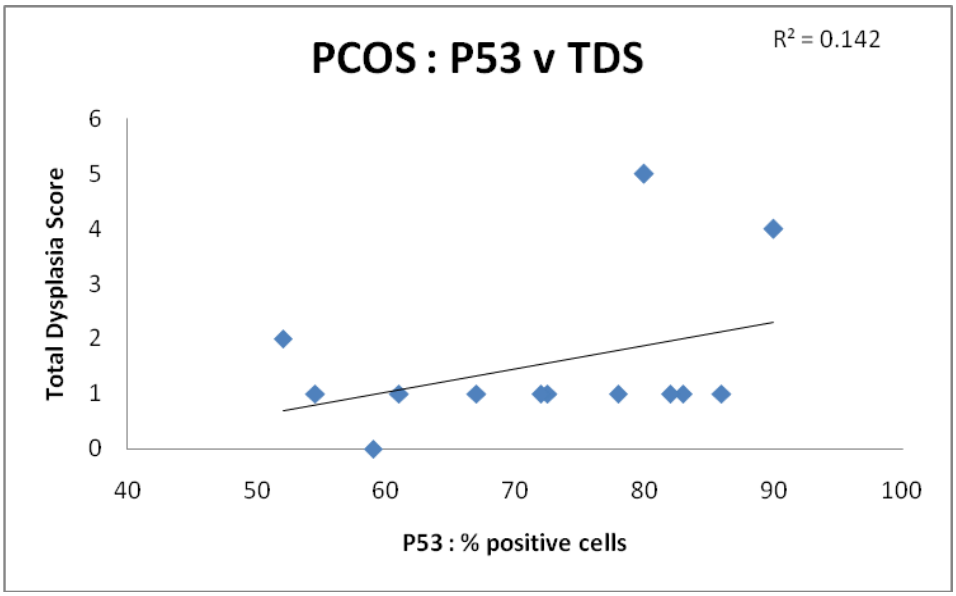


Figure 49: Mean of percentage of positive p53 stained cells in relation to total dysplasia score in PCOS

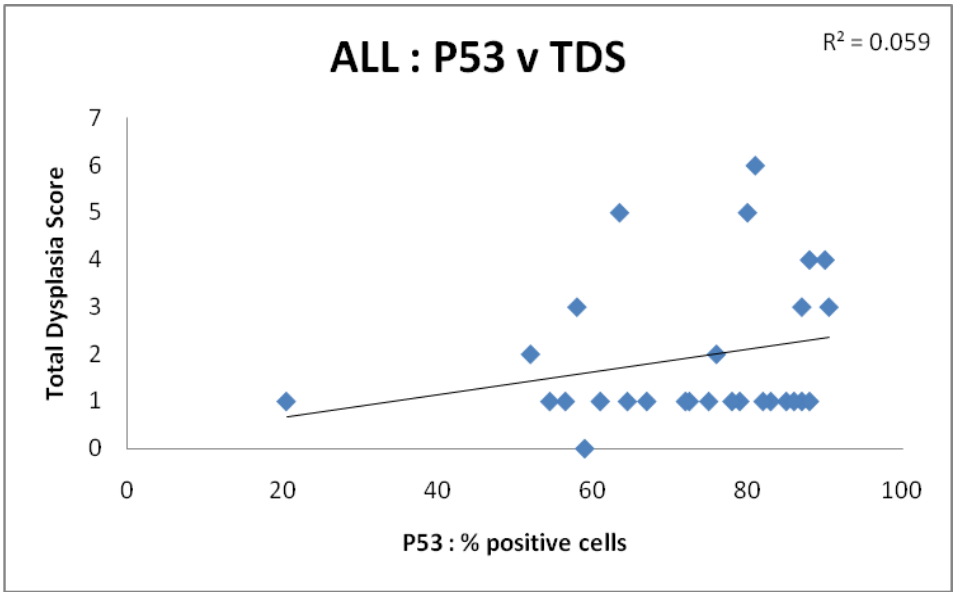


Figure 50: Mean of percentage of positive p53 stained cells in relation to total dysplasia score in PCOS subjects and controls

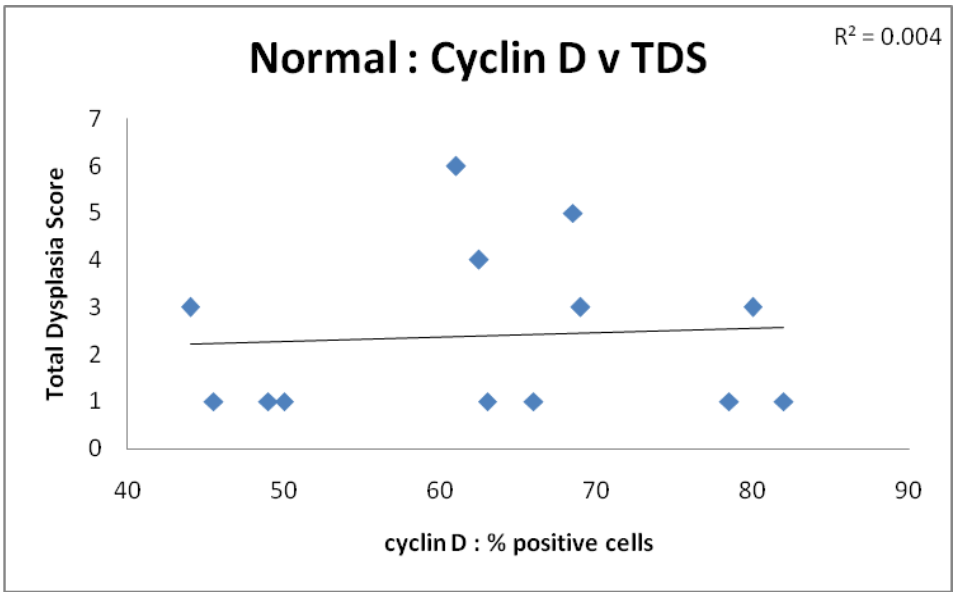


Figure 51: Mean of percentage of positive Cyclin D stained cells in relation total dysplasia score to control group

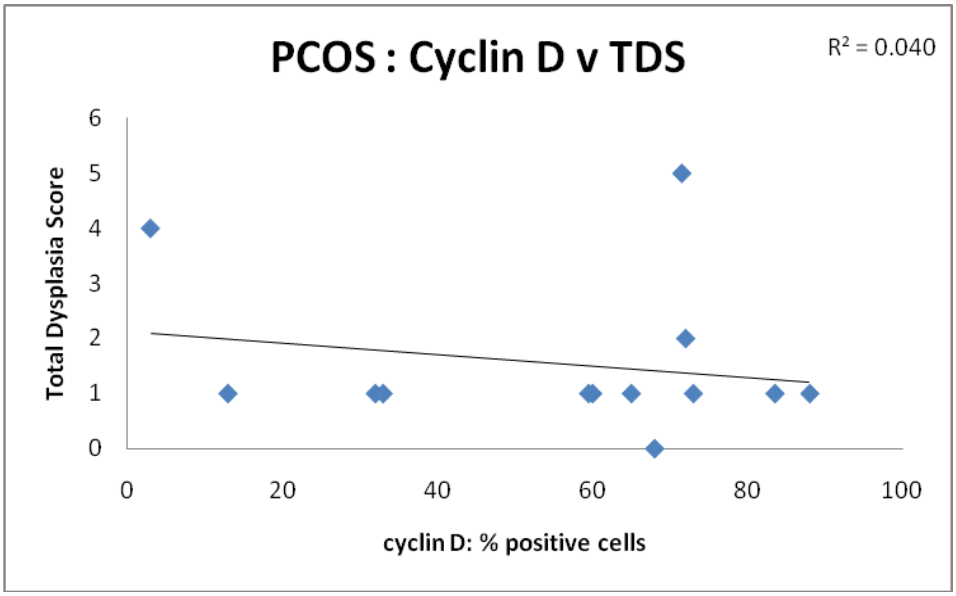


Figure 52: Mean of percentage of positive Cyclin D stained cells in relation to total dysplasia score in PCOS group

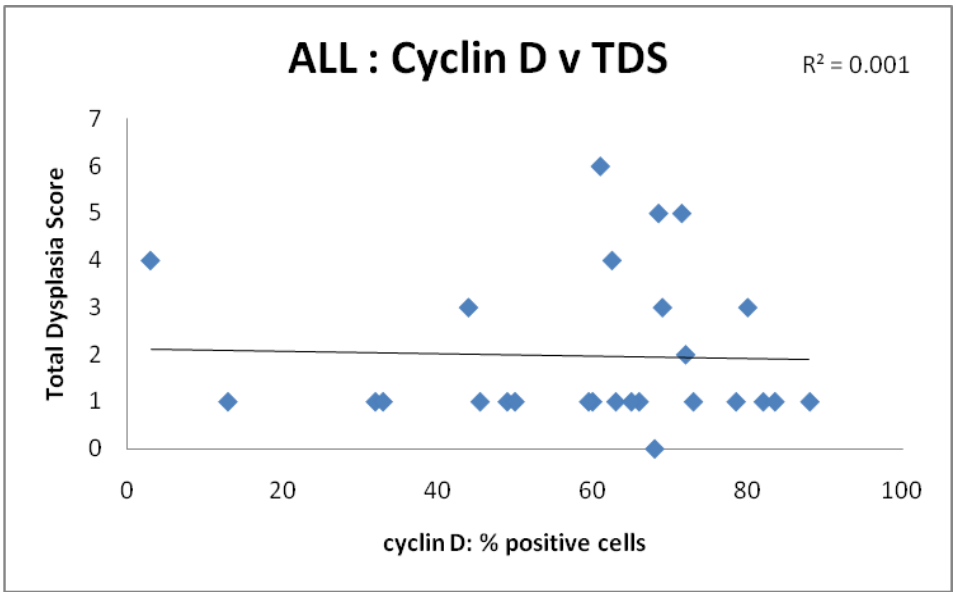


Figure 53: Mean of percentage of positive Cyclin D stained cells in relation to total dysplasia score in both groups

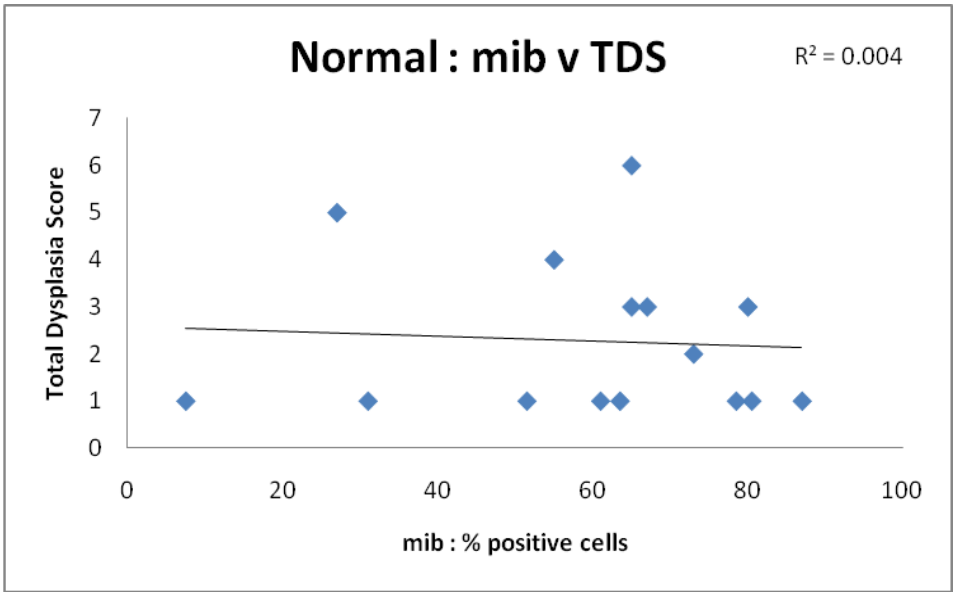


Figure 54: Mean of percentage of positive mib stained cells in relation to total dysplasia score in control group

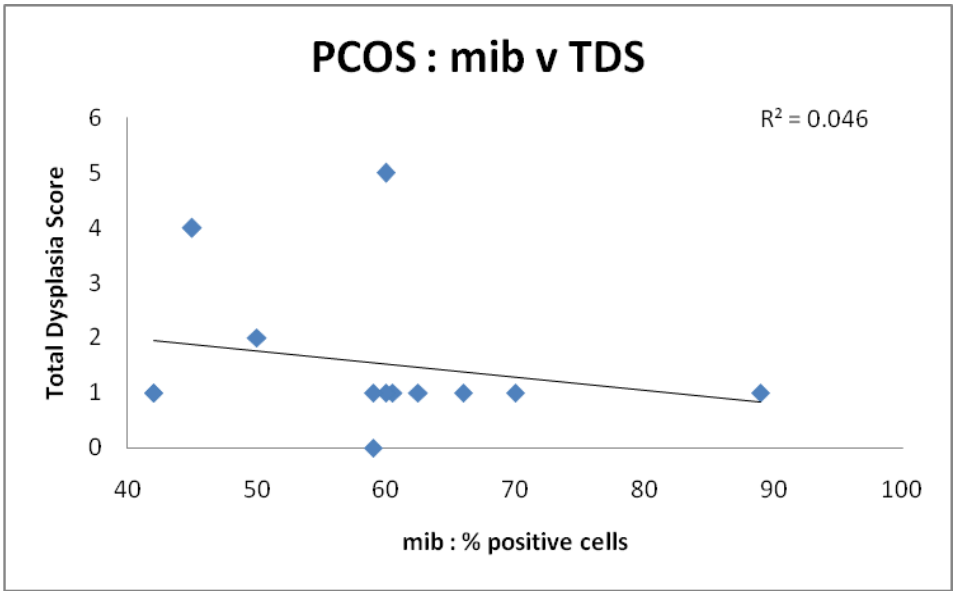


Figure 55: Mean of percentage of positive mib stained cells in relation to total dysplasia score in PCOS group

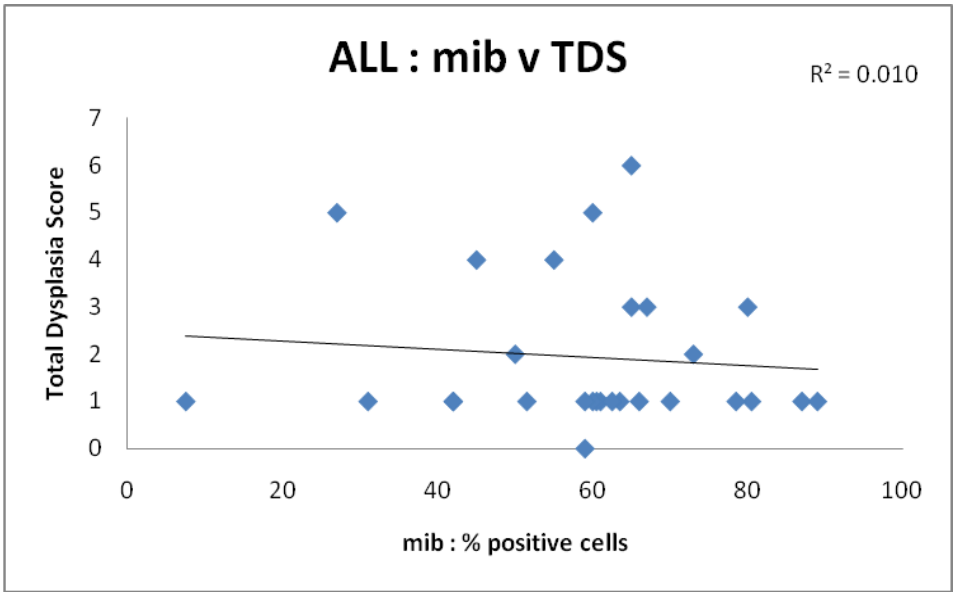


Figure 56: Mean of percentage of positive mib stained cells in relation to total dysplasia score in both groups

3.4 Gene expression

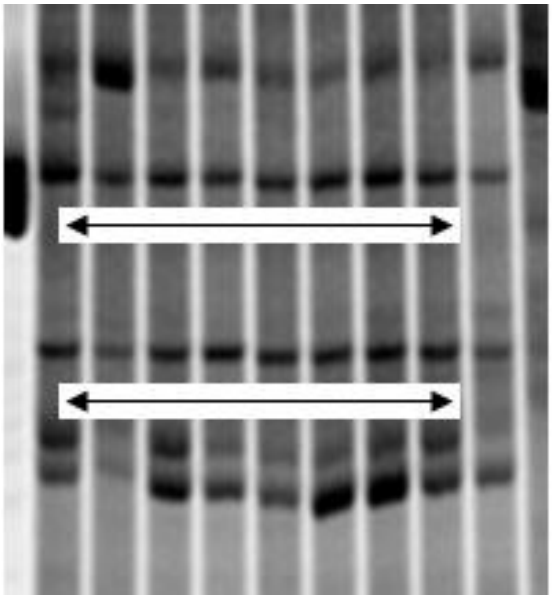
Although RNA extraction was initially carried out using 23 normal ovaries, 19 ovarian cancer (papillary serous adenocarcinoma) and 7 PCOS ovaries. RNA quantities and quality have been tested using the previously described methods chapter. Only three samples from each group were used for the study as the rest of the ovarian RNA samples were degraded evidenced by a lack of bands at 18s and 28s.

Spectrophotometeric determination of RNA concentration using A260 /A 280 ratio of 1.6-2.0 indicated reasonably pure RNA, relatively free of protein contamination Yang *et al.*, (2012). RNA integrity was confirmed by agarose gel electrophoresis. RNA samples were treated by DNase successfully from traces of contaminating genomic DNA.

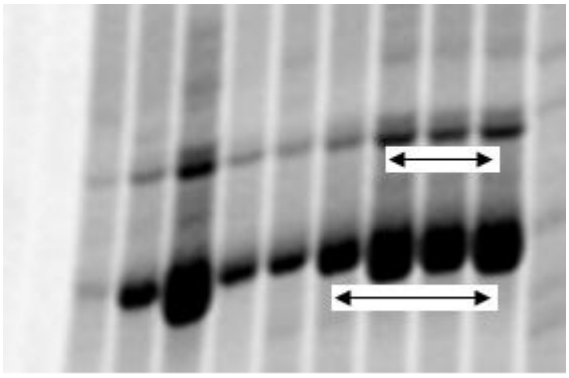
The extracted RNA has been purified and the concentration has been calculated subsequently (Appendix 6). After extracting the total RNA, Reverse transcription for both general PCR and Differential display was performed as described previously. This was followed by PCR amplification with arbitrary primers.

The Analysis of DDRT-PCR products using polyacrylamide gel electrophoresis was done. A total of 1700 PCR products (fragments) (Figure 57) were identified, isolated (Figure 58) and re-precipitated. Only 34products (2%) consistently varied in abundance between normal and PCOS samples. 12 products were over expressed in PCOS and 22 under expressed. Subsequently, cloning of these differentially expressed PCR fragments was carried out as described earlier by ligation of PCR fragments into cloning vector followed by Plasmid purification.

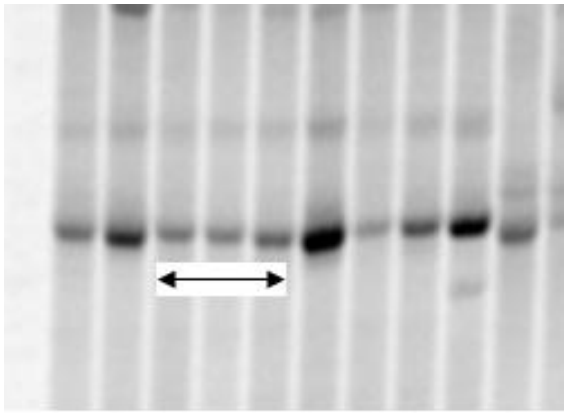
Provided that the expected fragment T3-T7 from BSII is 130 bp, when plasmid ligates closed with insert at the *Xho*I/*EcoR*I junction with fragment ligated the size of the fragment is 130 bp. Some PCR products indicated they contained plasmid i.e. P1.1, P2.1, P2.3, P2.5 and P4.1. P1.1, P2.1 and P2.5 PCR products suggest inserts of ca 130 bp which is what we expected. P2.3 products suggest this is not a pure clone, and those of P2.6 indicate no insert. P4.1 product suggests a larger insert of 180bp. Following plasmid purification plasmids were isolated successfully followed by sequencing of plasmids to identify the genes. A summary of clone sequences and related genes are in table 26.



No differentially expressed bands



Over expressed bands



Under expressed bands

Figure 57: Polyacrylamide gel electrophoresis for DD PCR shows the differentially expressed bands.

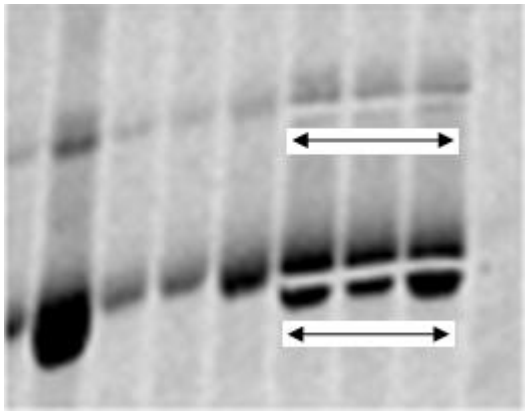


Figure 58: Polyacrylamide gel electrophoresis for DD PCR showing where differentially expressed bands are extracted.

One of the over expressed products was identified as being derived from the 3` terminal of the mRNA for human alpha 2 smooth muscle actin. RT-PCR analysis of alpha 2 actin mRNA abundance detected the expected 970 bp product, at levels calculated 15-fold higher in PCOS ovary than in normal ovary $p< 0.001$.

Western blotting of protein extracts from another set of normal and PCOS ovaries was performed (Figures 59&60). The alpha 2 actin antibody detected a protein of the expected size, the abundance of which was elevated approximately four fold in PCOS ovary compared to controls ($p<0.05$).

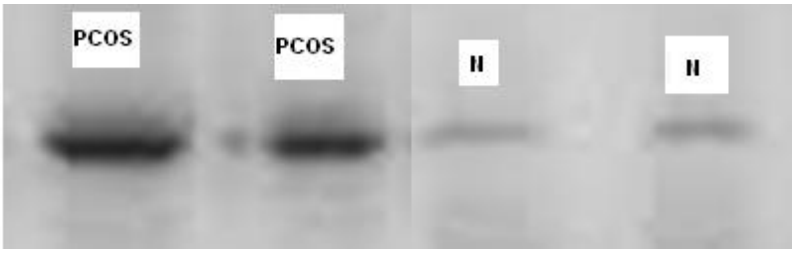


Figure 59: Western blot of 2µg protein from normal (N) and PCOS ovaries

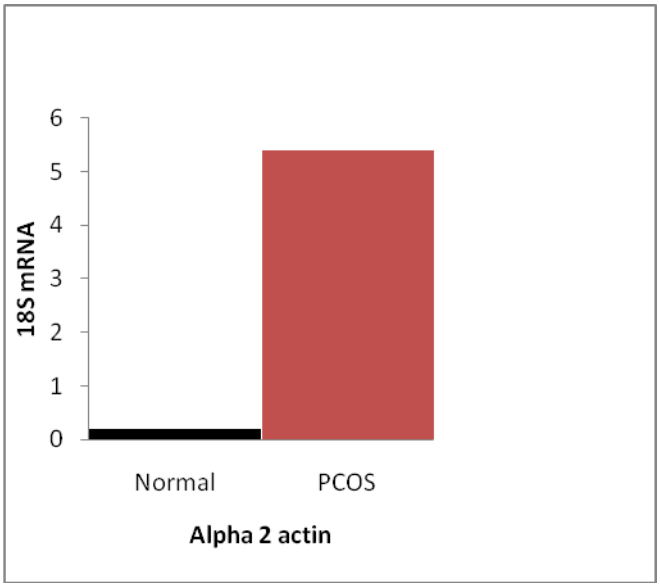


Figure 60: Alpha 2 actin mRNA abundance in normal and PCOS ovary

This preliminary differential display analysis indicates that ovarian gene expression is modified in PCOS. The abundance of 2% of PCR products was disturbed, thus up to 2% of expressed gene transcripts in the ovary may be altered in PCOS, and since several PCR products may be derived from a single transcript. Ovarian alpha 2 actin mRNA expressions were considerably up-regulated in PCOS, as was alpha 2 actin protein abundance.

Table 26: Summary of clone sequencing data with identities

Clones	Gene	Entrez Gene Number:
P1-1; P1-12; P1-13, P1-14	Human smooth muscle alpha2 actin	NM_001613.1
P2 PCR frag; P2-1; P2-5	Human actin, alpha2, SM, cds	BC017554
P3-21	Human mRNA VSM alpha2 actin	X13839.31/HSACTA
143 bases	Human DNA seq clone RP11-399019	AL157394.15
	Human aortic SM alpha2 actin	M33216.1/HUMSMAAA
	Human cDNA FLJ36021 fis	AK093340.1
P1-14 P3-13; P3-14 147 bases	Human clone RP11-155N3, chr 13	AL161420.1
P3-23	Human BAC RP11-736E3 from 7	AC073346.12
143 bases	Human BAC RP11-599B13 from 17	AC129492.6
	Human chr 17, clone CTD-3051C7	AC107913.7
	Human chr 17, clone RP11-769H22	AC104762.7
	Human clone RP4	HSJ543J19
P4-1	Human clone BAC 82621, chr19	AC007193
156 bases	Human BAC RP11-450E9, chr2	AC062029.7
P4.2	Human BAC RP11-461L13, chr 4	AC080078.7
158 bases	Human chr? (1e1-36)	AC010409
	Human clone RP11-169K16, chr1	AL450998.19
	Human clone D47-S479, chr21q22.2	AP000153.1
P4.4	Human clone FLB4816, PRO1252mRNA	AF130054
157 bases	Human clone RP11-497D24, chr2	AC012076.5
	Human clone RP11-255C15, chr3	AC007823.38
P5-1; P5-3; P5-5	Human clone RP13-98N21, chr 15	AC126339.6
P7-1	Human clone RP13-996F3, chr 15	AC139425.3
106 bases	Human clone RP13-262C2, chr 15	AC136698.6
	Human clone RP11-561C5, chr15	AC044860.11
	Human clone RP11-606M5, chr 15	AC126605.4
	Human PAC RP5-1129D5, chr15	AC005630.1

P6-1; P6-2; P6-3	Human genomic DNA, chr22q11.2	AP000344.1
P7-2; P7-3	Human hypothetical protein FLJ31568	NM_152509.1
100 bases except P7-3	Human cDNA FLJ35262 fis	AK092581.1
(102, CT insert)	Human cDNA FLJ35211 fis	AK092530.1
P8.2	Human clone RP1-34B21, chr6p12.1-21.1	AL031778.1
134 bases	Human clone RP11-57G22, chr 18	AC012123.8
	Human clone RP11-386P4, chr18?	AC025887

P8.3	TPA human chr 7	BL000001.1
136 bases	human chr 14 clone	AC025594
	human chr 7 clone	AC018639

P8.4 104 bases	Human clone RP11-387M24,	AL355385.15

Chapter four

Discussion

The studies described in this thesis were designed to test the hypothesis that women with PCOS are not at increased risk of developing epithelial ovarian cancer compared to controls that do not have PCOS.

4.1 The aims of the studies were to:

1. Assess the history of ovarian cancer in first degree relatives of PCOS women.
2. Assess the degree of morphological abnormality in the ovarian epithelium of women with PCOS.
3. Assess cell cycle and apoptotic protein expression in the ovarian surface epithelium of women with PCOS and healthy controls.
4. Assess gene expression in the ovaries of women with PCOS, healthy controls and women with serous epithelial ovarian cancer

4. 2 Questionnaire study

The results of this questionnaire study showed women with PCOS were more likely to have a first degree relative with a family history of breast cancer and heart disease. However, there was no increase in the family history of ovarian cancer in this group when compared to women without PCOS.

Conversely, Moni *et al,* (2009) conducted a similar study in 549 infertile Iranian women (273 with PCOS and 276 controls). A significantly higher proportion of the PCOS subjects reported a family history of breast cancer compared to the controls (4.35% vs. 1.30%, p=0.02). A possible explanation for the difference in

the results of our study compared to Moni *et al's,* (2009) could be that in my study the subjects were from different ethnic background. Likewise, the ages of the relatives in both studies were not considered as some women develop cancers at different stages of their lives.

The finding of an increased prevalence of breast cancer in the relatives of PCOS women is therefore rather surprising. This association could theoretically result from some genetic predisposition to breast cancer in PCOS women. The most well known genetic factors conferring an inherited susceptibility to breast and ovarian cancers are mutations of *BRCA1* and *BRCA2* genes (Brody *et al*, 1998). However, there is no available study to assess mutations of *BRCA1* and *BRCA2* genes in PCOS.

Although the genetics of PCOS have not yet been elucidated, studies of first-degree relatives of women diagnosed with PCOS reveal familial clustering of the disease, particularly hyperandrogenaemia. A prospective study (Strauss *et al*, 2003) of first-degree female relatives of PCOS women found that 46% of ascertainable sisters of PCOS women were hyperandrogenaemic. The serum bioavailable testosterone in the cohort of sisters showed a bimodal distribution. These observations suggest a dominantly inherited trait controlling androgen levels.

Nicolás Díaz-Chico *et al.*, 2007 and Travis *et al*, 2003 studies have found elevated blood concentrations of androgens were associated with increased risk of breast cancer in both pre and post-menopausal women and thus, androgens may be potential factors linking obesity and breast cancer (Travis *et al*, 2003).

An alternative explanation for the finding of breast cancer in relatives of PCOS women is that there is some predisposing familial endocrine or metabolic disturbance in women with PCOS. Liede *et al,* 2000, reported two families sharing an unusual clustering of pyloric stenosis, endometriosis, and breast cancer and that one of these families contained nine women with PCOS. They hypothesized that there could be a common genetic basis for these conditions and highlighted that pyloric stenosis has been linked to the locus of the neuronal nitric oxide synthase (*NOS1*) gene. Such a linkage could also explain the endothelial dysfunction identified in PCOS women (Lakhani *et al,* 2006). However, in the Baskol *et al.* (2012) study no significant difference was found between nitric oxide and thiol level activities of women with PCOS and controls ($p>0.05$).

An association between PCOS and a family history of breast cancer could also be related to increase BMI, on the basis that relatives of women with PCOS may themselves be overweight or obese. These conditions are associated with hyperinsulinaemia, high circulating levels of serum C-peptide, which are known to be a risk of post-menopausal breast cancer (Schairer *et al*, 2004, Goodwin *et al*, 2002, Yang *et al,* 2001, Suga *et al*, 2001, Toniolo *et al*, 2000 and Del Giudice *et al*, 1998). It is reasonable to conclude that women with PCOS might be more likely to develop breast cancer because obesity is more common in PCOS and most large epidemiological studies have found that overweight or obese women are at increased risk of developing postmenopausal breast cancer.

4. 3 Morphological assessment of ovarian surface epithelium

In this study, the results of the detailed morphological examination of the ovarian surface epithelium in PCOS women revealed an increased prevalence of psammoma bodies and mitoses were more common compared to controls.

These findings could have been the early morphological changes leading to ovarian cancer as Ferenczy *et al,* (1977) observed the formation of PBs which was initiated intracellulary in neoplastic epithelial cells and they concluded that PBs in ovarian papillary serous neoplasm was a consequence of dysmorphic calcification associated with cellular degeneration.

Kozlovskii *et al,* (1978) followed all stages of PBs formation and observed that it is formed as a result of continuous deposition of micro crystals of oxyapatites on accumulations of protein substances in ergastoplasma cisterns and on the mitochondrial cristae giving rise to intracytoplasmic inclusions. After convergence, these inclusions fill the cell resulting in cell death and liberation of small PBs (Das *et al,* 2009).

All women in this study were complaining of infertility and for this reason ovarian biopsy was performed and subsequently they were exposed to ovulation induction drugs. This allowed a comparison of these morphological changes in PCOS and controls. The findings of no significant difference in total dysplasia score suggest that these drugs were not responsible for these subtle, but potentially premalignant changes. This is in accordance with results of a study published by Dos Santos Silva *et al,* (2009). In that retrospective study to assess long-term health effects of ovarian-stimulation drugs, 7355 woman were followed up for over 20 years; 43% were prescribed ovarian-stimulation drugs, and 367 incident cancers and 274

deaths were identified. There were no significant differences in the risk of cancer of the ovary between women who had been prescribed ovarian-stimulation drugs and those who had not. It should be noted however, that the subjects for the studies in this thesis were also included in the cohort of women with ovulatory disorders studied by Dos Santos Silva *et al,* (2009). Histological examinations for early dysplasia changes were examined only in our study.

Like the Dos Santos Silva *et al,* (2009) study, studies conducted by Ness *et al,* (2002), Potashnik *et al* (1999), Modan *et al* (1998), and Mosgaard *et al* (1997) concluded that treatment with ovulation-inducing drugs did not appear to increase the risk for ovarian cancer. Conversely, Ness *et al* (2002) and Rossing *et al* (1994) noticed that fertility drug use was associated with a significantly increased risk of borderline serous ovarian tumours when analysed by histological subtypes of cancer.

4. 4 Cell cycle and apoptotic protein expression

The finding of this study demonstrated an increased p53 expression in PCOS ovaries which suggested that those lesions identified using morphological criteria, may have increased malignant potential.

Increased expression of p53 protein was found in approximately 50% of invasive epithelial ovarian tumours and in inclusion cysts and endometriosis adjacent to invasive tumours (Kihana *et al,* 1992). Although positive immunostaining can result from increased expression of wild-type p53, it usually reflects mutation of the p53 tumour suppressor gene resulting in abnormal protein expression which accumulates in the nucleus. Such mutations, which are very uncommon in normal

ovarian epithelium, are believed to be a crucial step in the development of ovarian malignancy.

In the light of the results, future work will be required to sequence p53 in these ovaries to obtain evidence of gene mutation. It can be hypothesized that such mutations will be more prevalent in ovarian epithelial cells which exhibit signs of dysplasia. Confirmation of p53 mutations in p53 positive ovarian epithelium would challenge the ethics of any future prospective study on the progression and behaviour of ovarian epithelial dysplasia, if a method was available to diagnose dysplasia in vivo.

It is perhaps surprising that there was no significant difference in expression of proliferation marker, Ki-67 and the anti-apoptotic protein, bcl-2 in control and PCOS ovaries (p= 0.89 and 0.57 respectively).

The expression of the human protein Ki-67 is associated with cell proliferation. During interphase, the antigen can only be detected within the nucleus, whereas in mitosis, the majority of the protein is trans-located to the surface of the chromosomes. The fact that Ki-67 protein is present during all the active phases of the cell cycle (G1, S, G2 and mitosis), and absent from the G0 phase, has made it an excellent marker for determining the growth fraction of a determined cell population (normal or tumoural) (Scholzen *et al,* 2000).

The results did show marginally increased expression of Cyclin in the surface ovarian epithelium of PCOS women. The D type Cyclins are a family of proteins which regulate the activity of Cyclin dependent kinases. These phosphorylate the retinoblastoma protein, which undergoes conformational change to release

transcription factors which coordinate expression of G1 and early S phase genes required for cell cycle progression.

In summary, the results suggest that there is an abnormality of cell cycle regulation in ovarian epithelial dysplasia. In view of these results, it would be important to study later ovarian cancer incidence in these women.

4.5 Gene expression

In the RT-PCR DD study, smooth muscle α2-actin (αSMA) was found to be over expressed in PCOS which is a novel finding. α-SMA is one of three actin isoforms expressed in fibroblast cells and is also expressed in vascular smooth muscle cells. Its expression is regulated by hormones, cell proliferation and pathological conditions including oncogenic transformation (Bushel *et al,* 1995, Kumar *et al,* 1992, Owens *et al,* 1986 & Leavitt *et al,* 1985). The αSMA promoter is highly conserved between human, mouse and chicken genes (Reddy *et al,* 1990). αSMA promoter activity is represented in transformed cell lines (Bushel *et al,* 1995 and Kumar *et al,* 1992). Its activity is regulated by cell proliferation. Actively proliferating fibroblast cells and smooth muscle cells have low levels of αSMA; inhibition of cell proliferation, by density arrest or treatment with antimitotic agents has been found to induce αSMA promoter activity (Kumar *et al,* 1995, Owen *et al,* 1986 & Leavitt *et al,* 1985).

Increased stromal blood flow was observed in the PCOS group by transvaginal colour Doppler evaluation (Abd El Aal *et al*., 2005 and Loverro *et al.*, 2001). In the Abd El Aal study serum levels of vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and hormonal profile were measured in fifty women diagnosed with PCOS compared with twenty healthy and fertile women

with regular menstrual cycles (Abd El Aal *et al*., 2005). Significantly higher serum levels of VEGF and IGF-1 in PCOS women (P <0.001 and P <0.01, respectively) were noted. This may be related to the increased vascularity that underlies the increased blood flow demonstrated by Doppler blood flow measurements in these women.

Czernobilsky *et al* (1989) conducted an immunohistochemical investigation of alpha-smooth muscle actin (α -SMA) using the monoclonal anti α -SMA -1 antibody in 15 normal ovaries in three ovaries with stromal hyperplasia and in 27 neoplastic ovaries. The pattern of actin isoforms was examined by using 2 D-gel electrophoresis. In normal ovaries, α -SMA was found in the inner cortex and in the theca externa. In ovarian stromal hyperplasia expression of α -SMA was minimal or absent. In primary and metastatic epithelial tumours there was positive stromal staining for α -SMA, especially in the vicinity of epithelial elements which was more widespread in malignant neoplasm. Thecomas did not express α -SMA and could be differentiated from leiomyomas which stained intensely for α -SMA. Only focal stromal staining of α -SMA was observed in granulosa and germ cell tumours.

In all the tissues studied blood vessels were strongly positive for α -SMA. Desmin, although present in the stroma of most of the specimens, was less abundant than α –SMA. Therefore, it was concluded that α -SMA is a component of the normal human ovary and contributes to the contractility of its stroma. Its absence in the normal outer cortex, theca interna and in stromal hyperplasia and thecoma suggests that sex hormones do not stimulate α -SMA production in the ovary. Among neoplasm it is most widely represented in the stroma of epithelial tumours reflecting stromal stimulation mediated by neoplastic epithelium.

Cortón *et al,* (2007) carried out a similar case-control study to examine gene expression profiles of omental fat. Biopsy samples obtained from morbidly obese women with or without PCOS at the time of bariatric surgery. Eight PCOS patients and seven controls were recruited for the study. Cortón *et al,* (2007) identified changes in the expression patterns of 63 genes between PCOS and control samples. α -SMA was found to be a down-regulated gene in PCOS. The disagreement in findings could be due to the nature of the samples used as in this study, ovarian samples were used compared to omental fat in Cortón *et al*'s (2007) study.

Kobayashi *et al,* (1993) compared the expression of α -SMA between benign and malignant human ovarian tissues by immunohistochemical staining and Western blot analysis using the monoclonal antibody specific to α -SMA. In normal human ovaries, α -SMA was found in the blood vessel walls, muscle fibres, and stromal cells surrounding the follicles. The main source of α -SMA in the benign ovarian tumours was the blood vessel walls which highly expressed α -SMA throughout the tumour. In malignant tumour tissues, however, the vessels located in or close to the cluster of cancer cells did not express α -SMA. It corresponded with the results of the Western blot analysis, indicating the amount of α -SMA in malignant ovarian tumour tissues was less than that in benign tumour tissues. Ther alteration in α -SMA expression may reflects the qualitative difference in vessels between benign and malignant ovarian tissues and therefore α -SMA could be a histopathologically useful marker for indicating the malignant potential of ovarian tumours.

4.6 Conclusion

The hypothesis of this study was to establish whether or not there is an association between PCOS and ovarian cancer. Having reviewed the finding of all four areas (family history in patients with PCOS, ovarian surface epithelial morphology, cell cycle and apoptotic protein expression and gene expression), the evidence is not strong enough to link PCOS to ovarian cancer, directly or indirectly. In the questionnaire study, the relationship between the familiar association and PCOS and breast cancer could be incidental or due to other factors, such as raised BMI or genetic predisposition, which may have no direct link to PCOS. In the second area of this study which examined the ovarian epithelial changes, only two of eight features of the dysplasia criteria were identified. Consequently, there is inadequate evidence to make a link to ovarian cancer and therefore the findings are inconclusive. In the cell cycle changes study only p53 expression was a statistically significant finding which could be explained as due to the nature of PCOS and hyper androgenic state of the syndrome. This finding was also inconclusive as other parameters did not reveal any significant alterations in the cell cycle. Lastly, in the RT-PCR DD study, α-SMA was one of the genes traced but this gene is a normal component of the ovary and its high expression is due to the nature of the syndrome.

Therefore, in conclusion, there is no direct link between PCOS and cancer of the ovaries. The association that Fthalla (1971) made between anovulation and ovarian cancer may be outdated, and newer theories linking infertility, tubal changes with ovarian cancer may be the direction to investigate.

4.7 Suggestions for future work

1. Follow up of the biopsy patients who have been flagged on the NHS Central Register (as part of CR UK funded study published by Dos Santos Silva *et al,* (2009) to assess the incidence of ovarian cancer in relation to the degree of morphology abnormality and cell cycle/ apoptotic protein expression data.
2. To micro dissect and laser capture p53 positive cells from the ovarian biopsies, extract and sequence the DNA.
3. Quantitative study (using an ELISA) of α smooth muscle actin in serum of PCOS women and healthy controls, to investigate a possible role as a biomarker for PCOS.
4. To test the hypothesis that NOS1 is a susceptibility locus for PCOS, perform linkage analysis and the transmission disequilibrium test using NOS single nucleotide polymorphisms in PCOS subjects and first degree relatives.
5. Immunohistochemistry using antibody to alpha smooth actin or in FISH to identify anatomical site of increased expression of this protein.

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Appendices

Appendix 1: Invitation letter to the family history study

Royal Free & University College Medical School

UNIVERSITY COLLEGE LONDON

DEPARTMENT OF OBSTETRICS & GYNAECOLOGY

Royal Free Campus

Rowland Hill Street

London NW3 2PF

Tel: (020) 7794 0500

Fax: (020) 7830 2261

Head of Department Professor A B MacLean (3736)

Patient`s address

Date:

Dear **Patient`s name**

According to my records you were attending my Gynaecology clinic at the North Middlesex Hospital, and I am writing to you to ask for your help in a research project which has been designed to investigate a possible association between some hormonal conditions and certain health problems. This project has been approved by Enfield and Haringay Health Authority, Local Research Ethics Committee

I would be very grateful if you could fill in the short form attached, and return to us in the envelope provided. Your participation in this project is voluntary and your care will not be affected if you decide not to take part. However your reply will be treated in the strictest confidence, and will provide important information which will help us to improve treatment for women with fertility problems.

Yours Sincerely

Mr Paul Hardiman MD FRCOG

Senior Lecturer, Honorary Consultant in Obstetrics and Gynaecology

Appendix 2: Reminder letter to the invitation to the family history study

Royal Free & University College Medical School

UNIVERSITY COLLEGE LONDON

DEPARTMENT OF OBSTETRICS & GYNAECOLOGY

Royal Free Campus

Rowland Hill Street

London NW3 2PF

Tel: (020) 7794 0500

Fax: (020) 7830 2261

Head of Department Professor A B MacLean (3736)

Patient`s address

Date:

Dear **Patient`s name**

At the end of January, I wrote to you asking for your help in a research project designed to investigate a possible association between some hormonal conditions and certain health problems. According to my records I have not received a reply to this letter and I am concerned that you may not have received it. I therefore enclose a copy of the original questionnaire and I would be very grateful if you could complete it and return to me in the postage-paid envelope provided.

As mentioned in the previous letter; your participation in this project which has been approved by Enfield and Haringey Health authority, local research ethics Committee, is voluntary and your care will not be affected if you decide not to take part. The information, which you give, will be treated confidentially; and will help us to improve treatment for women with fertility problems.

Your participation in this project will be much appreciated.

Yours Sincerely

P Hardiman MD FRCOG

Senior lecturer,

Appendix 3: Immunohistochemistry protocols

Full name: *Dewax
ID: 9100
Version: 8(current)
Type: Preparation
Created by: VBS
Creation time: 2/06/2008 3:36 PM
Facility: UCL-AD

Protocol: *D

Step	Reagent	Supplier: Vision BioSystems
1	*Bond Dewax Solution	

Step type: Reagent step | Incubation time: 30s | Temperature: 72 | Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
2	*Bond Dewax Solution	

Step type: Reagent step | Incubation time: 0s | Temperature: 72 | Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
3	*Bond Dewax Solution	

Step type: Reagent step | Incubation time: 0s | Temperature: Ambient | Dispense type: 150 µL

Step	Reagent	Supplier:
4	*Alcohol	

Step type: Wash step | Incubation time: 0s | Temperature: Ambient | Dispense type: 150 µL

Step	Reagent	Supplier:
5	*Alcohol	

Step type: Wash step | Incubation time: 0s | Temperature: Ambient | Dispense type: 150 µL

Step	Reagent	Supplier:
6	*Alcohol	

Step type: Wash step | Incubation time: 0s | Temperature: Ambient | Dispense type: Intermediate

Step	Reagent	Supplier: Vision BioSystems
7	*Bond Wash Solution	

Step type: Wash step | Incubation time: 0s | Temperature: Ambient | Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
8	*Bond Wash Solution	

Step type: Wash step | Incubation time: 0s | Temperature: Ambient | Dispense type: 150 µL

Protocol: *D

Full name: *Dewax
ID: 9100
Version: 8(current)
Type: Preparation
Created by: VBS
Creation time: 2/06/2008 3:36 PM
Facility: UCL-AD

Step	Reagent	*Supplier: Vision BioSystems*
9	*Bond Wash Solution	

Step type: Wash step Incubation time: 5min0s Temperature: Ambient Dispense type: Intermediate

Protocol: UCL O/C

Full name: UCLAD 15.8.8 O/C
ID: 10163
Version: 3(current)
Type: IHC staining
Created by: BondPowerUser
Creation time: 17/10/2008 1:44 PM
Facility: UCL-AD
Double-staining status: Single

Step	Reagent	Supplier: Vision BioSystems
1	*Peroxide Block	

Step type: Reagent step Incubation time: 5min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
2	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
3	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Open

Step	Reagent	Supplier: Vision BioSystems
4	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Not applicable
5	Primary	

Step type: Reagent step Incubation time: 15min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
6	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
7	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
8	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Protocol: UCL O/C

Full name: UCLAD 15,8,8 O/C
ID: 10163
Version: 3(current)
Type: IHC staining
Created by: BondPowerUser
Creation time: 17/10/2008 1:44 PM
Facility: UCL-AD
Double-staining status: Single

Step	Reagent	Supplier: Vision BioSystems
9	*Post Primary	

Step type: Reagent step Incubation time: 8min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
10	*Bond Wash Solution	

Step type: Wash step Incubation time: 2min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
11	*Bond Wash Solution	

Step type: Wash step Incubation time: 2min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
12	*Bond Wash Solution	

Step type: Wash step Incubation time: 2min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
13	*Polymer	

Step type: Reagent step Incubation time: 8min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
14	*Bond Wash Solution	

Step type: Wash step Incubation time: 2min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
15	*Bond Wash Solution	

Step type: Wash step Incubation time: 2min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
16	*Bond Wash Solution	

Step type: Wash step Incubation time: 2min0s Temperature: Ambient Dispense type: Closed

Protocol: UCL O/C

Full name: UCLAD 15,8,8 O/C
ID: 10163
Version: 3(current)
Type: IHC staining
Created by: BondPowerUser
Creation time: 17/10/2008 1:44 PM
Facility: UCL-AD
Double-staining status: Single

Step	Reagent	*Supplier:*
17	*Deionized Water	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier:*
18	Pre Mixed DAB	

Step type: Reagent step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier:*
19	Pre Mixed DAB	

Step type: Reagent step Incubation time: 10min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier:*
20	*Deionized Water	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier:*
21	*Deionized Water	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier:*
22	*Deionized Water	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier: Vision BioSystems*
23	*Bond DAB Enhancer	

Step type: Reagent step Incubation time: 5min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	*Supplier: Vision BioSystems*
24	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Protocol: UCL O/C

Full name: UCLAD 15.8.8 O/C
ID: 10163
Version: 3(current)
Type: IHC staining
Created by: BondPowerUser
Creation time: 17/10/2008 1:44 PM
Facility: UCL-AD
Double-staining status: Single

Step	Reagent	Supplier: Vision BioSystems
25	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
26	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
27	*Hematoxylin	

Step type: Reagent step Incubation time: 1min0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier:
28	*Deionized Water	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier: Vision BioSystems
29	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Step	Reagent	Supplier:
30	*Deionized Water	

Step type: Wash step Incubation time: 0s Temperature: Ambient Dispense type: Closed

Protocol: *H1(30)

ID: 9204
Version: 8(current)
Type: Pretreatment
Created by: VBS
Creation time: 2/06/2008 3:36 PM
Facility: UCL-AD

Step	Reagent	Supplier: Vision BioSystems
1	*Bond ER Solution 1	

Step type: Reagent step Incubation time: 0s Temperature: Ambient Dispense type: Open

Step	Reagent	Supplier: Vision BioSystems
2	*Bond ER Solution 1	

Step type: Reagent step Incubation time: 0s Temperature: Ambient Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
3	*Bond ER Solution 1	

Step type: Reagent step Incubation time: 30min0s Temperature: 100 Dispense type: Intermediate

Step	Reagent	Supplier: Vision BioSystems
4	*Bond ER Solution 1	

Step type: Reagent step Incubation time: 12min0s Temperature: Ambient Dispense type: Intermediate

Step	Reagent	Supplier: Vision BioSystems
5	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: 35 Dispense type: Open

Step	Reagent	Supplier: Vision BioSystems
6	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: 35 Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
7	*Bond Wash Solution	

Step type: Wash step Incubation time: 0s Temperature: 35 Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
8	*Bond Wash Solution	

Step type: Wash step Incubation time: 3min0s Temperature: Ambient Dispense type: Intermediate

Protocol: *H2(30)

Full name: *HIER 30 min with ER2
ID: 9205
Version: 8(current)
Type: Pretreatment
Created by: VBS
Creation time: 2/06/2008 3:36 PM
Facility: UCL-AD

Step	Reagent	Supplier: Vision BioSystems
1	*Bond ER Solution 2	

Step type: Reagent step | Incubation time: 0s | Temperature: Ambient | Dispense type: Open

Step	Reagent	Supplier: Vision BioSystems
2	*Bond ER Solution 2	

Step type: Reagent step | Incubation time: 0s | Temperature: Ambient | Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
3	*Bond ER Solution 2	

Step type: Reagent step | Incubation time: 30min0s | Temperature: 100 | Dispense type: Intermediate

Step	Reagent	Supplier: Vision BioSystems
4	*Bond ER Solution 2	

Step type: Reagent step | Incubation time: 12min0s | Temperature: Ambient | Dispense type: Intermediate

Step	Reagent	Supplier: Vision BioSystems
5	*Bond Wash Solution	

Step type: Wash step | Incubation time: 0s | Temperature: 35 | Dispense type: Open

Step	Reagent	Supplier: Vision BioSystems
6	*Bond Wash Solution	

Step type: Wash step | Incubation time: 0s | Temperature: 35 | Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
7	*Bond Wash Solution	

Step type: Wash step | Incubation time: 0s | Temperature: 35 | Dispense type: 150 µL

Step	Reagent	Supplier: Vision BioSystems
8	*Bond Wash Solution	

Step type: Wash step | Incubation time: 3min0s | Temperature: Ambient | Dispense type: Intermediate

Appendix 4: Immunohistochemistry statistics

	p53	Cyclin D	mib	bcl	TDS
N	1.94	1.53	1.18	96.21	1
N	31.63	0	15.21	89.24	1
N	0	0	0	44.02	3
N	30.23	0	9.67	72.61	1
N	0	0	94.02	100	6
N	0	0	1.68	0	3
N	2.4		0.03	0	2
N	20.4	0		100	5
N	0	0.68	0	100	3
N	21.14	0	2.12	100	1
N	12.44	3.25	0	98.24	1
N	38	1.01	0	100	1
N	14.12	21.97	0	82.52	1
N	11.11		0	100	1
N	13.33	0	0	100	4
Mean	13.1	2.2	8.9	78.9	2.3

PCOS	44.12	1.08	1.42	78.75	1
PCOS	19.23	3.78	5	98.75	1
PCOS	20.48	3.03	23.17	30.61	1
PCOS	77.44	0	9.16	100	5
PCOS	84.94	10.4	6.89	85.14	4
PCOS	0	14.67	0	92.63	1
PCOS	77.06	3.02	40.91	92.76	1
PCOS	7.35	0	8.2	76.4	1
PCOS	57.59	17.04	25.75	100	1
PCOS	28.26	20	4.35	96.07	1
PCOS	35.3	9.56	3.26	62.66	1
PCOS	19.04	2.7	0	92.45	0
PCOS	46.31	9.25	0	100	2
Mean	39.8	7.3	9.9	85.1	1.5
T test	0.002545	0.053672	0.897063	0.579269	0.225025

Mean percentage cells stained positive for cyclin D1, mib, p53 and bcl in the ovarian surface epithelium of PCOS subjects and controls

	p53	Cyclin D	mib	bcl	Clomid	TDS
N	1.94	1.53	1.18	96.21	NO	1
N	31.63	0	15.21	89.24	NO	1
N	0	0	94.02	100	NO	6
N	20.4	0		100	NO	5
N	0	0.68	0	100	NO	3
PCOS	20.48	3.03	23.17	30.61	NO	1
PCOS	77.44	0	9.16	100	NO	5
PCOS	57.59	17.04	25.75	100	NO	1
PCOS	28.26	20	4.35	96.07	NO	1
PCOS	35.3	9.56	3.26	62.66	NO	1
Mean	**27.3**	**5.2**	**19.6**	**87.5**		**2.5**
N	0	0	0	44.02	YES	3
N	30.23	0	9.67	72.61	YES	1
N	0	0	1.68	0	YES	3
N	2.4		0.03	0	YES	2
N	21.14	0	2.12	100	YES	1
N	12.44	3.25	0	98.24	YES	1
N	38	1.01	0	100	YES	1
N	14.12	21.97	0	82.52	YES	1
N	11.11		0	100	YES	1
N	13.33	0	0	100	YES	4
PCOS	44.12	1.08	1.42	78.75	YES	1
PCOS	19.23	3.78	5	98.75	YES	1
PCOS	84.94	10.4	6.89	85.14	YES	4
PCOS	0	14.67	0	92.63	YES	1
PCOS	77.06	3.02	40.91	92.76	YES	1
PCOS	7.35	0	8.2	76.4	YES	1
PCOS	19.04	2.7	0	92.45	YES	0
PCOS	46.31	9.25	0	100	YES	2
Mean	**24.5**	**4.4**	**4.2**	**78.6**		**1.6**
T test	0.778883	0.793027	0.052621	0.445574		0.152468

Cell cycle protein expression (% positive cells) and total dysplasia score in subject expose according to previous exposure to clomiphene citrate

	p53	Cyclin D	mib	bcl	hMG	TDS
N	31.63	0	15.21	89.24	NO	1
PCOS	20.48	3.03	23.17	30.61	NO	1
PCOS	77.44	0	9.16	100	NO	5
PCOS	57.59	17.04	25.75	100	NO	1
PCOS	28.26	20	4.35	96.07	NO	1
PCOS	35.3	9.56	3.26	62.66	NO	1
N	0	0	0	44.02	NO	3
N	0	0	1.68	0	NO	3
N	21.14	0	2.12	100	NO	1
N	12.44	3.25	0	98.24	NO	1
N	38	1.01	0	100	NO	1
N	14.12	21.97	0	82.52	NO	1
N	11.11		0	100	NO	1
N	13.33	0	0	100	NO	4
PCOS	44.12	1.08	1.42	78.75	NO	1
PCOS	19.23	3.78	5	98.75	NO	1
PCOS	84.94	10.4	6.89	85.14	NO	4
PCOS	77.06	3.02	40.91	92.76	NO	1
PCOS	19.04	2.7	0	92.45	NO	0
PCOS	46.31	9.25	0	100	NO	2
N	1.94	1.53	1.18	96.21	NO	1
N	0	0	94.02	100	NO	6
N	30.23	0	9.67	72.61	NO	1
Mean	**29.7**	**4.9**	**10.6**	**83.5**		**1.8**

N	2.4		0.03	0	YES	2
PCOS	0	14.67	0	92.63	YES	1
PCOS	7.35	0	8.2	76.4	YES	1
N	20.4	0		100	YES	5
N	0	0.68	0	100	YES	3
Mean	**15.6**	**1.6**	**28.6**	**64.0**		**2.7**

T test	0.049974	0.781109	0.43099	0.508516		0.466868

Cell cycle protein expression (% positive cells) and total dysplasia score in subject expose according to previous exposure to human menopausal gonadtrophins

	p53	Cyclin D	mib	bcl	GnRH	TDS
N	31.63	0	15.21	89.24	NO	1
PCOS	20.48	3.03	23.17	30.61	NO	1
PCOS	77.44	0	9.16	100	NO	5
PCOS	57.59	17.04	25.75	100	NO	1
PCOS	28.26	20	4.35	96.07	NO	1
PCOS	35.3	9.56	3.26	62.66	NO	1
N	0	0	0	44.02	NO	3
N	0	0	1.68	0	NO	3
N	2.4		0.03	0	NO	2
N	21.14	0	2.12	100	NO	1
N	12.44	3.25	0	98.24	NO	1
N	38	1.01	0	100	NO	1
N	14.12	21.97	0	82.52	NO	1
N	11.11		0	100	NO	1
N	13.33	0	0	100	NO	4
PCOS	44.12	1.08	1.42	78.75	NO	1
PCOS	19.23	3.78	5	98.75	NO	1
PCOS	84.94	10.4	6.89	85.14	NO	4
PCOS	0	14.67	0	92.63	NO	1
PCOS	77.06	3.02	40.91	92.76	NO	1
PCOS	7.35	0	8.2	76.4	NO	1
PCOS	19.04	2.7	0	92.45	NO	0
PCOS	46.31	9.25	0	100	NO	2
Mean	**28.8**	**5.8**	**6.4**	**79.1**		**1.7**

N	1.94	1.53	1.18	96.21	YES	1
N	0	0	94.02	100	YES	6
N	20.4	0		100	YES	5
N	0	0.68	0	100	YES	3
N	30.23	0	9.67	72.61	YES	1
Mean	**10.5**	**0.4**	**26.2**	**93.8**		**3.2**

T test	0.137539	0.117067	0.059857	0.314871		0.042168

Cell cycle protein expression (% positive cells) and total dysplasia score in subject expose according to previous exposure to gonadotrophin releasing hormone.

	p53	Cyclin D	mib	bcl	Any treatment	TDS
N	31.63	0	15.21	89.24	NO	1
PCOS	20.48	3.03	23.17	30.61	NO	1
PCOS	77.44	0	9.16	100	NO	5
PCOS	57.59	17.04	25.75	100	NO	1
PCOS	28.26	20	4.35	96.07	NO	1
PCOS	35.3	9.56	3.26	62.66	NO	1
Mean	**41.8**	**8.3**	**13.5**	**79.8**		**1.7**

N	0	0	0	44.02	YES	3
N	0	0	1.68	0	YES	3
N	21.14	0	2.12	100	YES	1
N	12.44	3.25	0	98.24	YES	1
N	38	1.01	0	100	YES	1
N	14.12	21.97	0	82.52	YES	1
N	11.11		0	100	YES	1
N	13.33	0	0	100	YES	4
PCOS	44.12	1.08	1.42	78.75	YES	1
PCOS	19.23	3.78	5	98.75	YES	1
PCOS	84.94	10.4	6.89	85.14	YES	4
PCOS	77.06	3.02	40.91	92.76	YES	1
PCOS	19.04	2.7	0	92.45	YES	0
PCOS	46.31	9.25	0	100	YES	2
N	1.94	1.53	1.18	96.21	YES	1
N	0	0	94.02	100	YES	6
N	30.23	0	9.67	72.61	YES	1
N	2.4		0.03	0	YES	2
PCOS	0	14.67	0	92.63	YES	1
PCOS	7.35	0	8.2	76.4	YES	1
N	20.4	0		100	YES	5
N	0	0.68	0	100	YES	3
Mean	**22.1**	**3.9**	**8.6**	**84.1**		**2.0**

T test	**0.067483**	**0.147673**	**0.566064**	**0.853533**		**0.651773**

Cell cycle protein expression (% positive cells) and total dysplasia score in subject expose according to previously exposure to any ovulation induction treatment

Appendix5: Ethical Approval

Royal Free Hampstead NHS
NHS Trust
Clinical Governance Support Centre
Royal Free Hospital
Pond Street
London NW3 2QG
Tel:020 7830 2816
Fax:020 7830 2233
zoe.spywee@rfh.nthames.nhs.uk

27 June 2002

Dr Essam El-Mahdi
Dept of Obstetrics and Gynaecology
Royal Free Hampstead NHS Trust
Pond Street
Hampstead
London
NW3 2QG

Dear Dr El-Mahdi

Gene expression in polycystic ovaries and epithelial ovarian cancer

Project ID: **5880** **(Please quote on ALL correspondence)**
Ethics ID: **5880**

I am pleased to inform you that following submission of your R&D registration form your project has been approved by the R&D department. This letter ensures that you and the researchers working with you holding trust contracts are indemnified by the trust, under department of health HSG (96) 48, for non commercial research only. This means you can now proceed with your project.

In addition to ensuring your study complies with good clinical research practice as outlined in the ICH GCP guidelines we require the following:

Patient contact - only trained researchers holding a trust contract (honorary or full) are allowed to make contact with patients.

Informed Consent – Only the lead researcher or other trained researcher should obtain signed consent and in accordance with the ethics committee requirements. The original signed consent form should be kept on file and informed consent will be monitored by the trust at intervals and you will be required to provide the relevant documentation.

Confidentiality - All those involved in the study appreciate the importance of maintaining confidentiality and that they comply with the Data Protection Act 1988.

Amendments – The R&D office needs to be kept informed of any changes to the project for example regarding patient recruitment, funding, personnel changes or your project status. If changes are made to the protocol they will need to be considered by the ethics committee.

Progress report – A progress report will need to be completed annually.

Publications – Any publication resulting from your project needs to be reported to the R&D office. This

Royal Free Hampstead NHS
NHS Trust

Clinical Governance Support Centre
Royal Free Hospital
Pond Street
London NW3 2QG
Tel:020 7830 2816
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zoe.spyvee@rfh.nthames.nhs.uk

is vital in ensuring the quality and output of research across the trust.

NHS Funding - If the project uses any trust resources any publication must include the following statement.
'This work was undertaken by [investigator's name] with the Royal Free Hampstead NHS Trust who received [funding or a proportion of its funding] from the NHS Executive; the views expressed in this publication are those of the authors and not necessarily those of the Trust or NHS Executive.'

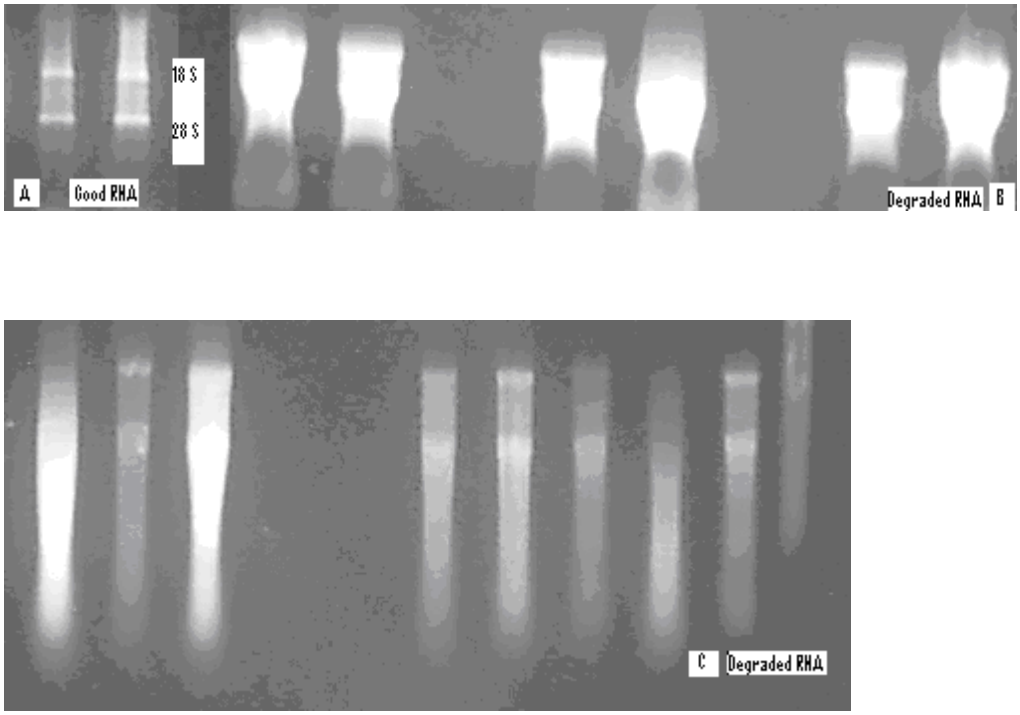
This approval is subject to your consent for information to be extracted from your project for inclusion in NHS project registration/ management databases and, where appropriate, the National Research Register and the UCL Clinical Research Network register.

Should you have any queries please contact the R&D office quoting the ID number.

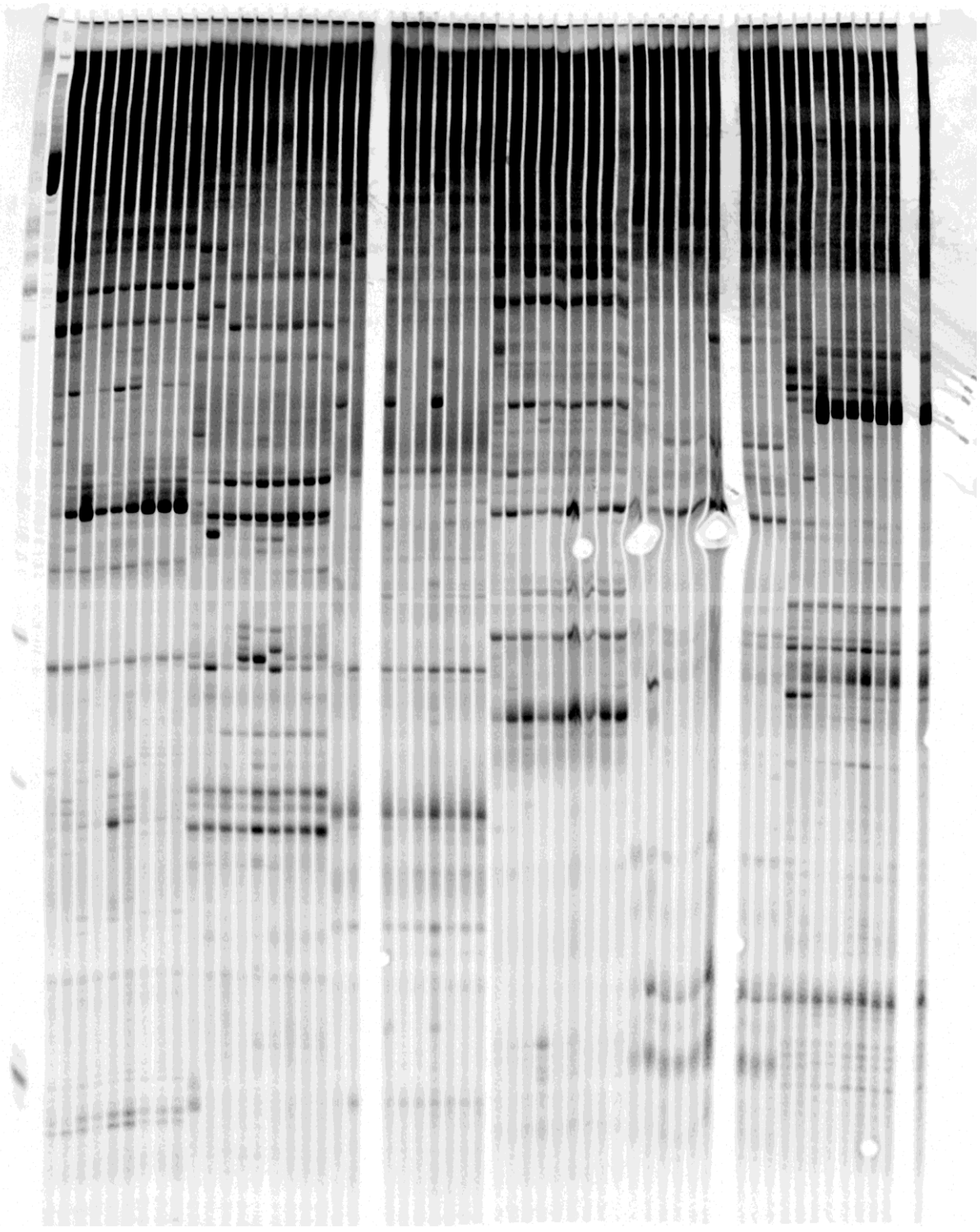
Yours sincerely,

[redacted]
Zoe Spyvee
Research and Development Officer

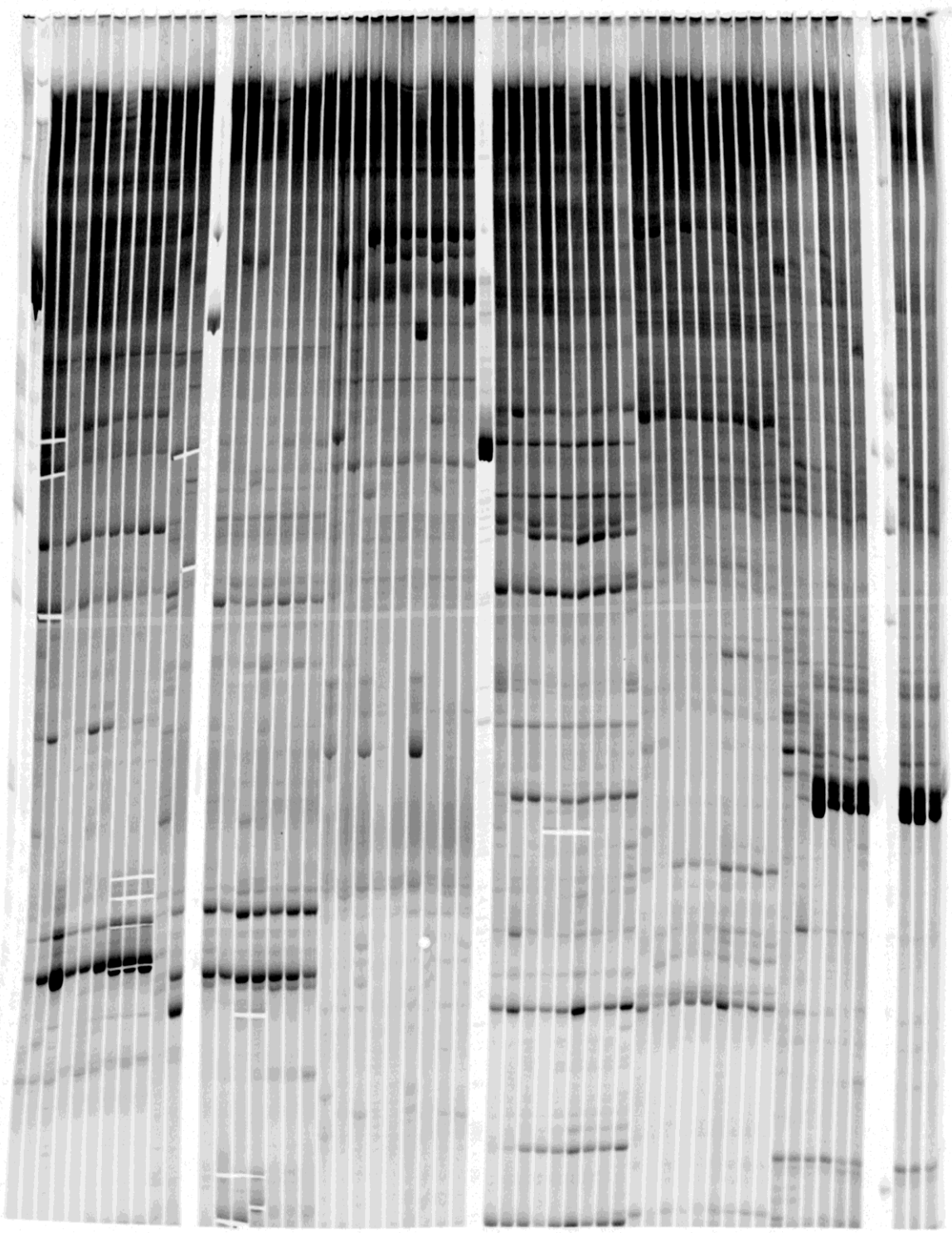
Appendix 6: Differential Display



Extracted RNA: (A) intact RNA, S18 and 28S are clearly seen (B&C) degraded RNA.



Polyacrylamide gel electrophoresis for DD PCR (after 18 exposure). Upstream primers EPA 1 and 2. Arbitrary primer ET 12. After 18 hours exposure. Showing where differentially expressed bands



Polyacrylamide gel electrophoresis for DD PCR (after 18 exposure). Upstream primers EPA 1 and 2. Arbitrary primer ET 12. After 18 hours exposure. Showing where differentially expressed bands are extracted.

CODE	RNA	DiffD
N1	NO	
N2	NO	
N3	NO	
N4	NO	
N5	YES	YES
N6	NO	
N7	YES	YES
N8	NO	
N9	YES	YES
N10	NO	
N11	YES	
N12	YES	
N14	YES	
N15	NO	
N17	YES	
N18	YES	
N20	NO	
N21	NO	
N23	NO	
C1	NO	
C2	YES	
C3	NO	
C4	NO	
C5	YES	YES
C6	YES	YES
C7	NO	
C8	NO	
C9	YES	YES
C11	YES	
C12	YES	
C13	YES	
C14	NO	
C15	YES	
C16	YES	
C18	YES	
C19	YES	
P5	YES	
P5	YES	
P1	YES	YES
P2	YES	YES
P2/B	YES	YES
P4	NO	
P6	NO	
P7	NOT USED	

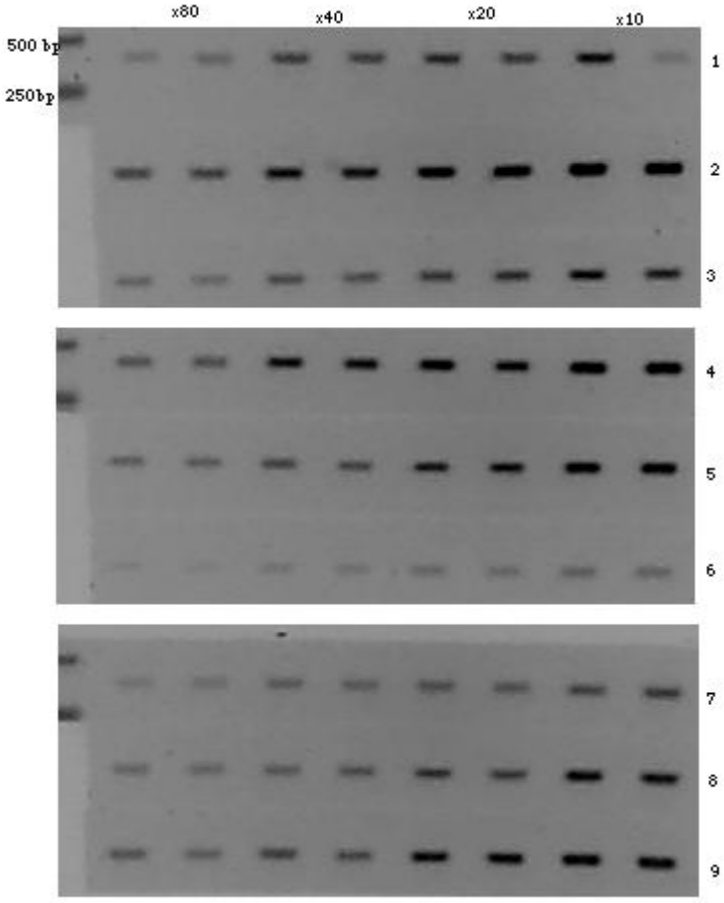
Ovarian samples used in the study ;(N) Normal, (C) cancer, (P) PCOS

			Un purified			Purified	
CODE	Date.pro	ul vol	ug/ul	tot ug	µl vol	µg/µl	total µg
C2	13.02.03	27	3.08	86.24			
C5	19.11.02				178	6.92	1231.76
C11	13.02.03	45	12.64	44.82			
C12	13.02.03	43	1.06	12.92			
C15	13.02.03	77	9.57	746.4			
C18	13.02.03	77	9.269	723			
C6	19.11.02				402	2.42	972.84
C9	02.12.02				8.46	1.8	15.228
C16	10.03.03	26	9.560	248.56			
C13	10.03.03	26	12.36	321.4			
C20	13.02.03	17	0.76	12.92			
C19	10.03.03	26	1.18	30.68			
N5	19.11.02				20	5.04	100.8
N9	02.12.02				4.56	1.2	5.472
N14	10.03.03	26	2.02	52.52			
N18	13.02.03	43.8	1.42	62.2			
N11	08.02.03	22.2	1.02	55.68			
N12	13.02.03	43.1	1.04	44.82			
N17	13.02.03	43.5	1.28	55.68			
N7	02.12.02	24	3.24	44.82	9.5	2.98	28.31
N21	10.03.03	26	2.46	63.96			
P1	02.12.02	14	1.72	12.92	9	0.96	8.64
P2	02.12.02	19	1.6	55.68	11.35	1.74	19.749
P2/B	02.12.02	24	2.96	62.2	8.8	2.5	22
P5	10.03.03	26	4.72	122.7			
P5/B	10.03.03	26	6.02	156.5			

RNA extracted quantification. (N) Normal, (C) cancer, (P) PCOS

Spectrophotometeric determination of RNA concentration

	µl vol	RNA	diluent	diln x	280 nm	260 nm	280 nm	260 nm	260/280	µg/µl	260/280	µg/µl	total µg
N3	**9**	**1**	**999**	**1000**	**0.069**	**0.088**	**0.069**	**0.088**	**1.28**	**3.52**	**1.48**	**3.440**	**30.96**
		1	**999**	**1000**	**0.050**	**0.084**	**0.050**	**0.084**	**1.68**	**3.36**			
N5	**22**	**1**	**999**	**1000**	**0.102**	**0.177**	**0.102**	**0.177**	**1.74**	**7.08**	**1.31**	**5.900**	**129.80**
		1	**999**	**1000**	**0.134**	**0.118**	**0.134**	**0.118**	**0.88**	**4.72**			
N9	**9**	**1**	**999**	**1000**	**0.035**	**0.056**	**0.035**	**0.056**	**1.60**	**2.24**	**1.64**	**2.640**	**23.76**
		1	**999**	**1000**	**0.045**	**0.076**	**0.045**	**0.076**	**1.69**	**3.04**			
C4/A	**9**	**1**	**999**	**1000**	**0.150**	**0.207**	**0.150**	**0.207**	**1.38**	**8.28**	**1.37**	**8.120**	**73.08**
		1	**999**	**1000**	**0.147**	**0.199**	**0.147**	**0.199**	**1.35**	**7.96**			
C6/A	**44**	**1**	**999**	**1000**	**0.232**	**0.344**	**0.232**	**0.344**	**1.48**	**13.76**	**1.63**	**13.620**	**599.28**
		1	**999**	**1000**	**0.189**	**0.337**	**0.189**	**0.337**	**1.78**	**13.48**			
C4/B	**24**	**1**	**999**	**1000**	**0.042**	**0.060**	**0.042**	**0.060**	**1.43**	**2.40**	**1.43**	**2.320**	**55.68**
		1	**999**	**1000**	**0.039**	**0.056**	**0.039**	**0.056**	**1.44**	**2.24**			
C5	**44**	**1**	**999**	**1000**	**0.141**	**0.208**	**0.141**	**0.208**	**1.48**	**8.32**	**1.47**	**8.540**	**375.76**
		1	**999**	**1000**	**0.129**	**0.219**	**0.150**	**0.219**	**1.46**	**8.76**			
C6/B	**94**	**1**	**999**	**1000**	**0.152**	**0.217**	**0.152**	**0.217**	**1.43**	**8.68**	**1.44**	**8.800**	**827.20**
		1	**999**	**1000**	**0.153**	**0.223**	**0.153**	**0.223**	**1.46**	**8.92**			
P1	**9**	**1**	**999**	**1000**	**0.035**	**0.043**	**0.035**	**0.043**	**1.23**	**1.72**	**1.22**	**2.580**	**23.22**
		1	**999**	**1000**	**0.071**	**0.086**	**0.071**	**0.086**	**1.21**	**3.44**			
P2	**24**	**1**	**999**	**1000**	**0.071**	**0.086**	**0.071**	**0.086**	**1.21**	**3.44**	**1.26**	**3.580**	**85.92**
		1	**999**	**1000**	**0.071**	**0.09**	**0.071**	**0.093**	**1.31**	**3.72**			
P2/B	**24**	**1**	**999**	**1000**	**0.181**	**0.266**	**0.181**	**0.266**	**1.47**	**10.64**	**1.45**	**9.840**	**236.16**
		1	**999**	**1000**	**0.159**	**0.226**	**0.159**	**0.226**	**1.42**	**9.04**			
P4	**40**	**1**	**999**	**1000**	**0.022**	**0.026**	**0.022**	**0.026**	**1.18**	**1.04**	**1.18**	**1.200**	**48.00**
		1	**999**	**1000**	**0.029**	**0.034**	**0.029**	**0.034**	**1.17**	**1.36**			
N5\B	**8**	**1**	**999**	**1000**	**0.123**	**0.18**	**0.123**	**0.177**	**1.44**	**7.08**	**1.16**	**5.900**	**47.20**
		1	**999**		**0.134**	**0.118**	**0.134**	**0.118**	**0.88**	**4.72**			



PCR of cDNA used in DD: (1-3) Cancer (4-6) Normal and (7-9) PCOS using 1.5µl/15µl reaction at x10, x20, x40 and x80 dilution

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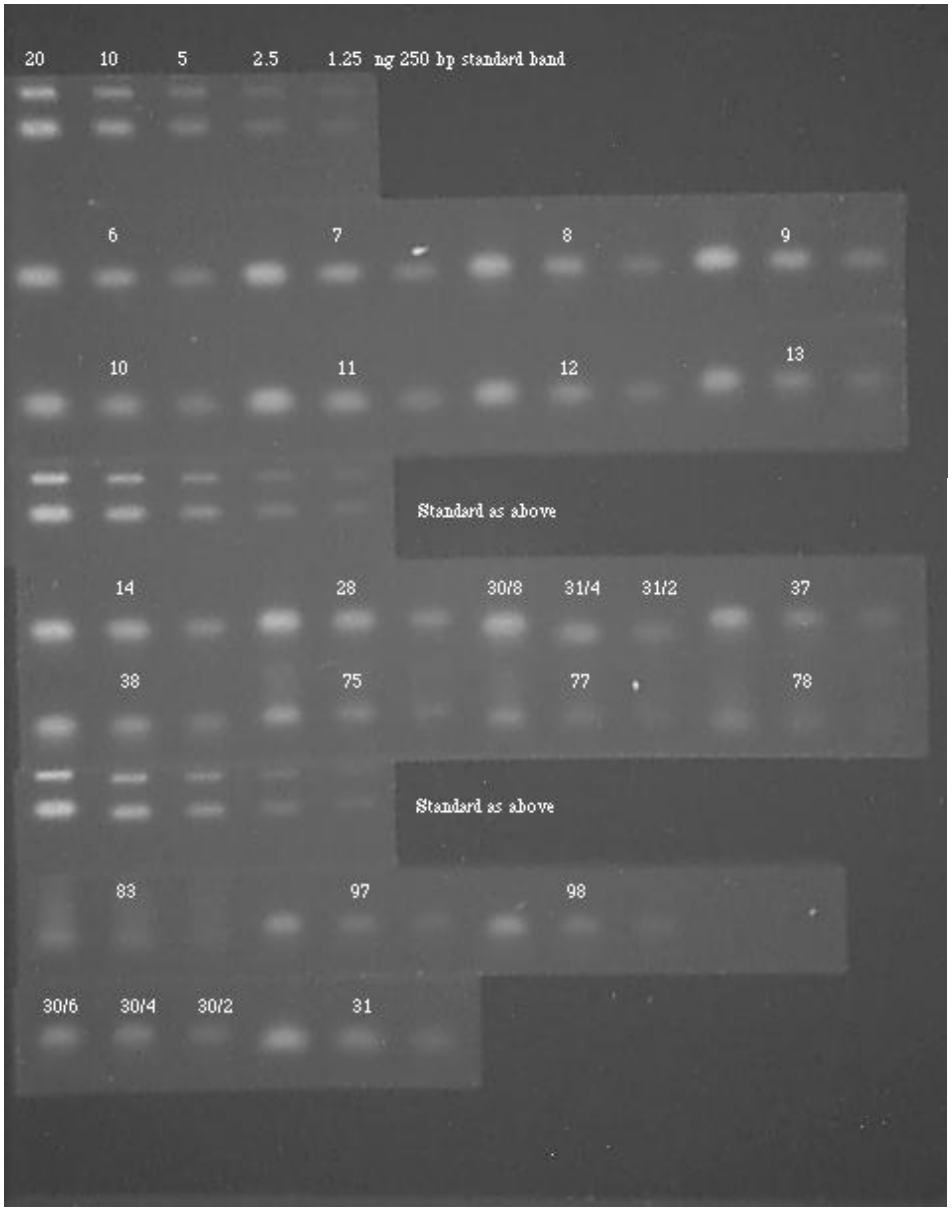
PCR NO.	BAND	effect	PCR NO.	BAND	effect	PCR NO.	BAND	effect
1	C1	C>N	62	C31	1C>N	13	P8	P<N
2	C2	C>N	71	C32	2C>N	14	P9	P<N
3	C3	C>N	72	C33	2C>N	32	P10	P<N
4	C4	C>N	73	C34	1C>N	33	P11	P<N
5	C5	1C>N	75	C35	3C<N	34	P12	P<N
21	C6	1C>N	76	C36	2C>N	35	P13	P<N
22	C7	2C>N	77	C37	1C>N	36	P14	P>N
23	C8	1C>N	79	C38	2C>N	37	P15	P<N
24	C9	1C>N	80	C39	1C>N	38	P16	P<N
25	C10	1C>N	81	C40	2C>N	43	P17	P>N
26	C11	1C>N	82	C41	2C>N	44	P18	P<N
27	C12	1C>N	83	C42	1C>N	45	P19	P<N
28	C13	2C>N	84	C43	1C>N	49	P19	P<N
29	C14	2C>N	86	C44	3C>N	50	P20	P<N
30	C15	1C>N	88	C45	2C>N	51	P21	P<N
31	C16	1C>N	89	C46	2C>N	55	P22	P>N
41	C17	1C>N	90	C47	1C>N	56	P23	P<N
42	C18	1C>N	91	C48	2C>N	63	P24	P>N
46	C20	1C>N	95	C49	1C>N	74	P25	P>N
47	C21	1C>N	96	C50	1C>N	78	P26	P<N
48	C22	1C>N	6	P1	P>N	85	P27	P>N
52	C23	2C>N	7	P2	P>N	87	P28	P>N
53	C24	1C>N	8	P3	P>N	92	P29	P<N
54	C25	1C>N	9	P4	P>N	93	P30	P<N
57	C26	1C>N	10	P5	P<N	94	P31	P>N
58	C27	2C>N	11	P6	P<N	97	P32	P<N
59	C28	1C>N	12	P7	P<N	98	P33	P<N
60	C29	1C>N						
61	C30	1C>N						

Summary of gene expression of the DDRT-PCR products



Quantitative gel electrophoresis of re purified PCR products which shows only single bands with few primer dimmers. All run at 8, 4, and 2 µl.
Cancer C, P Polycystic ovary, N Normal ovaries.

sample	aka	µl load	µl DNA	peak	peak/µl	fmol/µl	av	sd	cv	corr. for 200 bp vs 250 bp stan	DNA µl	H2Oµl	fmol/6µl
6	P1	8	2	75.74	37.87	47.47	56.99	10.8	18.9	71.24	3.0	33.0	35.6
		4	1	43.76	43.76	54.85							
		2	0.5	27.39	54.78	68.66							
7	P2	8	2	70.87	35.44	44.42	55.27	11.2	20.2	69.08	3.0	33.0	34.5
		4	1	43.62	43.62	54.67							
		2	0.5	26.61	53.22	66.71							
8	P3	8	2	67.90	33.95	42.55	47.51	4.4	9.4	59.39	3.0	33.0	29.7
		4	1	40.80	40.80	51.14							
		2	0.5	19.48	38.96	48.83							
9	P4	8	2	80.61	40.31	50.52	56.18	5.0	8.9	70.23	3.0	33.0	35.1
		4	1	47.86	47.86	59.99							
		2	0.5	23.15	46.30	58.03							
10	P5	8	2	74.75	37.38	46.85	50.71	3.7	7.2	63.39	3.0	33.0	31.7
		4	1	43.20	43.20	54.15							
		2	0.5	20.40	40.80	51.14							
11	P6	8	2	71.72	35.86	44.95	51.40	5.6	10.9	64.25	3.0	33.0	32.1
		4	1	43.41	43.41	54.41							
		2	0.5	21.88	43.76	54.85							
12	P7	8	2	55.76	27.88	34.95	36.27	4.0	11.1	45.34	4.5	31.5	34.0
		4	1	32.54	32.54	40.79							
		2	0.5	13.20	26.40	33.09							

Band intensity quantitation for single PCR products after purification (continued).

13	**P8**	**8**	**2**	**55.13**	**27.57**	**34.55**	**32.81**	**3.3**	**10.0**	**41.02**	**4.5**	**31.5**	**30.8**
		4	**1**	**27.81**	**27.81**	**34.86**							
		2	**0.5**	**11.58**	**23.16**	**29.03**							
14	**P9**	**8**	**2**	**80.12**	**40.06**	**50.21**	**57.60**	**6.4**	**11.1**	**71.99**	**3.0**	**33.0**	**36.0**
		4	**1**	**48.93**	**48.93**	**61.33**							
		2	**0.5**	**24.43**	**48.86**	**61.24**							
28	**C13**	**8**	**2**	**68.67**	**34.34**	**43.04**	**47.79**	**4.3**	**8.9**	**59.74**	**3.0**	**33.0**	**29.9**
		4	**1**	**40.87**	**40.87**	**51.23**							
		2	**0.5**	**19.59**	**39.18**	**49.11**							
30	**C15**	**8**	**2**	**54.92**	**27.46**	**34.42**							
31	**C16**	**4**	**1**	**28.96**	**28.96**	**36.30**							
31	**C16**	**2**	**0.5**	**17.36**	**34.72**	**43.52**							
37	**P15**	**8**	**2**	**47.86**	**23.93**	**29.99**	**26.59**	**3.0**	**11.2**	**33.24**	**6.0**	**30.0**	**33.2**
		4	**1**	**20.13**	**20.13**	**25.23**							
		2	**0.5**	**9.79**	**19.58**	**24.54**							
38	**P16**	**8**	**2**	**64.22**	**32.11**	**40.25**	**40.57**	**0.9**	**2.2**	**50.71**	**4.5**	**31.5**	**38.0**
		4	**1**	**31.80**	**31.80**	**39.86**							
		2	**0.5**	**16.59**	**33.18**	**41.59**							
75	**C35**	**8**	**2**	**30.11**	**15.06**	**18.87**	**18.93**	**0.8**	**4.3**	**23.66**	**9.0**	**27.0**	**35.5**
		4	**1**	**14.47**	**14.47**	**18.14**							
		2	**0.5**	**7.89**	**15.78**	**19.78**							

Band intensity quantitation for single PCR products after purification (continued).

77	**C37**	**8**	**2**	**14.90**	**7.45**	**9.34**	**10.39**	**1.0**	**9.6**	**12.98**	**18.0**	**18.0**	**39.0**
		4	**1**	**8.37**	**8.37**	**10.49**							
		2	**0.5**	**4.52**	**9.04**	**11.33**							
78	**P26**	**8**	**2**	**13.06**	**6.53**	**8.18**	**8.45**	**0.8**	**8.9**	**10.57**	**18.0**	**18.0**	**31.7**
		4	**1**	**7.42**	**7.42**	**9.30**							
		2	**0.5**	**3.14**	**6.28**	**7.87**							
83	**C42**	**8**	**2**	**33.77**	**16.89**	**21.16**	**19.95**	**1.1**	**5.3**	**24.94**	**9.0**	**27.0**	**37.4**
		4	**1**	**15.37**	**15.37**	**19.27**							
		2	**0.5**	**7.75**	**15.50**	**19.43**							
97	**P32**	**8**	**2**	**34.88**	**17.44**	**21.86**	**19.62**	**2.0**	**10.2**	**24.52**	**9.0**	**27.0**	**36.8**
		4	**1**	**15.11**	**15.11**	**18.94**							
		2	**0.5**	**7.20**	**14.40**	**18.05**							
98	**P33**	**8**	**2**	**37.91**	**18.96**	**23.76**	**21.59**	**2.6**	**11.8**	**26.99**	**9.0**	**27.0**	**40.5**
		4	**1**	**14.98**	**14.98**	**18.78**							
		2	**0.5**	**8.87**	**17.74**	**22.24**							
30	**C15**	**6**	**1.5**	**44.72**	**29.81**	**37.37**	**37.34**	**3.4**	**9.1**	**46.67**	**4.5**	**31.5**	**35.0**
		4	**1**	**33.60**	**33.60**	**42.12**							
		2	**0.5**	**14.14**	**28.28**	**35.45**							
31	**C16**	**8**	**2**	**64.35**	**32.18**	**40.33**	**38.60**	**3.8**	**9.8**	**48.25**	**4.5**	**31.5**	**36.2**
		4	**1**	**31.23**	**31.23**	**39.14**							
		2	**0.5**	**13.44**	**26.88**	**33.69**							

(continued) Band intensity quantitation for single PCR products after purification.

			corr							3x12 µl sequencing rxn (36 µl)			
sample	aka	fmol/µl	fmol/µl	DNA µl	H2Oµl	fmol/6µl		Up	Lp	DNA µl	H2Oµl	fmol/6µl	
6	P1	56.99	71.24	3.00	33.00	35.62114829	*P+	1	A	3.0	33.0	35.6	
7	P2	55.27	69.08	3.00	33.00	34.541363	***P+	1	A	3.0	33.0	34.5	
8	P3	47.51	59.39	3.00	33	29.69343	*P+	1	A	3.0	33.0	29.7	
9	P4	56.18	70.23	3.00	33	35.11324466	*P+	1	A	3.0	33.0	35.1	
10	P5	50.71	63.39	3.00	33.00	31.6950141	**P-	1	C	3.0	33.0	31.7	
11	P6	51.40	64.25	3.00	33.00	32.12718916	**P-	1	C	3.0	33.0	32.1	
12	P7	36.27	45.34	4.50	31.50	34.00734654	**P-	1	C	4.5	31.5	34.0	
13	P8	32.81	41.02	4.50	31.50	30.76211657	*P-	1	C	4.5	31.5	30.8	=P16
14	P9	57.60	71.99	3.00	33.00	35.99718	**P-	2	A	3.0	33.0	36.0	
28	C13	47.79	59.74	3.00	33.00	29.86969465	***C+(2)	2	C	3.0	33.0	29.9	
30	C15	37.33783643	46.6723	4.50	31.50	35.00	*C+(1)	2	C	4.5	31.5	35.0	
31	C16	38.59712406	48.2464	4.50	31.50	36.18	***C+(1)	2	C	4.5	31.5	36.2	=P9
37	P15	26.58960343	33.237	6.00	30.00	33.24	*P-	2	C	6.0	30.0	33.2	
38	P16	40.56543992	50.7068	4.50	31.50	38.03	**P-	2	A	4.5	31.5	38.0	
75	C35	18.92900665	23.66	9.00	27.00	35.49	***C-	5	C	9.0	27.0	35.5	tumour suppressor?
77	C37	10.38682497	12.9835	18.00	18.00	38.95	*C+(1)	5	C	18.0	18.0	39.0	
78	P26	8.452351938	10.5654	18.00	18.00	31.70	*P-	5	C	18.0	18.0	31.7	
83	C42	19.95264789	24.9408	9.00	27.00	37.41	***C+(3),*P+	5	G	9.0	27.0	37.4	
97	P32	19.61630862	24.52	9.00	27.00	36.78	*P+	6	G	9.0	27.0	36.8	
98	P33	21.59047387	26.9881	9.00	27.00	40.48	**P-	6	G	9.0	27.0	40.5	

Differential displayfragments characterisation

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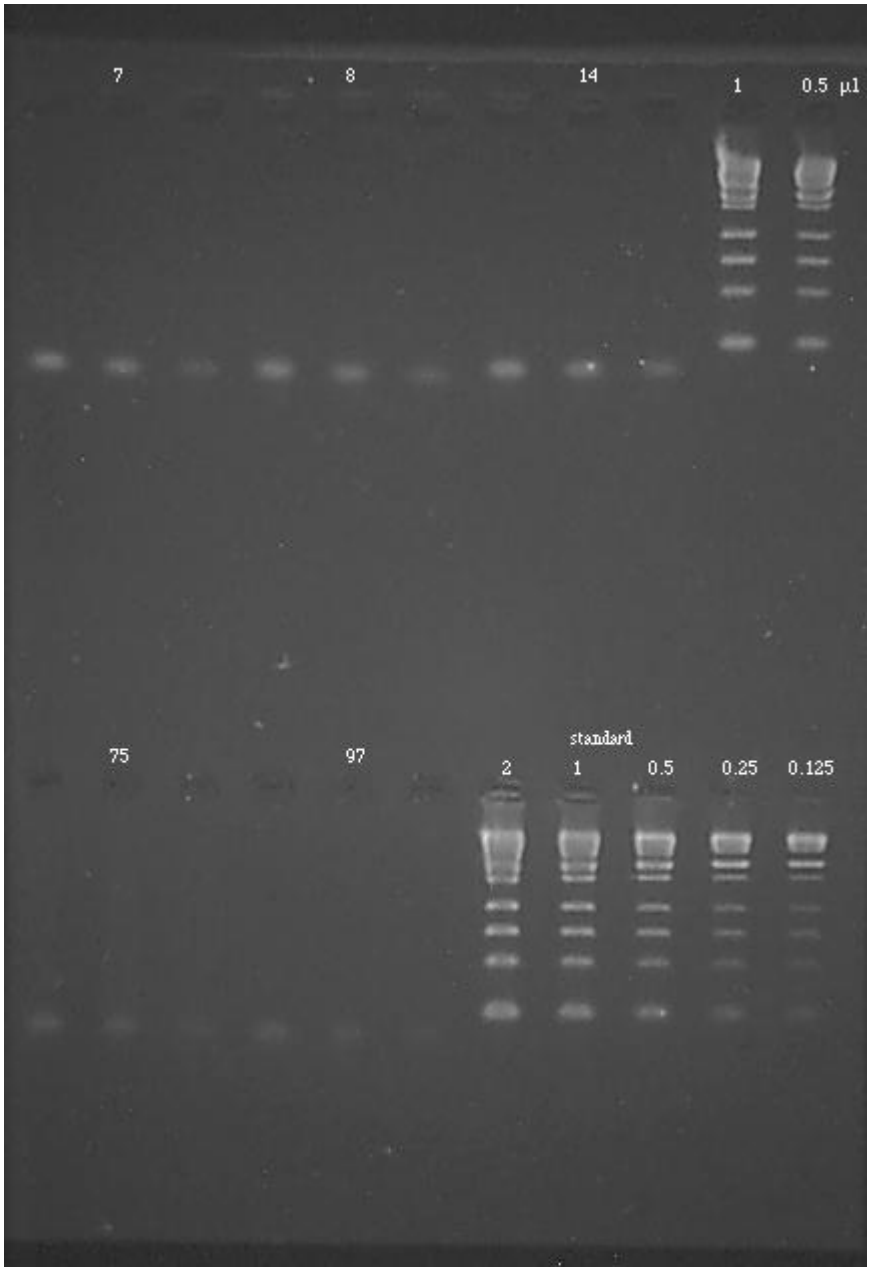
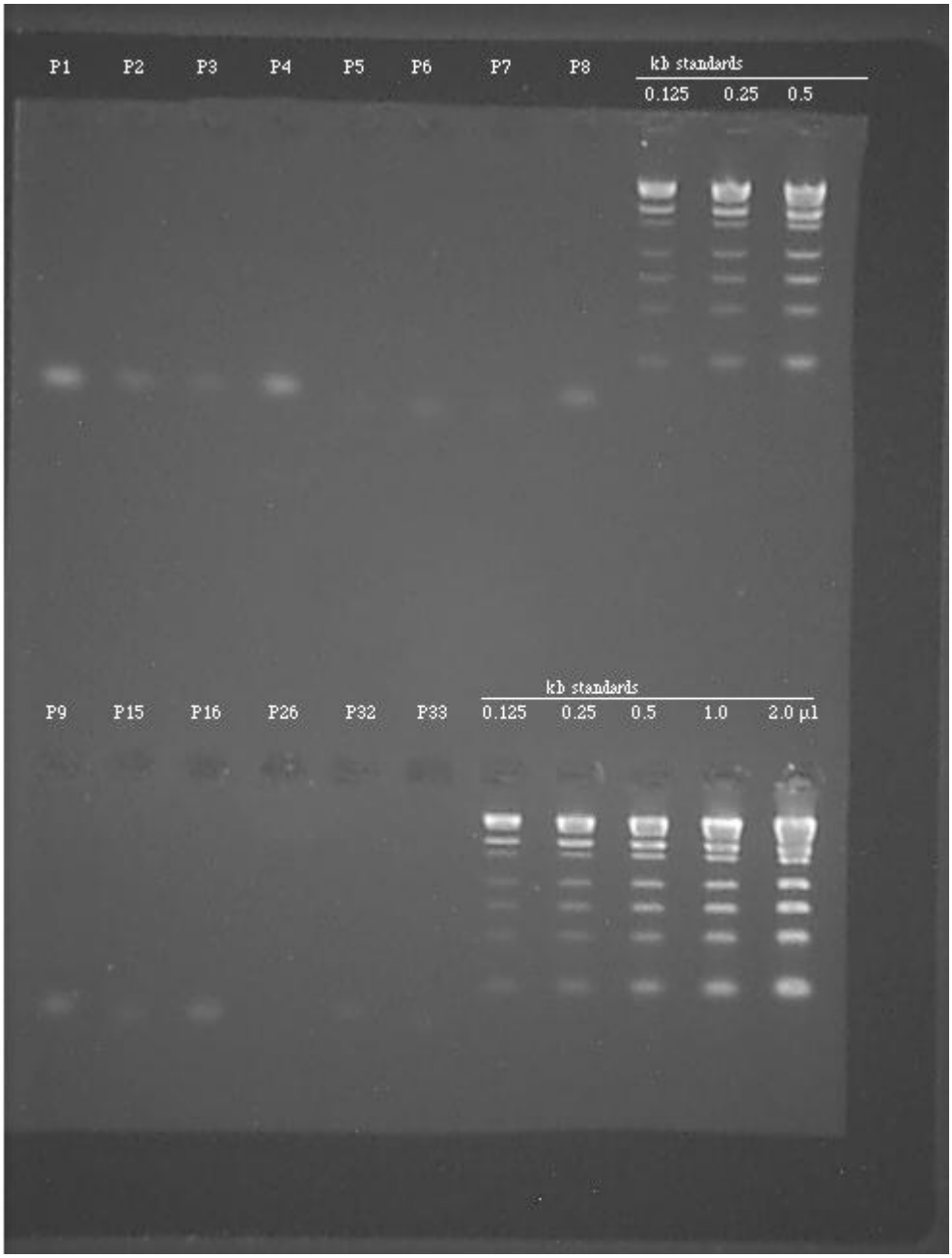
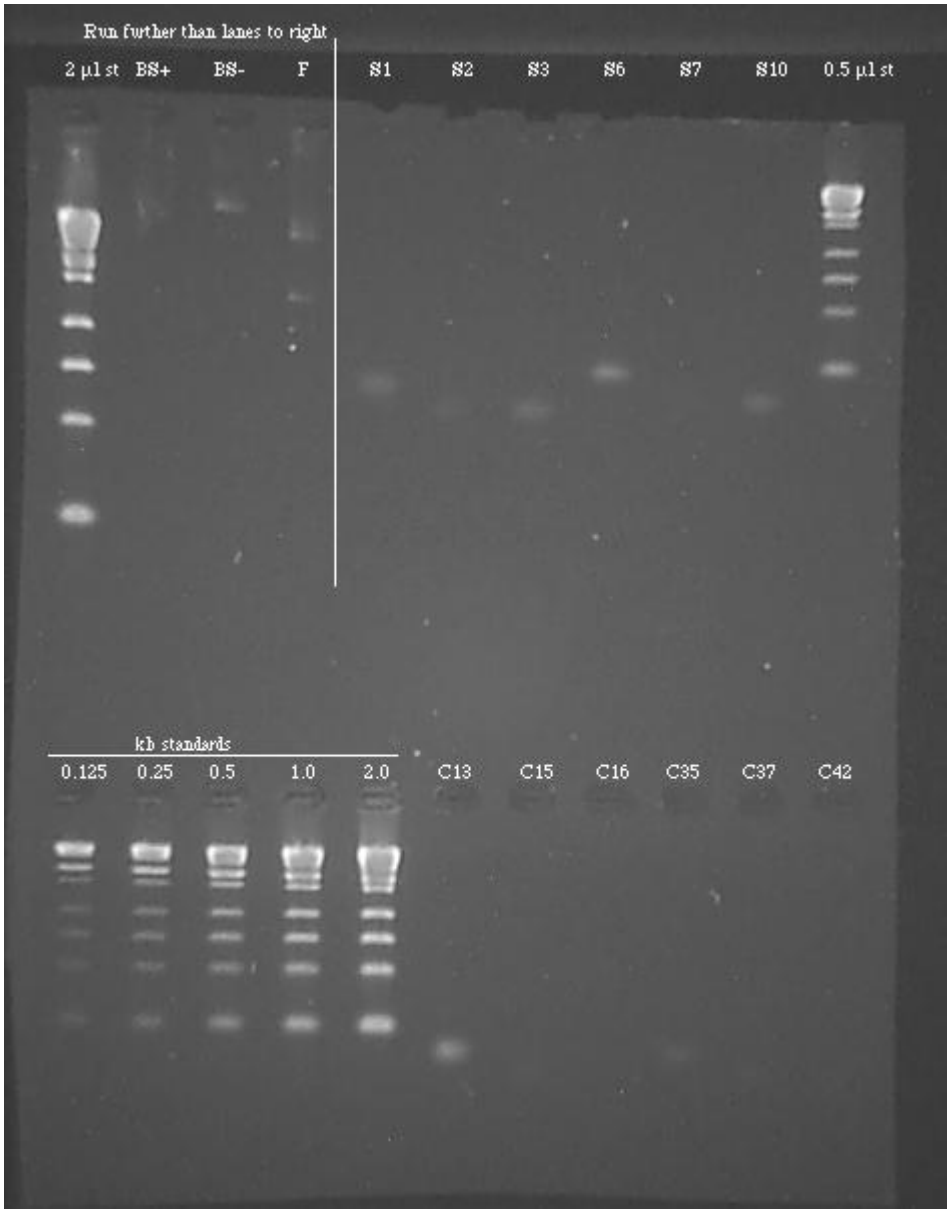


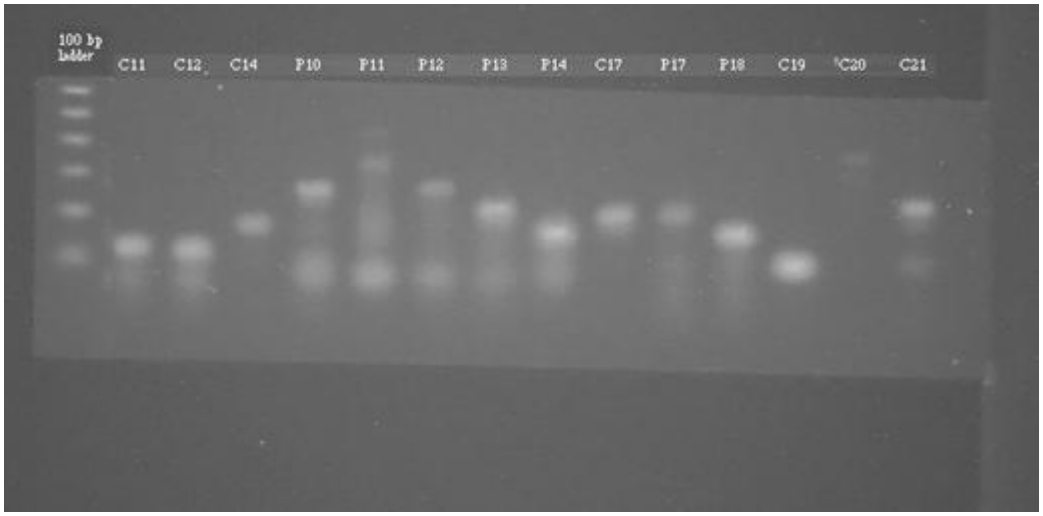
DNA quantitation in Hybaid column purified samples (30µl elutes) each loaded at 2.25, 1.5 and 0.75 µl.
7=P2 8=P3 14=P9 75=C35 97=P32. P(PCOS), C(Cancer)

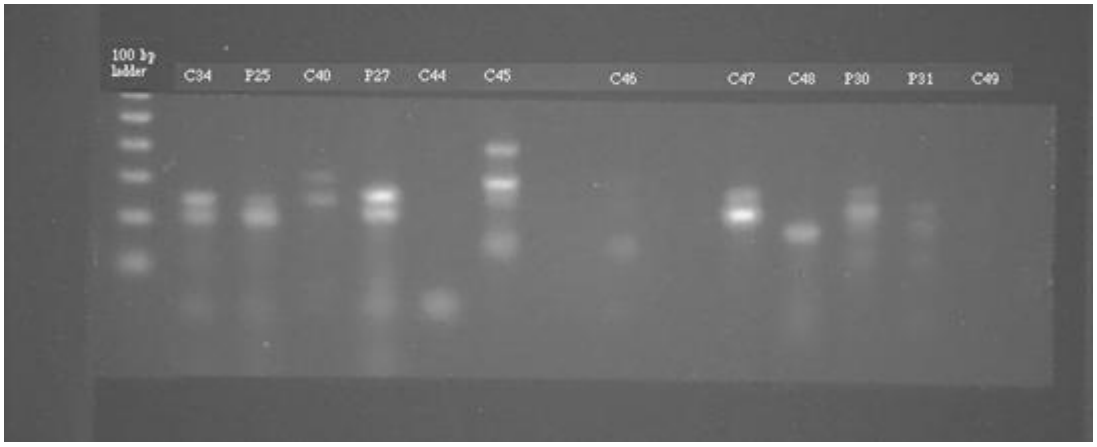


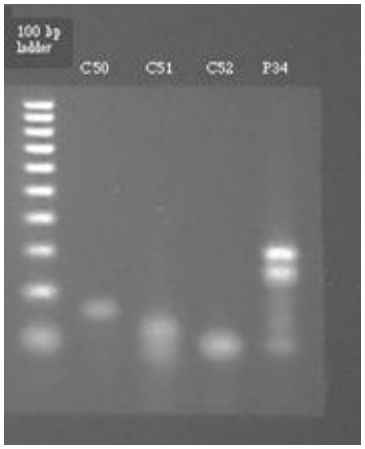
2% agrose electrophoresis of hybaid purified *EcoR*I and *Xho*I cleaved differentially displayed fragments (P1-P33).



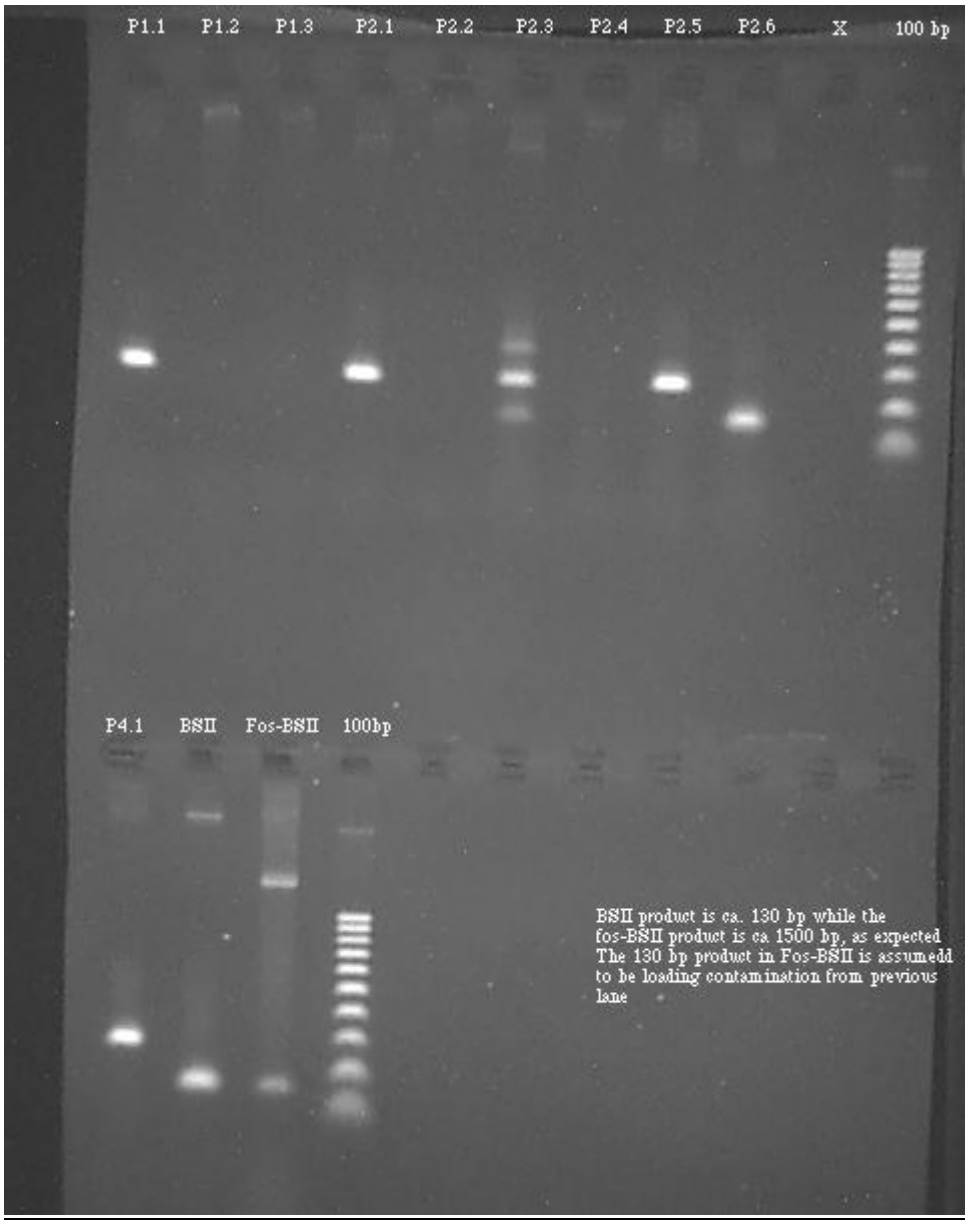
2% agrose electrophoresis of hybaid purified *Eco*RI and *Xho*I cleaved differentially displayed fragments (C13-C42). S (Standred).



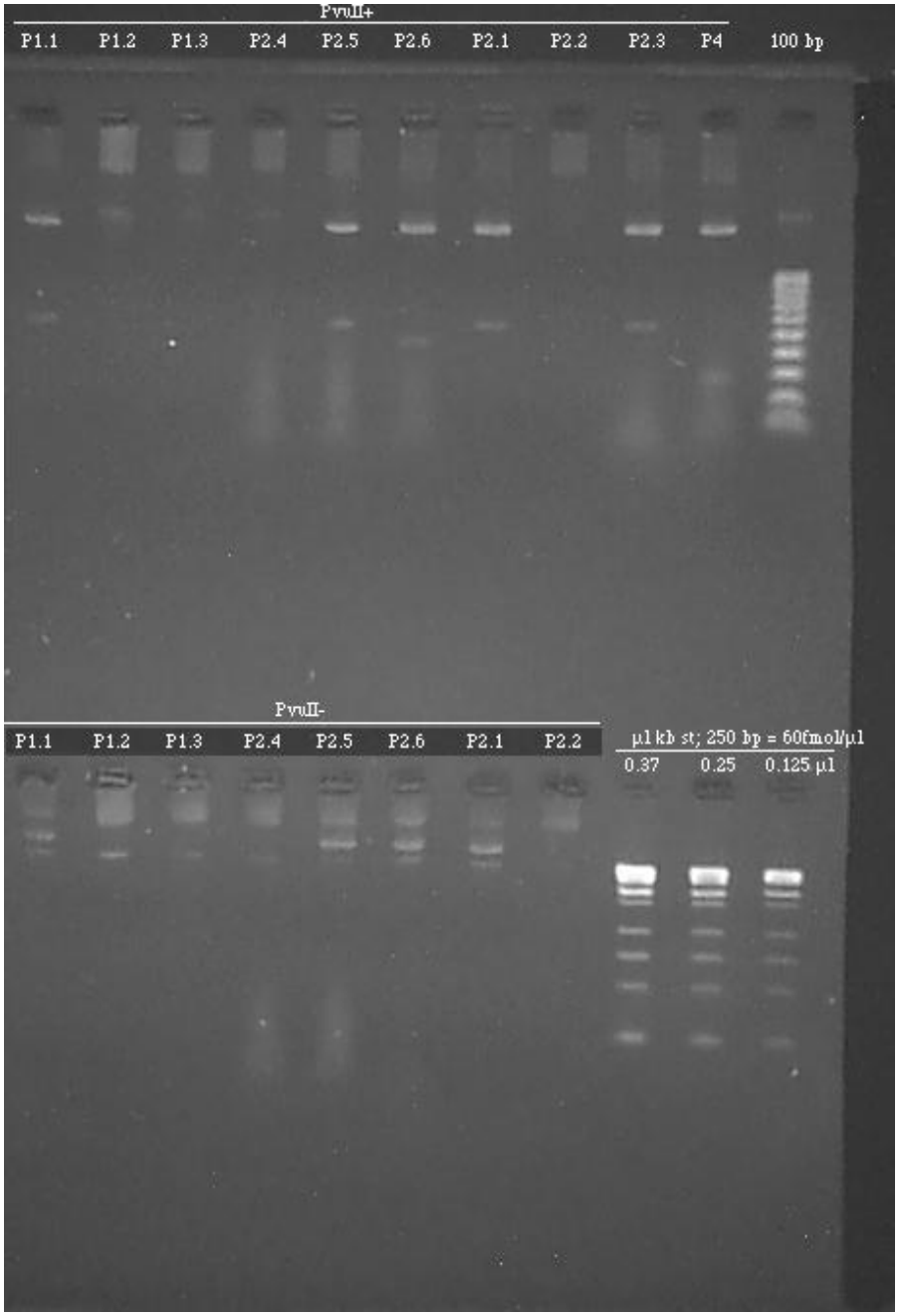




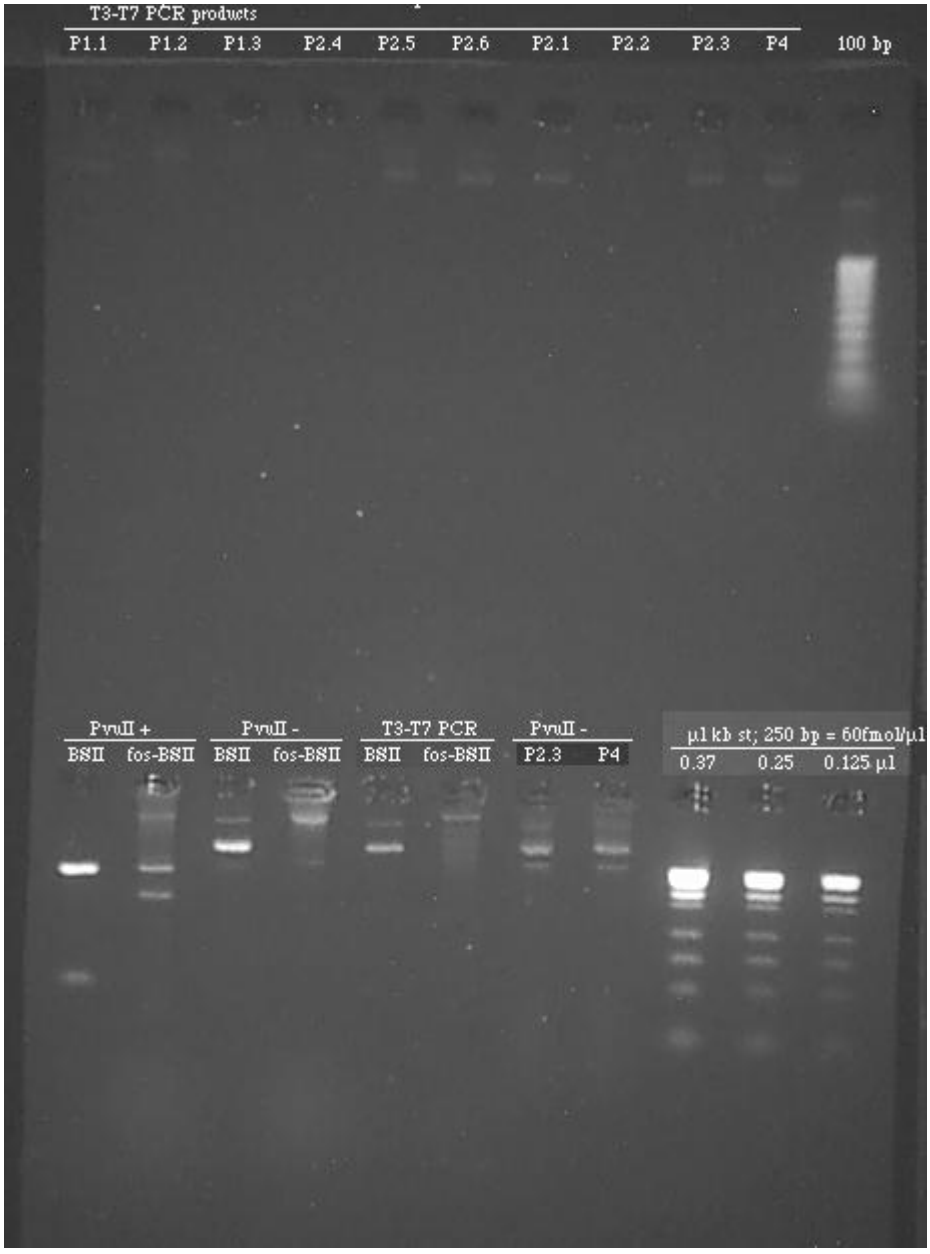
Estimation of DNA content in re purified restriction enzyme digested PCR fragment preparations. 100bp marker on the left side for size estimation.



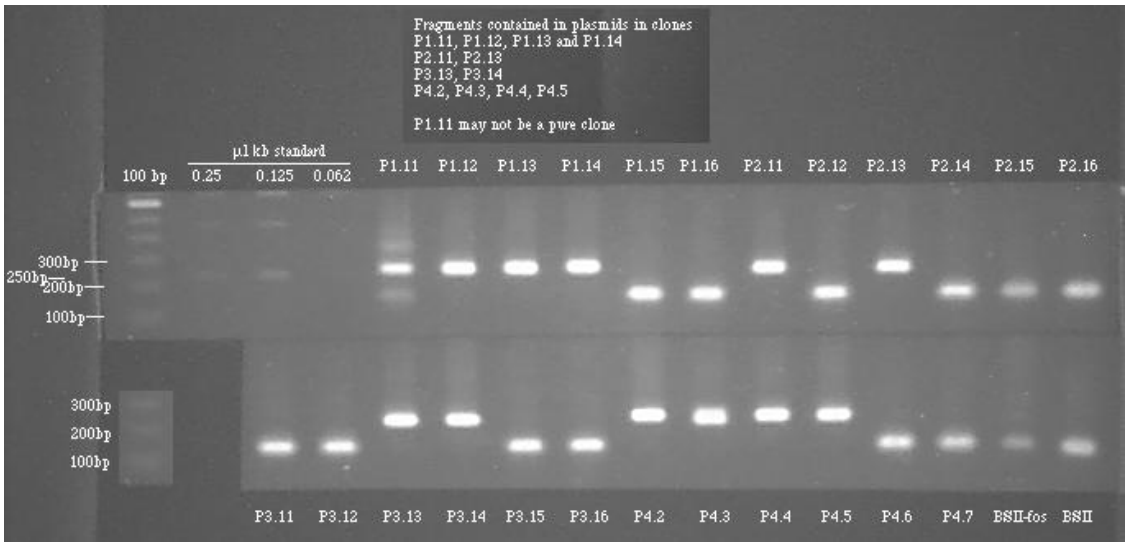
T3-T7 PCR of plasmid preparations.



PvuII digest of plasmid DNA isolated from first set of clones

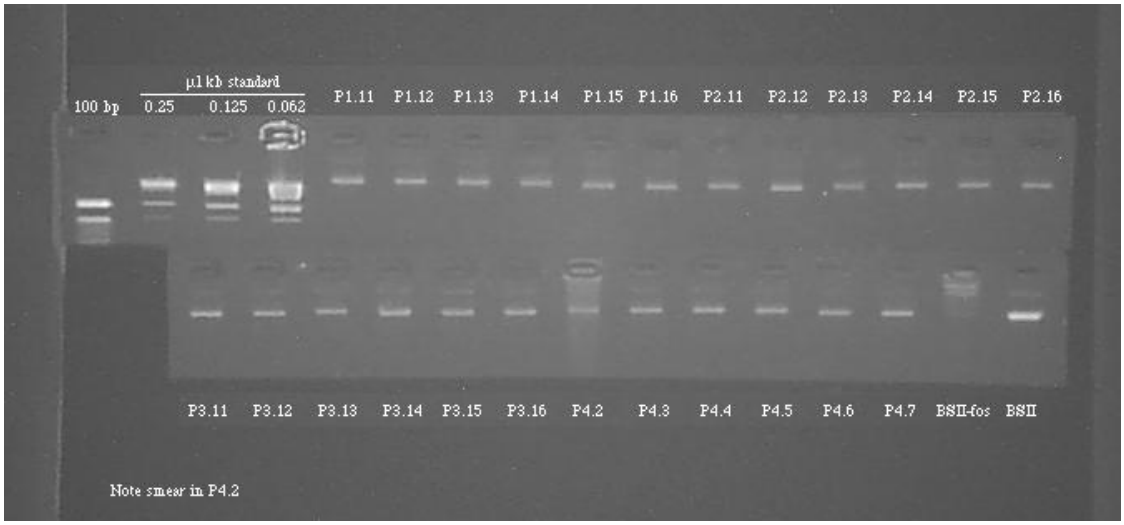


T3-T7 PCR products from the first plasmid batch and also PvuII digest and PCR controls. BS=blue script II SK+ and fos-BSII is same plasmid with the 1-1349 fos cDNA insert in the *Xho*I/*Eco*RI .

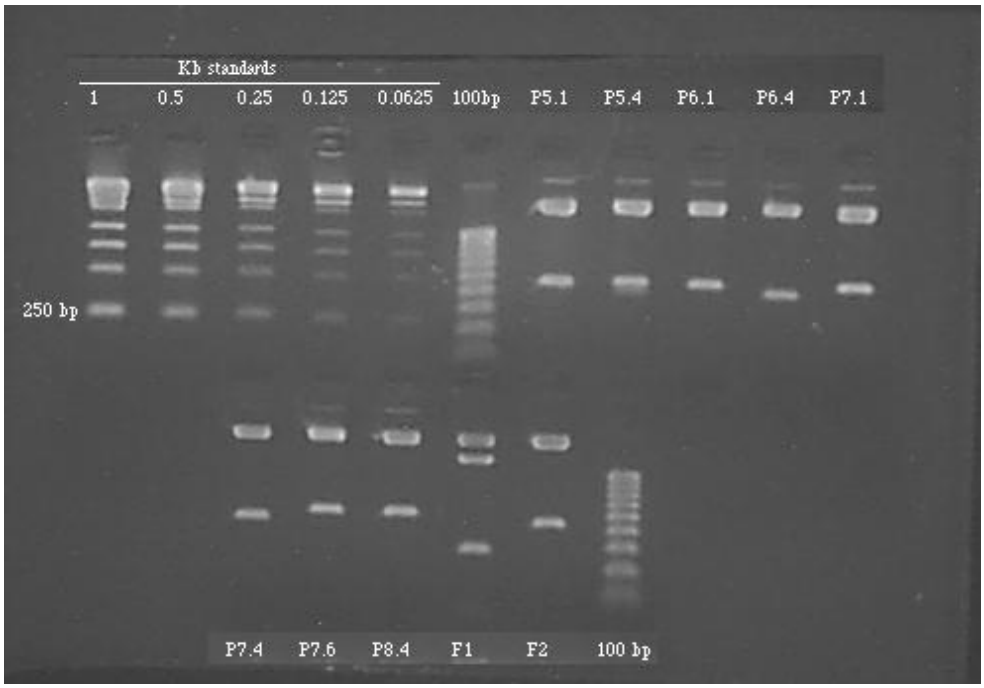


T3-T7 PCR of plasmid second transformation and plasmid isolation using P1, P2, P3 DD fragments ligated in bluescript II.
T3-T7 PCR product in *Xho*I/*EcoR*I linearised ligated bluscript is from bases 791-626 in vector without the 33 bp *Xho*I/*EcoR*I fragment ie 133bp.

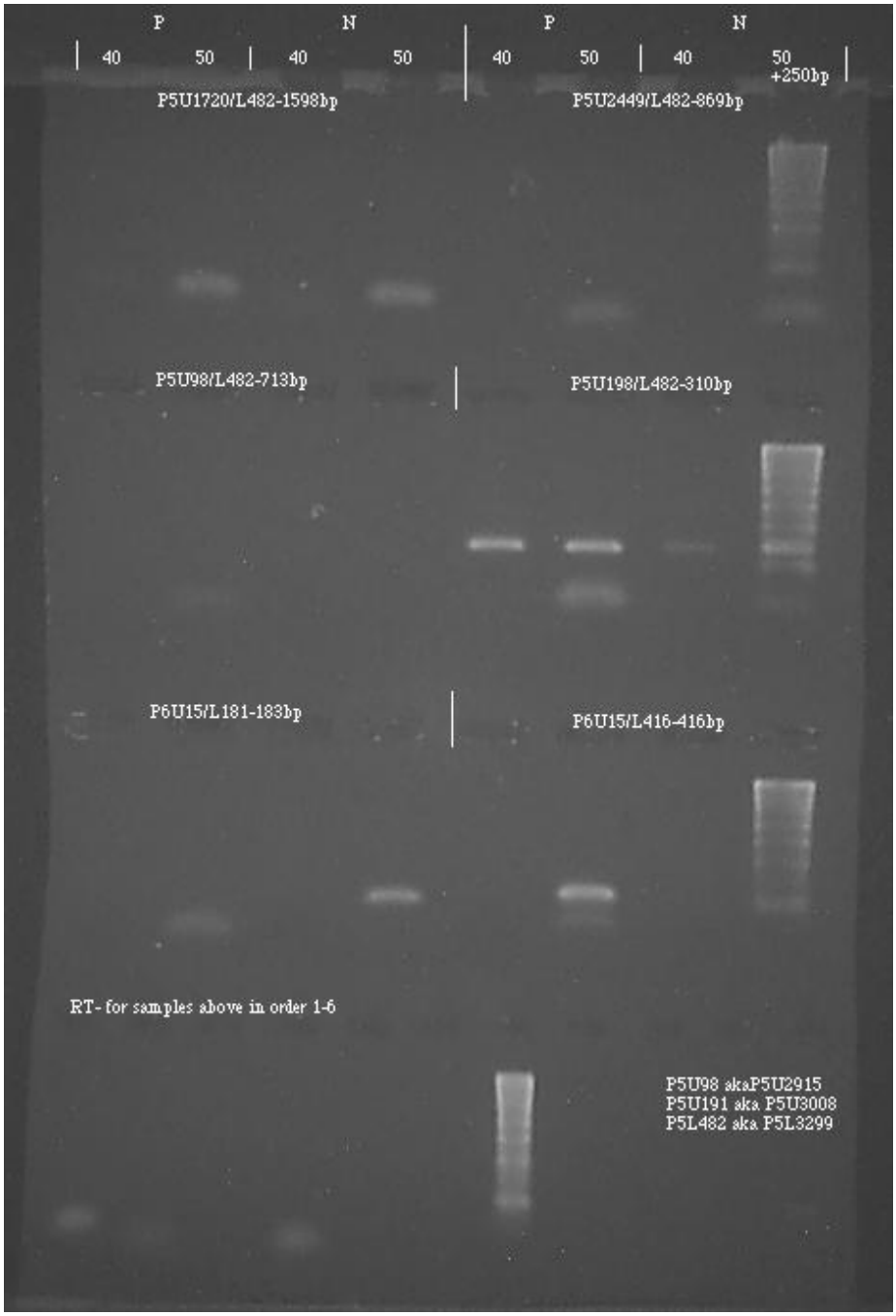
Pvu II digest of plasmids, second transformation and plasmid isolation using P1, P2, P3 and P4 DD fragments ligated in bluscript II . Expected Pvu II fragments without fragment insert are 2513 and 415, the area photographed shows only 415 bp area.



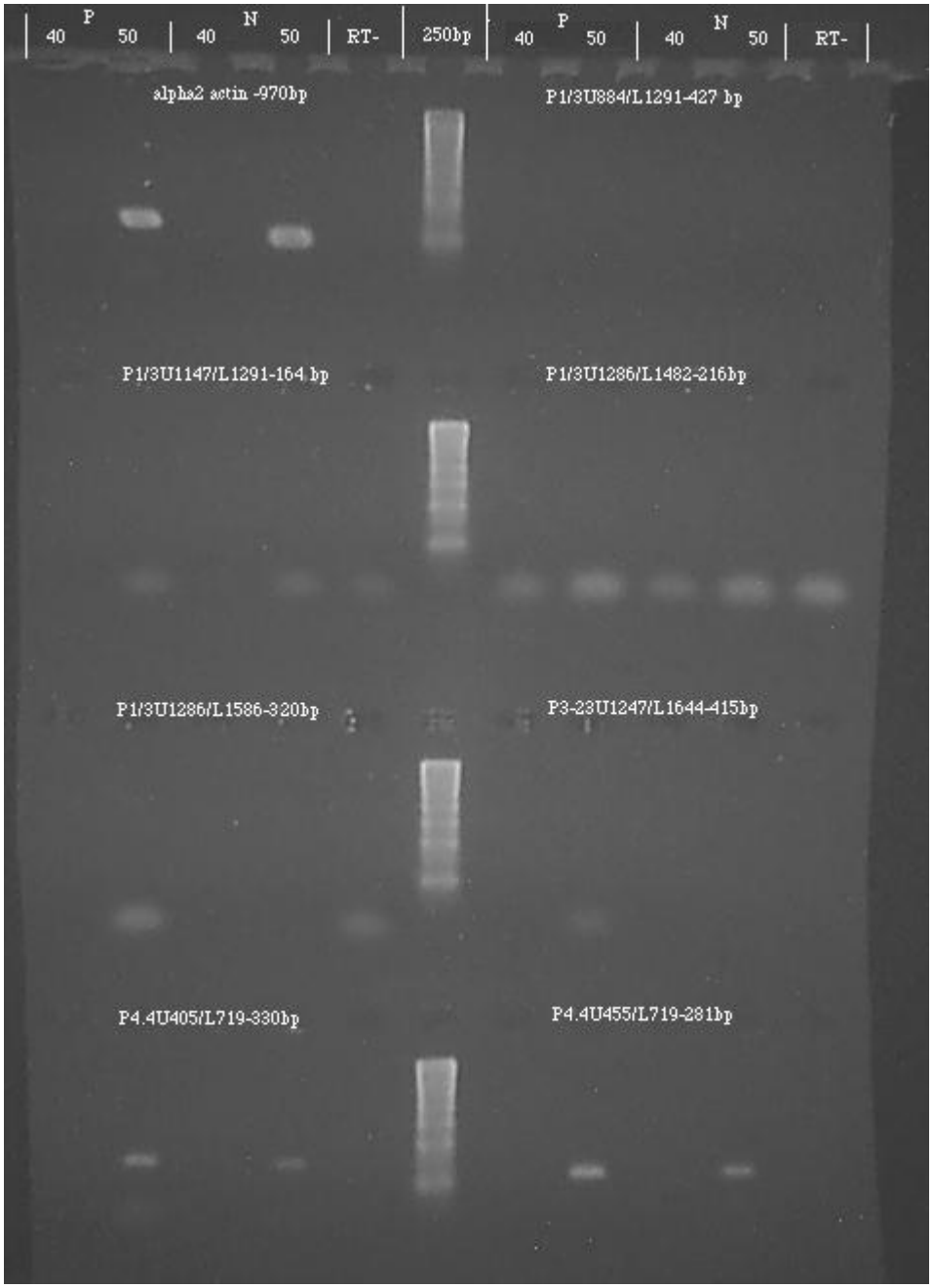
undigested plasmid from second transformation and plasmid isolation Using P1, P2, P3 and P4 DD fragments ligated in bluscript II.



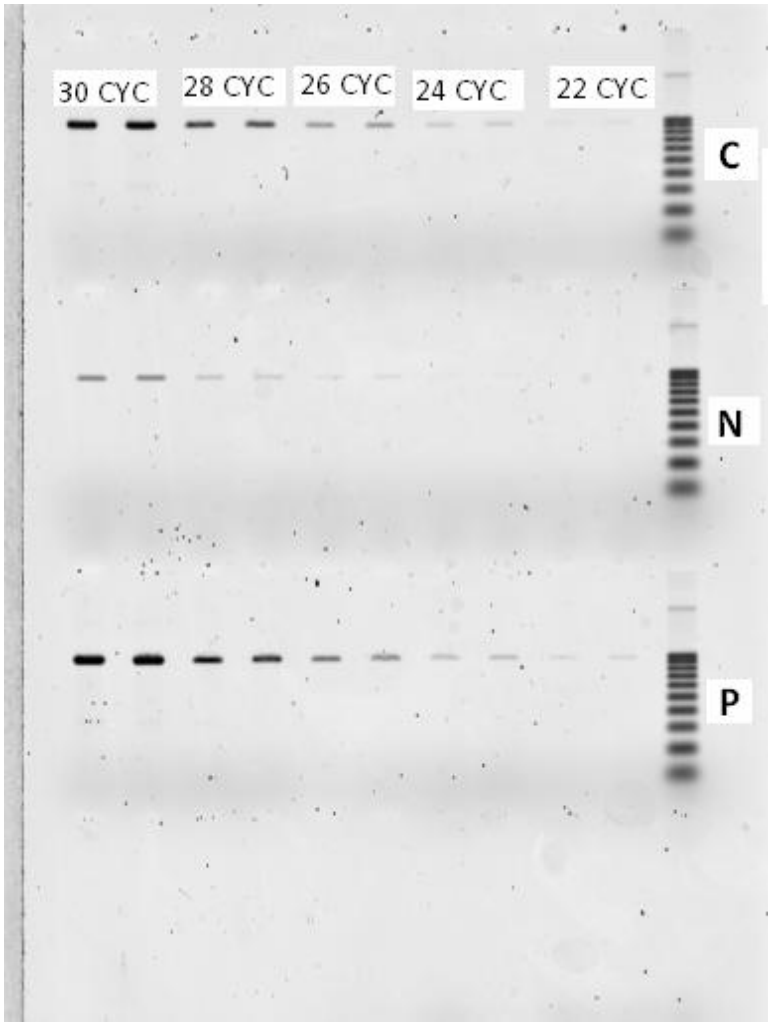
PvuII digests of plasmid products from second transformation and plasmid isolation using P5.1, P5.4, P6.1, P6.4, P7.1, P7.4, P7.6 and P8.4 DD fragments ligated in bluscript II . Expected product 133bp: insert in P5.1,P6.1, P7.1, P7.6, P8.4. F1 shows the expected 1500 bp product, F2 no insert. Clone P5.4 appears to be a mixed up clone.



PCR detection of differential display targets using designed primers(1).



PCR detection of differential display targets using designed primers (2)



PCR for cDNA used in DD using α actin. Good products in cancer and PCOS at 28 and 32 cycles (CYC)

Designed Primers used for the study. Upper and lower primer sequences, expected product size, and annealing temperature for housekeeping genes and target gene fragments. Primers were designed using Oligo 5.0 software and synthesized by Sigma Genosys Ltd. NCBI Genbank accession numbers for target gene sequences have been indicated, as have the sequence for upper (U) and lower (L) primers.

Clone	Target Gene	Primer sequences (5' - 3')	Nucleotides from start of coding sequence	Expected product size (bp)	Annealing temperature (°C)
P1-1, P1-12, P1-13, P1-14, P2-1, P2-5, P3-21:	Human alpha2 smooth muscle actin mRNA	U: CTCCAGCTATGTGTGAAGAAGAGG L: CCGTGATCTCCTTCTGCATT	59-82 1008-1028	970 bp	58
P1-14, P3-13, P3-14	Chr 13, BAC RP11-155N3, AL161420.1	U:CATGGGGGATCGTTTGTAT L:TCACAGGTGTATGCGTGTCT	884-902 1291-1310	427	61
		U:TTGGGAGGCTGAGGAAC L:TCACAGGTGTATGCGTGTCT	1147-1163 1291-1310	164	61
		U:AGAGCAGACACGCATACA L:AAAACATGTTAGTTGCCT	1286-1303 1482-1500	216	61

		U:AGAGCAGACACGCATACA **L:GGTTCTGAAGCCAAATCTAT**	**1286-1303** **1586-1605**	**320**	**57**
P3-23	**Chr 7, BAC RP11-736E3,** **AC073346.12**	**U: AAATGGCAACAAACATACA** **L: ATATTAACTTGGGAGACAACA**	**973-992** **1253-1273**	**301**	**55**
		U: AAAGGCCTACTGAGTCAAA **L: ATATTAACTTGGGAGACAACA**	**845-864** **1253-1273**	**421**	**56**
		U:TGTTTTGTTGTCTCCCAAGT **L:CCTTAATGCTGCCCAGTT**	**1247-1266** **1644-1661**	**415**	**59**
P4-1: **P4-2** **P4-4**	**Chr 19 BAC82621** **AC007193,** **Chr 4 BAC RP11-461L13** **Human clone FLB4816 PRO1252 mRNA**	**U: CCTGGCCTCAAGCAA** **L: GCCAAATAGCAAATAGGTCA**	**101-115** **795-814**	**714**	**57**
		U: CTATTTGCTATTTGGCTGTT **L: TCAGTGTGAAGGGGTCTTT**	**799-819** **1272-1290**	**493**	**59**

P4-4:	**Compilation of AC012076 genomic complementary sequence and AF130054 1653**	**L:CTTGAGGCCAGGAGTT** **U:GATATTTTCTTCAGGCAGAT**	**719-734** **405-424**	**330**	**55**
		U:TGGGACATAACAAATGAAAA **U:GATATTTTCTTCAGGCAGAT**	**455-474** **405-424**	**281**	**58**
P5-1, P5-3, P5-5, P7-1:	**Chr 15 AC005630.1 and others**	**U: CTGCCTTCCCCTCCACTT** **L: GGCATGATCTTGGCTCACTAA**	**98-115** **790-810**	**713**	**63**
		U: CATTTCATTGCACGCTTTCTA **L: GGCATGATCTTGGCTCACTAA**	**499-520** **790-810**	**312**	**62**
P5-1, P5-3, P5-5, P7-1:	**Chr 15 AC005630.1 and others**	**U:ATATAAAAGGGCCAGGCACA** **L: TGGGCATTTTCACAGTCGT**	**1720-1739** **482-500**	**1598**	**63**
		U:CACCTGAGTCCCCAGAGAAT **L: TGGGCATTTTCACAGTCGT**	**2449-2468** **482-500**	**869**	**63**

		U: CTGCCTTCCCCTCCACTT L: TGGGCATTTTCACAGTCGT	98-115 482-500	403	63
		U: AGGCCAGTTTTGCTACCTCT L: TGGGCATTTTCACAGTCGT	191-210 482-500	310	63
P6-1, P6-2, P6-3, P7-2, P7-3	Chr 22q11	U: CCACCCCTGCGATGAAGA L: TGTAGGACAGGCTGCGATTGT	175-192 731-751	577	66
		U: GAGACCTGGCATTTCTTTGAT L: CTGTCACCAGGCTGGAGTA	1881-1901 2309-2327	447	61
		U: TCCAGCCTGGTGACAG L: GATTCGCCTTCCTCCAA	15-32 181-197	183	62
		U: TCCAGCCTGGTGACAG L: GTCCCGGCTGCTCAG	15-32 416-431	416	62

Appendix 7: Summary of clone sequencing data

Analysis for clones P1-1, P1-12, P1-13, P2-1, P2-5, P3-21

P1.1

GAATTCACACGACTGT**GAATGTCCTGTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAG**
CCAAAGCTCTGACTCGTTACCTATGTGTTTTTTAATAAATCTGAAATAGGCTACTGGTAAAAAAA
AAAAACTCGAG

P1.12

GAATTCACACGACTGT**GAATGTCCTGTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAG**
CCAAAGCTCTGACTCGTTACCTATGTGTTTTTTAATAAATCTGAAATAGGCTACTGGTAAAAAAA
AAAAACTCGAG

P1.13

GAATTCACACGACTGT**GAATGTCCTGTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAG**
CCAAAGCTCTGACTCGTTACCTATGTGTTTTTTAATAAATCTGAAATAGGCTACTGGTAAAAAAA
AAAAACTCGAG

P2.1

GAATTCACACGACTGT**GAATGTCCTGTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAG**
CCAAAGCTCTGACTCGTTACCTATGTGTTTTTTAATAAATCTGAAATAGGCTACTGGTAAAAAAA
AAAAACTCGAG

P2.5

GAATTCACACGACTGT**GAATGTCCTGTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAG**
CCAAAGCTCTGACTCGTTACCTATGTGTTTTTTAATAAATCTGAAATAGGCTACTGGTAAAAAAA
AAAAACTCGAG

P3-21

GAATTCACACGACTGT**GAATGTCCTGTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAG**
CCAAAGCTCTGACTCGTTACCTATGTGTTTTTTAATAAATCTGAAATAGGCTACTGGTAAAAAAA
AAAAACTCGAG

Homology with several entries for human alpha2 actin from smooth muscle mRNA (NM-00163.1; X138389,HSACTA; AL157394.15; M33216.1, HUMSMAAA; AK093340.1; BC017554). 119/120 fit with most, though occasionally the insert sequence was a couple of bases shorter.

BC017554, human alpha2 actin, smooth muscle, aorta, clone MGC:9221. Sub 21/11/01

CCCACGCGTCCGGTGTGGAGCAGCCCAGCCAAGCACTGTCAGGAATCCTGTGAAGCAGC^{59}TCCAG
CTATGTGTGAAGAAGAGGACAGCACTGCCTTGGTGTGTGACAATGGCTCTGGGCTCTGTAAGGCCG
GCTTTGCTGGGGACGATGCTCCCAGGGCTGTTTTCCCATCCATTGTGGGACGTCCCAGACATCAGG
GGGTGATGGTGGGAATGGGACAAAAAGACAGCTACGTGGGTGACGAAGCACAGAGCAAAAGAGGAA
TCCTGACCCTGAAGTACCCGATAGAACATGGCATCATCACCAACTGGGACGACATGGAAAAGATCT
GGCACCACTCTTTCTACAATGAGCTTCGTGTTGCCCCTGAAGAGCATCCCACCCTGCTCACGGAGG
CACCCCTGAACCCCAAGGCCAACCGGGAGAAAATGACTCAAATTATGTTTGAGACTTTCAATGTCC
CAGCCATGTATGTGGCTATCCAGGCGGTGCTGTCTCTCTATGCCTCTGGACGCACAACTGGCATCG
TGCTGGACTCTGGAGATGGTGTCACCCACAATGTCCCCATCTATGAGGGCTATGCCTTGCCCCATG
CCATCATGCGTCTGGATCTGGCTGGCCGAGATCTCACTGACTACCTCATGAAGATCCTGACTGAGC
GTGGCTATTCCTTCGTTACTACTGCTGAGCGTGAGATTGTCCGGGACATCAAGGAGAAACTGTGTT
ATGTAGCTCTGGACTTTGAAAATGAGATGGCCACTGCCGCATCCTCATCCTCCCTTGAGAAGAGTT

ACGAGTTGCCTGATGGGCAAGTGATCACCATCGGAAATGAACGTTTCCGCTGCCCAGAGACCCTGT
TCCAGCCATCCTTCATCGGGATGGAGTCTGCTGGCATCCATGAAACCACCTACAACAGCATCATGA
AGTGTGATATTGACATCAGGAAGGACCTCTATGCTAACAATGTCCTATCAGGGGGCACCACTATGT
ACCCTGGCATTGCCGACCGA^{1008}ATGCAGAAGGAGATCACGGCCCTAGCACCCAGCACCATGAAGA
TCAAGATCATTGCCCCTCCGGAGCGCAAATACTCTGTCTGGATCGGTGGCTCCATCCTGGCCTCTC
TGTCCACCTTCCAGCAGATGTGGATCAGCAAACAGGAATACGATGAAGCCGGGCCTTCCATTGTCC
ACCGCAAATGCTTCTAAAACACTTTCCTGCTCCTCTCTGTCTCTAG*CACAC*^{A}*ACTGTGAATGTCCT*
GTGGAATTATGCCTTCAGTTCTTTTCCAAATCATTCCTAGCCAAAGCTCTGACTCGTTACCTATGT
***GTTTTTTAATAAATCTGAAATAGG*CTACTGGTAA**AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Italics shows insert. Bold shows variance between insert and published sequence.

Note that this fit was 109/110. All other fits were 119/120 with 10 bases more (italics-shadow box; 2 are as in the polyA tail) in the clones fitting with the inserts, these being 3' before the polyA tail (ie ATAGG-CTACTGGTAA-AAAAAAAAAACTCGAG). BC017554 is probably a splice variant featuring premature polyA tail formation. All other clones are for the longer, probably more common, mRNA species.

The alpha2 mRNA differs at 29 bases from the alpha1 form, in the first 120 bases of the cds (67.1200). This leads to 6 residue differences, 4 in the first 7 residues.

The ordered primers are boxed - CTCCAGCTATGTGTGAAGAAGAG (23 mer, 59-82) and CCGTGATCTCCTTCTGCATT (20 mer, 1008-1028) – and differ slightly from published primer sequences (underlined), to ensure primer comparability of Tm. The Lp complimentary sequence is:

AATGCAGAAGGAGATCACGG.

Up: CTCCAGCTATGTGTGAAGAAGAGG (23mer, 59-82) 970 bp
58C
Lp: CCGTGATCTCCTTCTGCATT (20mer, 1008-1028)
19/3/3: Full quantitative PCR analysis performed, P>>>>N
22/5/3: N and P pools, 32-50 cyc @60C. P>>>N
27/5/3: N and P pools, 32,35 cyc @60C. <u>No N products</u>
24/6/3: N/P pool, 22-32 cyc @55C. Product at 32 cyc only and this was ca 500bp. <u>18S failed</u>
4/7/3: N/P pool, 30-55 cyc @57C. Products from 35 cyc
10/7/3: 32,34,36 cycles @59C, N and P pools, <u>a2actin failed in P</u>

15/7/3: 32,34,36 cycles @59C, N and P pools, a2actin P>N, 18S N just above P as expected

Analysis of P1-14 clone, also P3-13 and P3-14

P1.14

GAATTCACACGACTGT**ACTCCAGCCTAGGTGACAAGTGAGACTCTGTCTCAAAAAATAATAATAA**
TGTATACAATTTGATGAGAGCAGACACGCATACACCTGTGATACCATCACCACAACTGAGGTAAA
AAAAAAAAACTCGAG

P3.13

GAATTCACACGACTGT**ACTCCAGCCTAGGTGACAAGTGAGACTCTGTCTCAAAAAATAATAATAA**
TGTATACAATTTGATGAGAGCAGACACGCATACACCTGTGATACCATCACCACAACTGAGGTAAA
AAAAAAAAACTCGAG

P3.14

GAATTCACACGACTGT**ACTCCAGCCTAGGTGACAAGTGAGACTCTGTCTCAAAAAATAATAATAA**
TGTATACAATTTGATGAGAGCAGACACGCATACACCTGTGATACCATCACCACAACTGAGGTAAA
AAAAAAAAACTCGAG

AL161420. 120/121 +/- fit. Human DNA clone RP11-155N3 from chr 13. Sub 1/2/1. The NCBI report talks about genes and expressed sequences in this clone but not involving the sequence corresponding to the insert in P1-14 - nuc 14337 – 14456.

CCCTGTGTGTCATGGAGAATTGGTAGGTAGAGTCAGAGGCTTGATCTGAGTCAGATGCAAGTTTTT
GGCAAGACTACTTCATAGGTGGTGGTGTGTACTTCTACCAGGAGACACGAGAACGCTTTGTCTTTT
TTGATAATATCAACTCCTTCAATGGTTACCAAGCAGTTTTCCTTTCATTATGAACTCATAGACTTA
ATTACATACTTTCAGTCCGTTGCAATTATTCTTTTTTTTTTTTTTTTTTTTTTTTTGAGATGGGG
TCTGGCTCTGTCACCCAGGCTGGAGTGCAGTGGCATGATTACTGCTCACTGCATTGCAATTATTCT
TACTGATGCCCATCCTGCACCTTGCTAGTGGGAGCTTCTTTAAAATCCTTCTCACACGACCCCAGA
GTGTTTTAATTTAACCCTGCTGGTCTCATAGACACATCTTTTCCTCTCCCATGTAAGAACCTGGTT
CCCGATGATACCAACATAAATACTCATTTACTCTCTGGCAATAAACATAAAACAGTCTCAGAATAA
AAACCAATGCAACACTTAAACAAAACAATTACTGAAACTAGTTTTTGTCAATAAACCACTAATAAA
TAAGTGGTTGCCACTTATTTGGCTGGGCGTGGTGGTTCACACCTGTAATCCCAGCACTTTGGGAGG
CCGAGGTGGGCAGATCACTTGAGGTCAGAAGTTTGAGACCAGCCTGGCCAACATGGTGGAATTCTG
TCTCTACCAAAAATTCAAAAATTAATAAATAATAAATAATTGATTGCCACTCTTCTTGACCTGAGG
GCATATCACACTAAGAAAGCACAGTAAAACTGCTGTAGTTTAAACTTGTTTGAAATAATTCCTTTC
TGTATGTTGCCAACATAATATTATATGGTTAGGTTCATTTATCACATTTTGCCTTCAATTTCTTAG
AAATTTAAAAATTCATTTGCTAATGATGTAAATATTTGCATGGTTCTGAAGCCAAATCTATAAAGC
AAATGATATTAGACAAGTTTATCTTCTAACCCTGTCCCCTGCACGTCATTCCCTCTCCCTTGCTGT
AGGTAACAACTATAAAACATGTTAGTTGCCTATATACGTTACTGTTATTGTGCACTTACAAATTTG
TTAAGAGGGTTGATCTCGTGTTAAATGTCCTTACCACAAAACAAGAGGGGAGCACGAGGAAACTTC
TGGAAGTGGTGGATATATTTTACTGTCCTTTTTTTTTTTTTTTTTTTTTTT*ACCTCAGTTGTGGTG*
ATGGTATCACAGGTGTATGCGTGTCTGCTCTCATCAAATTGTATACATTATTATTATTTTTTGAGA
***CAGAGTCTCACTTGTCACCTAGGCTGGAGTACAGT*G*GTGTG*GATTTCGGCTCACTGCAACCTCTGCT**
TCCCGGGTTCAAGCGATTCTAGTTCCTCAGCCTCCCAAGTGGCTGGGATTACAGGCGCCCGCCAGC
ATGCCTGGCTAATTTTTGAATTTTTGGTAGAGACAGAATTCCACCATGTTGGCCAGGCTGGTCTCA
AACTTCTGACCTCAAGTGATCTGCCCACCTCGGCCTCCCAAAGTGCTGGGATTACAGGTGTGCGCC
ACCGCGCCCAGCCAAATTGTATACATTAAATGAGCAGATTTTTACAGCTGCACAGTATTCCACTGT
GTGGATGTACCATGGTTTATACAAACGATCCCCCATGGATGGAGATCACATTTTCTTGGAACGGAG
TCACTGCTTTGAGTTATATGATGAAACCATTCTGGATGGCTGCTTCTCGTCACTCAGGTTACCTTG
CCATCCTGCAGTCCTGCTCTCAGCAGCTGTCCTCTGTGCAAGGGTCTGGCTCTATTTGTTCCCACC
TGGCTAATATCCAGGACAAGGTCTTAACTAGAGTTTTAATTCCAGTGTTGCCTCAAAAGCAGTGTT
GTGAGGGGGAAGTAGGGAGTTCCTGTTTAATGAGTACTGAGTGTGAGTTTGGGAAGATGAGTATCT

GGAGATGGAGGGTGGCGATGGTTGCACAATGAGAATCTACTTGATGCCAATGAACCTTACACTTAA
AAATGGTTAAACTGGTACATTTTAGGCATATTTTACCTCAATTAAAAGAAAGAATAAGTAGGACCT
ATGGGAAAAAAAACCCCACATTTACTGCAGAATAGCAATACGAATTCCCAAGAAAATGCTCCACCT
GGCTTTGTACATAACGAAGGGAAGATTTAATTGATACGGATGTTTCCCTGCAGGATGTTCCCAACT
TAGAGATGGCTCCTCTCTCCACTCCTGTCATAAGCAAGGCTTGGTCGCCTTCCAAACTTACCTTTA
TCTGACATGGGCTCTTTCTAGACTTCTATTTATCTTACTACATGGTGACTTGAGTTTTATGGTTTC
TGGATGAGGCATTTATGACTAGACGATCCAGCTCAGCGCCAGGCTCTTCTCCCAGACATGCTGATG
TTTGCCTTAAGGTTTATTAGCCCCGGCTGGGATGTCAGCGACATGGAACAAACGTATGCTGGGTAA
GTCCCAATGGCGTCATGAGTGCATAAGTGGGCTGTGGATTCTGGTGGGTCGTGGCGACCCTT

Complementary strand with ca 500 bp surround. Insert italicised.

AAGGGTCGCCACGACCCACCAGAATCCACAGCCCACTTATGCACTCATGACGCCATTGGGACTTAC
CCAGCATACGTTTGTTCCATGTCGCTGACATCCCAGCCGGGGCTAATAAACCTTAAGGCAAACATC
AGCATGTCTGGGAGAAGAGCCTGGCGCTGAGCTGGATCGTCTAGTCATAAATGCCTCATCCAGAAA
CCATAAAACTCAAGTCACCATGTAGTAAGATAAATAGAAGTCTAGAAAGAGCCCATGTCAGATAAA
GGTAAGTTTGGAAGGCGACCAAGCCTTGCTTATGACAGGAGTGGAGAGAGGAGCCATCTCTAAGTT
GGGAACATCCTGCAGGGAAACATCCGTATCAATTAAATCTTCCCTTCGTTATGTACAAAGCCAGGT
GGAGCATTTTCTTGGGAATTCGTATTGCTATTCTGCAGTAAATGTGGGGTTTTTTTTCCCATAGGT
CCTACTTATTCTTTCTTTTAATTGAGGTAAAATATGCCTAAAATGTACCAGTTTAACCATTTTTAA
GTGTAAGGTTCATTGGCATCAAGTAGATTCTCATTGTGCAACCATCGCCACCCTCCATCTCCAGAT
ACTCATCTTCCCAAACTCACACTCAGTACTCATTAAACAGGAACTCCCTACTTCCCCCTCACAACA
CTGCTTTTGAGGCAACACTGGAATTAAAACTCTAGTTAAGACCTTGTCCTGGATATTAGCCAGGTG
GGAACAAATAGAGCCAGACCCTTGCACAGAGGACAGCTGCTGAGAGCAGGACTGCAGGATGGCAAG
GTAACCTGAGTGACGAGAAGCAGCCATCCAGAATGGTTTCATCATATAACTCAAAGCAGTGACTCC
GTTCCAAGAAAATGTGATCTCCATCCATGGGGGATCGTTTGTATAAACCATGGTACATCCACACAG
TGGAATACTGTGCAGCTGTAAAAATCTGCTCATTTAATGTATACAATTTGGCTGGGCGCGGTGGCG
CACACCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGCAGATCACTTGAGGTCAGAAGTTTGAG
ACCAGCCTGGCCAACATGGTGGAATTCTGTCTCTACCAAAAATTCAAAAATTAGCCAGGCATGCTG
GCGGGCGCCTGTAATCCCAGCCACT[1147]TGGGAGGCTGAGGAACTAGAATCGCTTGAACCCGGGAA
GCAGAGGTTGCAGTGAGCCGAAATCACACC*ACTGTACTCCAGCCTAGGTGACAAGTGAGACTCTGT*
CTCAAAAAATAATAATAATGTATACAATTTGATGAGAGCA[1291]GACACGCATACACCTGTGATACC
ATCACCACAACTGAGGTAAAAAAAAAAAAAAAAAAAAAAAAAGGACAGTAAAATATATCCACCACTTC
CAGAAGTTTCCTCGTGCTCCCCTCTTGTTTTGTGGTAAGGACATTTAACACGAGATCAACCCTCTT
AACAAATTTGTAAGTGCACAATAACAGTAACGTATATAGGCAACTAACATGTTTTATAGTTGTTAC
CTACAGCAAGGGAGAGGGAATGACGTGCAGGGGACAGGGTTAGAAGATAAACTTGTCTAATATCAT
TTGCTTTATAGATTTGGCTTCAGAACCATGCAAATATTTACATCATTAGCAAATGAATTTTTAAAT
TTCTAAGAAATTGAAGGCAAAATGTGATAAATGAACCTAACCATATAATATTATGTTGGCAACATA
CAGAAAGGAATTATTTCAAACAAGTTTAAACTACAGCAGTTTTACTGTGCTTTCTTAGTGTGATAT
GCCCTCAGGTCAAGAAGAGTGGCAATCAATTATTTATTATTTATTAATTTTTGAATTTTTGGTAGA
GACAGAATTCCACCATGTTGGCCAGGCTGGTCTCAAACTTCTGACCTCAAGTGATCTGCCCACCTC
GGCCTCCCAAAGTGCTGGGATTACAGGTGTGAACCACCACGCCCAGCCAAATAAGTGGCAACCACT
TATTTATTAGTGGTTTATTGACAAAAACTAGTTTCAGTAATTGTTTTGTTTAAGTGTTGCATTGGT
TTTTATTCTGAGACTGTTTTATGTTTATTGCCAGAGAGTAAATGAGTATTTATGTTGGTATCATCG
GGAACCAGGTTCTTACATGGGAGAGGAAAAGATGTGTCTATGAGACCAGCAGGGTTAAATTAAAAC
ACTCTGGGGTCGTGTGAGAAGGATTTTAAAGAAGCTCCCACTAGCAAGGTGCAGGATGGGCATCAG
TAAGAATAATTGCAATGCAGTGAGCAGTAATCATGCCACTGCACTCCAGCCTGGGTGACAGAGCCA
GACCCCATCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAGAATAATTGCAACGGACTGAAAGTATGTA
ATTAAGTCTATGAGTTCATAATGAAAGGAAAACTGCTTGGTAACCATTGAAGGAGTTGATATTATC
AAAAAAGACAAAGCGTTCTCGTGTCTCCTGGTAGAAGTACACACCACCACCTATGAAGTAGTCTTG
CCAAAAACTTGCATCTGACTCAGATCAAGCCTCTGACTCTACCTACCAATTCTCCATGACACACAG
GG

Italics shows insert. Bold shows variance between insert and published sequence. Primers (10/6/03) shown in boxes, Lps shaded (884,1291; 1147,1291; 1286, 1482; 1286,1586)

P1-14, P3-13, P3-14**: Chr 13, BAC RP11-155N3, AL161420.1; No EST**
+/- hit, primers designed to complementary strand
No first look, Second phase of investigation
Lp: TCACAGGTGTATGCGTGTCT (20mer, 1291-1310)

Up: CATGGGGGATCGTTTGTAT (19mer, 884-902) 427 bp 61C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/03: 30,35..50,55 cyc@61C, NP pool. No product though controls relatively unproductive
3/10/03: 48,50,52 cyc@59C N/P pool, No product, controls good
No products with these primers

Up: TTGGGAGGCTGAGGAAC (17mer, 1147-1163) 164 bp 61C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/03: 30-55 cyc@61C, NP pool, 170bp 55cyc only
10/7/3: 36,38,40 cycles @59C, N and P pools, variable output 170bp, a2actin failed in P
3/10/03: 36,38,40cyc@59C N/P pool, No product, controls good
Uncertain whether 170 bp product is consistent and quantitative

Up: AGAGCAGACACGCATACA (18mer, 1286-1303)

Lp: AAAACATGTTAGTTGCCT (20mer, 1482-1500) 216 bp 53C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
3/7/03: 30-55 cyc@59C, NP pool, No product though Ta was high
4/7/3: N/P pool, 30-55 cyc @57C. No products, though 18S and a2 actin are lower than normal
No products (should check once more)

Lp: GGTTCTGAAGCCAAATCTAT (20mer, 1586-1605) 320 bp 57C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/03: 30-55 cyc@57C, NP pool, No product though Ta was high and 18S and a2 actin are lower than normal
No products (should check once more)

Analysis of P3-23 clone

P3-23
GAATTCACACGACTGT**TCGGTTATAATCACTGCCTCCTGAATCGTTGAGGAGTCT**
TTTAAATTAGATTTTTGTTTTGTTGTCTCCCAAGTTAATATTATATTTAGATATCA
GAGAGTCAGGTAAAAAAAAAAAACTCGAG

AC073346.12, +/- fit, Submitted 01/03/02.
115/116 hit with clone carrying human male DNA (clone RP11-736E3) from chr 7
GATGCTTCTTCACTCCTTGTACACTTTAACCAAAACTTTCATGCAAGCCAAGTCAGCTCACTCTAG
AATTACACCATAAACAGGACTTTAGGTTGCTAGTGCCTGTTTTTGGATAAAAAATGAGGCTCCCAA
TGTCATCTGGAGAGCTACAGATTGGAAAATGCATTTTAGGAAAAAATTTCACTACTTACCTCCAGT
GTGACCCTAAAGTTTGATTTCCTTAGACTACTTTATGCTGGCTACAACTTGAGAATCAAATGAATC
ACTGAGGACCCTTGTTAGACATGGAGTTCCAGGCCCCATCTCACAGCTATACAACCAGGAGAAAGA
CCAGGGAATCAAAAAGTCTTATCAAGTGCCTGGAATGATTCTTGGGCTCAAGCAAGTTGGCAGTCT
TCTTAAATTTGTAATGTGCATACAAATTAACTTGGGCTTTGGAAACGAATGCTGTTCCTGATCCAA
TTGGTCTGGGTGGGGCCTGAGATTCTGCATTTCTAACAAGCCCCAGGTGATGTTGCTGGCTTGAAG
ACACTTTAAGTAGCTAGGCCAGAGAGGATATTTCGTTTGGCTGGCGTACAAATCAGTTTAAGCCAT
TTCATTTTCCTTCTATATTTCTAGATTTTCTATCAGGGGCTTCTCTGACCTTAATGCTGCCCAGTT

CTCATTATGTCTCTTCAAGCTACCGCAATCAGTTAACACAAGGCATCCTGCCCTTCTCCACTTAGC
TCATATAAAAATCCACAATGGATACAAACAGCCCCAAATAAGAAATGGACACGAAACATTAACTTA
TAACATAATGGGTTTATATTTAATATAGTACTTCTCATCAGGAGGATGTTACTCAGTTAATATAAA
GTTTTTTTTCAAAACATTAAATCTCTTTTCCATGTCAATGTCTATAGTTTTTTTTTAACATTAAAT
TTTTTTCCGCATTTCAGTATTAGATACACTGAATACATTTTTCTAAATGTTTTTTCCCTAGAGATA
AAAGTTTTCC*TTTTTGCCTGACTCTCTGATATCTAAATATAATATTAACTTGGGAGACAACAAAAC*
***AAAAATCTAATTTAAAAGACTCCTCAACGATTCAGGAGGCAGTGATTATAACCGAACAGT*GGTGAT**
TTCCTAAGATTCTGGGCAAGAACTTCCTTCTTCCTATTTCACATGCTTTGAGAGAATTCTAAGATT
ATGAAATATTACTTATGTAAATTTCAGCCATCTTACTTCTTTGACTACCTAACTAGCAAATTATAG
GCTGCATTTTTCTGTGCATTATTTGTTGTAAAGAAAACATTTTTTTTTCAATTTATAAATGTATGT
TTGTTGCCATTTCATGGAAACAATGAAAAATGTGAAACTCCCTTATTTACTGATTCTTGGAACCTT
TCACACACCAAATCTCAGGTTTAGCCTCTAAAGCGCAAAGGTTTTCAGGGTGAGGTTTGACTCAGT
AGGCCTTTCAAAGTCACATCTGTCCATTTCTTTTTCGTGCATATACCCCCAAGCAGGCACAAATGC
CTGTAATGCTGAGAACCACACCTAACAAAAGGGCGATTGCATCACCGGCTTCTACTGCTTCCACAA
CAGGCAGCACCAAAAGCAGTGACATGAGGACTAAGGACAACTGTGTTGAAACTGAGGTCATGATGT
TGGAATCTTGAGGGCTGAAGGTTCCTAGAAATTAAACATGGAAACAAAAAGGAACCATTAAAAATT
CCTCCCTTTTCAAATGACAAAATGAACACATGACAAATAACAGGCCAGCCAATCTTTTGGATACTT
TATGAATCATTTCCAATCCATCTCCTCATCTTATAGGTGGAAAAACAGAGGGACGTTAAAGACAGT
AATTTGAGGTTCATCTCCCTATCTCGTGATAACACTCTAAACCCAGGAATTTTCCACGATAATGCC
AACACCTCGTGCATCAACCAAACAAAAATTGAACAGGAGTTCATAATTAAGAACAAACAGAAAAAA
AGCACGAGCCACTAACTTGGAAACTTAAAATATCCTAGTGGGTAAAAATTGTACATTTTCTTAGGG
TCTCTCCCATCCAAGAAATGCAGACAAATTTGGCAAAAGTCACACAACATATAACTCTGCGAAGTT
GTGG

Insert comprises nuc 48282-48397 of this clone and several notes for ESTs were described in the NCBI report

```
misc_feature 48066..48496
 /note="match to EST AI061282 (NID:g3336650) an25g11.x1"
misc_feature 48071..48705
 /note="match to EST AV753297 (NID:g10911145)"
misc_feature 48073..48295
 /note="match to EST BG054805 (NID:g12511889)"
misc_feature 48074..48754
 /note="match to EST AV700969 (NID:g10302940)"
misc_feature 48074..48507
 /note="match to EST R26096 (NID:g782231) yh49a02.s1"
misc_feature 48139..48476
 /note="match to EST AA248757 (NID:g1880196)"
misc_feature 48200..48955
 /note="match to EST BG619963 (NID:g13671334)"
misc_feature 48240..48950
    /note="match to EST BG030299 (NID:g12419442)"
misc_feature 48246..48273
    /note="match to EST BG289755 (NID:g13045863)"
misc_feature 48277..48955
    /note="match to EST BG501943 (NID:g13463460)"
misc_feature 48291..48844
    /note="match to EST BF921100 (NID:g12316988)"
misc_feature 48352..48961
    /note="match to EST BE568947 (NID:g9812667)"
misc_feature 48367..48955
    /note="match to EST BG289755 (NID:g13045863)"
misc_feature 48384..48955
    /note="match to EST BF247603 (NID:g11163210)"
misc_feature 48386..48955
    /note="match to EST BF028861 (NID:g10736573)"
misc_feature 48393..48955
    /note="match to EST BF215712 (NID:g11109298)"
```

Note other fits (AC129492.6; AC107913.7; AC104762.7, from chr 17) are very similar but likely to be gene duplications or pseudogenes. AC010102 from chr 2 is slightly less similar than these.

Complementary strand of AC073346.12 with fragment (italics) and surrounding ca 1000 bp 5' and 500 bp 3'. This should match the + mRNA strand, assuming RT from ET12X worked on ssRNAs, giving (-) cDNA in a 5' direction up the mRNA.

AAAATAATAATAGTAATAATAATTAATTTTTAAATCCTTCTCTCCGCCCCCTCCCCACCCCCCATG
CTAATAATAGGTCGTGTCACGTGGAACCTCTTAATCTCAGCATCCGGAGCTCCAGGAAGGGAAAAT
TTCAAGTCAGATAGAATTCTATATATACCATTTCTTTGGTAAGTCTTATTAATTCCCCACAACTTC
GCAGAGTTATATGTTGTGTGACTTTTGCCAAATTTGTCTGCATTTCTTGGATGGGAGAGACCCTAA
GAAAATGTACAATTTTTACCCACTAGGATATTTTAAGTTTCCAAGTTAGTGGCTCGTGCTTTTTTT
CTGTTTGTTCTTAATTATGAACTCCTGTTCAATTTTTGTTTGGTTGATGCACGAGGTGTTGGCATT
ATCGTGGAAAATTCCTGGGTTTAGAGTGTTATCACGAGATAGGGAGATGAACCTCAAATTACTGTC
TTTAACGTCCCTCTGTTTTTCCACCTATAAGATGAGGAGATGGATTGGAAATGATTCATAAAGTAT
CCAAAAGATTGGCTGGCCTGTTATTTGTCATGTGTTCATTTTGTCATTTGAAAAGGGAGGAATTTT
TAATGGTTCCTTTTTGTTTCCATGTTTAATTTCTAGGAACCTTCAGCCCTCAAGATTCCAACATCA
TGACCTCAGTTTCAACACAGTTGTCCTTAGTCCTCATGTCACTGCTTTTGGTGCTGCCTGTTGTGG
AAGCAGTAGAAGCCGGTGATGCAATCGCCCTTTTGTTAGGTGTGGTTCTCAGCATTACAGGCATTT
GTGCCTGCTTGGGGGTATATGCACGAAAAAGAAATGGACAGATGTGACTTTGA^{845}AAGGCCTACTG
AGTCAAACCTCACCCTGAAAACCTTTGCGCTTTAGAGGCTAAACCTGAGATTTGGTGTGTGAAAGG
TTCCAAGAATCAGTAAATAAGGGAGTTTCACATTTTTCATTGTTTCCATG^{973}AAATGGCAACAAAC
ATACATTTATAAATTGAAAAAAAAATGTTTTCTTTACAACAAATAATGCACAGAAAAATGCAGCCT
ATAATTTGCTAGTTAGGTAGTCAAAGAAGTAAGATGGCTGAAATTTACATAAGTAATATTTCATAA
TCTTAGAATTCTCTCAAAGCATGTGAAATAGGAAGAAGGAAGTTCTTGCCCAGAATCTTAGGAAAT
CAC*ACTGTTCGGTTATAATCACTGCCTCCTGAATCGTTGAGGAGTCTTTTAAATTAGATTTT*^{1247}T
***GTTT*^{1253}*TGTTGTCTCCCAAGTTAATAT*TATATTTAGATATCAGAGAGTCAGGCAAAAAGGAAAAC**
TTTTATCTCTAGGGAAAAAACATTTAGAAAAATGTATTCAGTGTATCTAATACTGAAATGCGGAAA
AAAATTTAATGTTAAAAAAAAACTATAGACATTGACATGGAAAAGAGATTTAATGTTTTGAAAAAA
AACTTTATATTAACTGAGTAACATCCTCCTGATGAGAAGTACTATATTAAATATAAACCCATTATG
TTATAAGTTAATGTTTCGTGTCCATTTCTTATTTGGGGCTGTTTGTATCCATTGTGGATTTTTATA
TGAGCTAAGTGGAGAAGGGCAGGATGCCTTGTGTTAACTGATTGCGGTAGCTTGAAGAGACATAAT
GAGA^{1644}ACTGGGCAGCATTAAGGTCAGAGAAGCCCCTGATAGAAAATCTAGAAATATAGAAGGAA
AATGAAATGGCTTAAACTGATTTGTACGCCAGCCAAACGAAATATCCTCTCTGGCCTAGCTACTTA
AAGTGTCTTCAAGCCAGCAACATCACCTGGGGCTTGTTAGAAATGCAGAATCTCAGGCCCCACCCA
GACCAATTGGATCAGGAACAGCATTCGTTTCCAAAGCCCAAGTTAATTTGTATGCACATTACAAAT
TTAAGAAGACTGCCAACTTGCTTGAGCCCAAGAATCATTCCAGGCACTTGATAAGACTTTTTGATT
CCCTGGTCTTTCTCCTGGTTGTATAGCTGTGAGATGGGGCCTGGAACTCCATGTCTAACAAGGGTC
CTCAGTGATTCATTTGATTCTCAAGT

Primers are shown in boxes, lower primers shaded (188, 1253 - not ordered; 845, 1253; 973, 1253; newer set = 1247, 1644). Note misc_feature 48200.48955. Match to EST BG619963 NID: g13671334, sequence shown underlined

Up: AAATGGCAACAAACATACA (19mer, 973-992) 301 bp
55C
Lp: ATATTAACTTGGGAGACAACA (21mer, 1253-1273)
10/6/3: P pool, 30,35,40,45, then further 20,30,40,50 cyc @53C. No products. Sigma said primers dodgy
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @57C. No products, though 18S and a2 actin are lower than normal
17/9/03: N and P pools, 40,45,50,55cyc @57C. From 40 cyc 300bp product, P>N ??
Recommend 40 cyc (??check signal at 40 cyc), 57C for x10-x80 cDNA dilutions

Up: AAAGGCCTACTGAGTCAAA (19mer, 845-864) 421 bp
56C
Lp: ATATTAACTTGGGAGACAACA (21mer, 1253-1273)
10/6/3: P pool, 30,35,40,45, then further 20,30,40,50 cyc @53C. No products. Sigma said primers dodgy
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @57C. Product from 35 cyc
17/9/03: N and P pools, 32,34,36,38 cyc @57C. From 36 cyc 420bp product, N>P??
Recommend 38 cyc,56C for x10-x80 cDNA dilutions

Second phase
Up: TGTTTTGTTGTCTCCCAAGT (20mer, 1247-1266)
Lp: CCTTAATGCTGCCCAGTT (18mer, 1644-1661) 415 bp
59C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/3: N/P pool, 30-55 cyc @57C. Products from 35 cyc onwards
10/7/3: 36,38,40 cyc @59C, N and P pools, No products, a2actin failed in P
17/9/03: 32,34,36,38cyc @57C. Product in N pool at 38cyc only, not in P pool, a2a OK
Recommend 40 cyc,57C for x10-x80 cDNA dilutions

P3-23: AC129492.6 CLONE RP11-599B13 FROM HUMAN MALE, CHR 17. +/- fit 58/60 and 47/47 broken by a 10 base insert (shadow box) which replaces TT in the dd cloned sequence (in this – strand) and in AC073346.12 from chr 7. Also CA (bold) differs.

TGTATTAGGTAATCTAGAAAAGATTTAAGGTACACAAGAGTTTGTACTTAGGTTACACACAAATAC

TTCTGCACCATTTTATATCAGGAACTTGAGCATCCTCAGATTTTGGTATCCAAGGGGGTCCCGGAA

CCAATCCCCTAAGGATACCCAAGGATAACTGTCTATTACACTTCTGTTTTACAGATGTTAACACTG

AGGCTTAGAAAGGTCGTTTGGACTGCCCAAGGTCACAAAAGCTACTAAATGAGCACTGGGACTCTA

CTTTTCCAAAGTCTTAAACTCTACACTATTTTTTTAATAACACAATGGGTTTATATTTAATATAGT

ACTTCTCATCAGGAGGATGTTACTCAGTTAATATAAAGTTTTTTTCAAAACACTAAATCTCTTTTC

CATGTCGATGTCAATAGTTTTTTTTTTTAACATTAAATTTTTTTCCGCATTTCAGTATTAGATACA

CTGAATACATTTTTCTAAATGTTTTTTCCCCAGAGATAAAAGTTTTCCTTTTTGG*CTGACTCTC*CA

ATATCTAAATATAATA

TGTATTAGGTAATCTAGAAAAGATTTAAGGTACACAAGAGTTTGTACTTAGGTTACACACAAATAC
TTCTGCACCATTTTATATCAGGAACTTGAGCATCCTCAGATTTTGGTATCCAAGGGGGTCCCGGAA
CCAATCCCCTAAGGATACCCAAGGATAACTGTCTATTACACTTCTGTTTTACAGATGTTAACACTG
AGGCTTAGAAAGGTCGTTTGGACTGCCCAAGGTCACAAAAGCTACTAAATGAGCACTGGGACTCTA
CTTTTCCAAAGTCTTAAACTCTACACTATTTTTTTAATAACACAATGGGTTTATATTTAATATAGT
ACTTCTCATCAGGAGGATGTTACTCAGTTAATATAAAGTTTTTTTCAAAACACTAAATCTCTTTTC
CATGTCGATGTCAATAGTTTTTTTTTTTAACATTAAATTTTTTTCCGCATTTCAGTATTAGATACA
CTGAATACATTTTTCTAAATGTTTTTTCCCCAGAGATAAAAGTTTTCCTTTTTGG*CTGACTCTC*CA
ATATCTAAATATAATATTAACTTGGGAGACAACAAAACAAAAATCTAAT*AAAGGAATGGAG*AAAA
***GACTCCTCAACGATTCAGGAGGCAGTGATTATAACCGAACAGT*GGTGATTTCCTAAGATTCTGGGC**
AAGAACTTCCTTCTTCCTATTTCACATGCTTTGAGAGAATTCTAAGATTATGAAATATTACTTATG
TAAATTTCAGCCATCTTACTTCTTTGACCGCCTTTCTAACTAGCAAATTATAGGCTGCATTTTTCT
GTGCATTATTTGTTGTAAAGAAAACATTTTTTTTTCTATTTATAAATGTATGTTTGTTCCCATTTC
ATGGAAACAATGAAAAATGTGAAACTCCCTTATTTACTGATTCTTGGAACCTTTCACACACCAAAG
CTCAGGTTTAGCCTCTATAGCACAAAGGTTTTCAGGGTGAGGTTTGACTCAGTAGGCCTTTCAAAG
TCACATCTGTCCATTTCTTTTTCATGCGTATACCCCCGGGCGATTGCATCACCGCCTTCTACTGCT
TCCACAACAGGCAGCACCAAAAGCAGTGACATGAGGACTAAGCACAACTGTGTTGAAACTGAGGTC
ATGATATTGGAATCTTGAGGGCTGAAGGTTCCAAAGAAATGGTATATATAGAATTCTATCTGACTT
GAAATTTTCCCTTCCTGGAGCTCCGGATGCTGAGATTAAGAGGTTCCACGTGACATGACCTTCCAG
GAAGCAG

Insert comprises nucleotides 18698-18757 and 18770-18816 in this clone

AC129492.6 and AC104762.7 are identical in this region. AC129492.6 shows very close homology (>95%) with AC073346.12 (above) for the 220 bases 5' to the insert area in sequence above and for 400 base 3'. Outside of this range little homology. The boundaries are discrete. This chr 17 gene is probably a gene duplication from the chr 7 form found in AC073346.12, though the latter is probably the gene related to P3-23

Appendix 8: Analysis of clones sequences

Analysis of P4 clones

All P4 clones (P4.1, .2, .4) contained a unique sequence

Clone P4.1

P4.1

GAATTCACACGACTGT**AATCCCAGCTGCCTGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCAGGAGGC**
ACATGTTGCAATGAGCCGAGATTGCGCCACTGCACTCCAGCCTAGTCGATAGAGTGAGACTCAGTCTAA
AAAAAAAAAACTCGAG

AC007193 146180 bp PRI 17-JUN-1999 Homo sapiens chromosome 19,

BAC 82621 (CIT-B-139a18). +/+ fit 129/130.

GGATTAGAAACCCAGATCCCTTGAGCCCTGCCAAGTCCTCACTCCCTGGGAGGTGCTGACAGGAGG
CTTGAGGCCAGGCAGGAGCAGCCCATCTGGCCCATCTTCTGCCACCACCCCCAACCCCACCGCACC
GCACCCTTCCCCTCCACGCTTGCCATGTTTTCCTCAGCAGGACAGATATTTAATGGTGACTTTGTG
GACCGAGGCTCCTTCTCTGTAGAAGTGATCCTCACCCTTTTCGGCTTCAAGCTCCTGTACCCAGAT
CACTTTCACCTCCTTCGAGGTGAGCTGGGAAGTGACAAGGTTTGGGTTCATTGTGGGGTCCTGAGT
GGGGCACTCACTCTGCAAGGATAATAGTTGCAGCTGTATTTATTTTGTGGACTCGGAGATCATGCA
CAGGGCGACCTTTTAAGATTGAGTATGTAGTGCACTGCACAAAAGCATCACACCAAAGGACGCGTT
TACATCATAGATAGTGTAGAAATGGATGTTTACTCTGACCATTTTTCAGCAGATGGCAAACAAGGG
TGTGAGGATGGGCCCCTTATTCTAATTTGCACAAGGGCATGAAACAGGCTCCTGGGTGGCCCTAAA
TCTGGCACTAAAGCTGAGTGACTGAGTGAACAGACCAGGGCTCGAGGGTGGGAGTAAGGAGACTGT
TGACGAGGCAGTTGTGCCCCCTCAGATTCAGGCTTGGTGTCAGATTTGGGTTCTCTTGATTGAAGT
AGAGCCCGGAGCTGAGTGTGCTGGTTCACACCTGTAATCCCAGCACTTTGGAAGGCCCAGGTAGGA
GGATCACTTCAGCCCAGGAGTTTGAGACCAGCCTGGGCAACACAGTGAGACTTTGTCTCCAGTAAA
CATTTTTTAAAAAGGCCAGGCGCAGTGGCTCATGCCTGTAATCCCAGCATTTTGGGAGGCCAAGGC
AGGTGGATCATCTGAGGTCAAGAGTTCGAAACCAGCCTGTCCAATGTGGTGAAACCCCGTCTCTAC
TAAAAATACAAAAATTAGCCGGGCATGGTGGT*ACACGCCTGTAATCCCAGCTGCCTGGGAGGCTGA*
GGCAGGAGAATCGCTTGAACCCAGGAGGCACATGTTGCAATGAGCCGAGATTGCGCCACTGCACTC
CAGCCTAGTCGATAGAGTGAGACTCAGTCTAAAAAAAAAAAAAAAAAAGCCAGGCATGGTGGCACCGT
GCACCCGTAGTCCCAGCTTCTTGGGTGGCCAAAGTGGAAGGATTGCTTGAGCCCAGGAGTGTAAGG
CTGCAGTGAGCCATGATCACACCGCTGCACTCCAGCGTGGGCAACAGAGCAAGACCCTGTCTCTTT
GAAAAAAAAAAAAAAAAGTAAGAAACTTGGAAGTAGAGCCCAGAGTATTTGCTGGAGGAACAGACAC
AGGTGTGAGACAAAGAGGGAACGAAAGGGGCCCTCTAGGGTTTTGGCCGGAGTATCTGGAGGGCTA
TGGCTGCCAGTGACGAAGACGGGAACATGTAGAGCAGGGTGTGGCCAGGAGCTGAGGAGCTCCCCT
TTGGATGTGGCTGAGACTCAGGAGACATGGGATGTGGGTGGGGTTCCAAGGGGTGTTGAAAATGTA
GGTTTCTTGGTGTCCAGATGAGATTAGACAAGAGGAGCTCACTGGGATGTGAGGGCCACACAGGGG
AGGCCCTGCCCATGGCGGGGCCGGGGTGAGTTGGGAGGTGAGTGATAGGCTGGGGCGAAGCCTGCA
TGCCCGTGTCCCTGGGCTCACAGGGTTCACGCAGCAGTGTCCAGCACAGGGTCAAAGCTCAGGAAG
CCCGACTGTGGGCCTCGTTGCAGGAGGCAACAAGTGCTCAGGCCGAGAAGTCAGACAAGCAGAGTC
TGAGCAGATGCGAGACTGGGCAGCCTTCTCCTTGCCCCCAAGCTCTGGAAGGGAAGAAGGAGGCCG
GGTGCTTGTGTCTGAGACAGAAGGATGGAGCTCAGGGTGGGGTGGGCTGGGGCACATGTTCTGTAG
ATGGGCCTACTTTTTGGTTTTGAGACGGTCTTGCTTTGCTCTGTTGCCCAGGCTGGAGTGCAGTAG
CATGAGCATAGCTCACTGCAGCCTCTGCTTCGTGGGCTCAAGTGATCCTCTGGCCTCAGCCTCCCA
AGTAGCTGGCACTATAGGCACGCACCACCATGCCCAGCTAATTTTTAA

Insert sequence is 130863 – 130992 and in italics. Bold is different in insert (A).

NCBI Note:

gene 92311..135795 /gene="PPP5C" "protein phosphatase 5, catalytic subunit"
mRNA join(92311..92431, 98962..99203, 120818..120965, 121667..121788, 128625..128690, 128994..129092, 130018..130123, 132307..132449, 132580..132667, 133622..133662,133767..133945, 135265..135346, 135498..135795)
CDS join(92311..92431,98962..99203,120818..120965, 121667..121788, 128625..128690, 128994..129092, 130018..130123, 132307..132449, 132580..132667, 133622..133662,133767..133945,135265..135346, 135498..135560) /codon_start=1 /product="PPP5_HUMAN"/ protein_id="AAD22669.1" /db_xref="GI:4558638"

This fragment appears to be primed from a stretch of 16As within the gene here but it does not arise from within the coding sequence or mature transcript, as detailed above. It may arise from an mRNA precursor. It may arise from a transcript within a transcript (cf rev-erbA) It may also arise from genomic DNA, though this is unlikely hopefully.

Clone P4.2
P4.2
GAATTCACACGACTGT**AATCCCAGCTACTTGGGAGGCTGAGTCATGAGAACCTCTTGAACCCGGGAAGT**
GGAGGTTGCAATGAGCCGAGATTGCGCCACTGCACTCTAGCATGGGCAACAGAGTATGACTATGTCTTT
AAAAAAAAAAAACTCGAG

AC080078.7 150567 bp PRI 16-APR-2002 Homo sapiens BAC clone RP11-461L13 from chr 4. +/- fit 131/132

ACAATCAGTACTAAAAAGTTTAACTCAACTAAAAAGTTTATAATTAGTGGAAAATACTATGAATAC
TATTAGTTCTTTTCTTTACCTTGCTAACATTTAAAAATACACTTCCATTTTATAATAGTAGGAGAA
TGTGTTTATATTCCTATGTCAGTGATTTTATTATTATCATGACAACACTTTGTACTCTAGAAAGGA
CTAGGAAACCACCACAGTTAGCTGTCTTGCCCTGAGCACAGAATTTAGATTACAGACAGTTCAAAA
TTACAGACAGTTCAAAATTTCAACATCAATGAAAGGAATGCCAAGCATGTTGAGACTGAAATACTT
TTTGAAATTTCACTAACTCACTGTCATTAACTTTCTGGGATGTGAGTGCTTATTAAAAGGTTTGCT
TATTAAAACTGGCAAACTAATAAACCTGTTCTATTACAGATATCAACTGGAATAGTATATATGCCC
AGCATTTACATTTTCACTAACACAAATAGGTGGCAGTATTGGCCAATAAGCAATTTTCCATATTTA
TTCATTCAGTAAGCATTTGTTTAGCACCTACTTTGTGTTGGGCACAGAATATTATATACATCAATT
GAAACTTTGTTCTTTCTCCATTGGAAATACAGTCAATTAACACAAGAGCATAGTGTAACGTAATAT
ACACAAACATCTGACTGTTAGAACTCATACTGAAATTAGTTCTAAAAAATTTTTGAGAACATGTAA
AATAAAACAAGATGAGCCAGCTTTAGTTCAAAACTACCATCTAAGTAAACAAATACTGTGACACTA
GTGCCTTAGAAAAAAATCCCAAACACTAAACACTGTAATAGATAATCTTACTTTACTTAAACTTAT
TTTTTGGGGGGAAGGGGATGTTTATAATATATATAATCCAAACCTTTGTAAATCAAGCTGCAAATC
AAAATGTTATAGCTGACTGCTTTTAAATAAATTTTAATATAATTACAGAAGTAGTAGTTTTTTCTT
TTTTTTTTTTAAAGACATAGTCATACTCTGTTGCCCATGCTAGAGTGCAGTGGCGCAATCTCGGCT
CATTGCAACCTCCACTTCC*T*GGGTTCAAGAGGTTCTCATGACTCAGCCTCCCAAGTAGCTGGGATT
***ACAGTCGTGT*GCCACCACACCCCACTAATTTTTCTATTTTTAGTAGAGATGGGGTTTCACCATGTT**
GGCCAGGCTAGTCTCGAACTCCTGGCCTCAAGTAATCCGCCCGCCTCAGCCTCCCAAGGTGCTGGG

ATTACAGGGGTAAGCCACCGTGCCTGGCCAGAAGTTTTAATTCTTGAAGTTTAAGCTATTTAAAAC
TCTCCTTACAAAGACACAGCCTCTGATTAGTGTGACACACTAATCAACTGCAGTATTAGTTCCAAC
AGTGTAAGTTTTTTCATTCCTTCAGATTGGGCCAAATTAGTCATCTGATGGACACATGCACAAATG
TATTTATCTACAAATATATAAATATACACACATACATAATTCATACCCAATAGTGCAAGTTAAGAG
TTCTATTTATGCCAGCCACAGAATAAAATAATATGGGGGTTTTATTCGCTCCTCTAAAATTTATAT
TGTTACAGAGCTGTCAGACTAATACTAAGTCAACATCCTCTACTTTTTCACAGACAGCCGGCATCA
GTGCAATAATTTGACAACAAAGTTAGCAAGTCACCCTGGAGTTTACTGATTTATCCAGAAAGCAAT
GAATCTTTCATGCTTAAAATCCAAAATAAACTACCAATAAGCATTTGGAATTCATAAGTTTCCTCT
CAAAATTTTATTGTGAATTTCTTATAGTAATACCTTCAGTAAAGTAAGACAGATATCATGGAAAAA
AAGTATAAAATGTAAGTTTGACTTCAAGAAAAATGAAACCACAAATATGACCCAGGAAAGAATTTA
ACATGGAGAAAGAATGAAATCAAATTTCCCTCAATATAAGGCCTAAACCCTATTCACTCTTCTGCT
TTCTTTACACAAATCCATATTTATTGCTGCTGTTTGTGATTTTCAAATATATTTGCCTGACTTGAG
AAGAATTTCCCATAGGTGTGATATATTAATCTTGTATTCATTTCAAGAACTGTTTAAAATTCTGGG
ATTAATTTCCTAAATTAGTGATTAGTCATGATAAAAAAGTTTTGAGAA

Insert sequence is 83681 - 83812 and in italics. Bold is different in insert (C)

Complementary strand

AAAAAGTAGAGGATGTTGACTTAGTATTAGTCTGACAGCTCTGTAACAATATAAATTTTAGAGGAG
CGAATAAAACCCCCATATTATTTTATTCTGTGGCTGGCATAAATAGAACTCTTAACTTGCACTATT
GGGTATGAATTATGTATGTGTGTATATTTATATATTTGTAGATAAATACATTTGTGCATGTGTCCA
TCAGATGACTAATTTGGCCCAATCTGAAGGAATGAAAAAACTTACACTGTTGGAACTAATACTGCA
GTTGATTAGTGTGTCACACTAATCAGAGGCTGTGTCTTTGTAAGGAGAGTTTTAAATAGCTTAAAC
TTCAAGAATTAAAACTTCTGGCCAGGCACGGTGGCTTACCCCTGTAATCCCAGCACCTTGGGAGGC
TGAGGCGGGCGGATTACTTGAGGCCAGGAGTTCGAGACTAGCCTGGCCAACATGGTGAAACCCCAT
CTCTACTAAAAATAGAAAAATTAGTGGGGTGTGGTGG*CACACGACTGTAATCCCAGCTACTTGGGA*
GGCTGAGTCATGAGAACCTCTTGAACCC*A*GGAAGTGGAGGTTGCAATGAGCCGAGATTGCGCCACT
***GCACTCTAGCATGGGCAACAGAGTATGACTATGTCTTT*AAAAAAAAAAAAAGAAAAAA**CTACTACTT
CTGTAATTATATTAAAATTTATTTAAAAGCAGTCAGCTATAACATTTTGATTTGCAGCTTGATTTA
CAAAGGTTTGGATTATATATATTATAAACATCCCCTTCCCCCCAAAAAATAAGTTTAAGTAAAGTA
AGATTATCTATTACAGTGTTTAGTGTTTGGGATTTTTTTCTAAGGCACTAGTGTCACAGTATTTGT
TTACTTAGATGGTAGTTTTGAACTAAAGCTGGCTCATCTTGTTTTATTTTACATGTTCTCAAAAAT
TTTTTAGAACTAATTTCAGTATGAGTTCTAACAGTCAGATGTTTGTGTATATTACGTTACACTATG
CTCTTGTGTTAATTGACTGTATTTCCAATGGAGAAAGAACAAAGTTTCAATTGATGTATATAATAT
TCTGTGCCCAACACAAAGTAGGTGCTAAACAAATGCTTACTGAATGAATAAATATGGAAAATTGCT
TATTGGCCAATACTGCCA

This fragment appears to be primed from a stretch of 16As within the genomic sequence (underlined) but it does not arise from within the coding sequence or mature transcript, as detailed in the NCBI notes. It may arise from an mRNA precursor. It may arise from a transcript within a transcript (cf rev-erbA) It may also arise from genomic DNA, though this is unlikely hopefully!.

Clone P4.4
P4.4
GAATTCACACGACTGT**AGTCCTAGCACATTGGGAAGCCAAGGTGGGAGGATTGCTTGAGGCCAGGAGTT**
CAAGGCTGCAGTGAGCTATGATTGCACACTGTACTCTAGCCTGGGAGACAGAGTGAGACACTGTCTCTA
AAAAAAAAAAACTCGAG

AF130054 1653 bp mRNA 08-MAY-2001, Homo sapiens clone FLB4816 PRO1252 mRNA, complete cds. Expressed in human fetal liver. +/- fit (should be +/+ if the hit is an mRNA sequence given the direction of sequencing ie seq end in poly A from ET12C primer)

GCTTTTTTTTTTTTTTTTTTTTTTTTTT*AGAGACAGTGTCTCACTCTGTCTCCCAGGCTAGAGTAC*
AGTGTGCAATCATAGCTCACTGCAGCCTTGAACT*CCTGGCCTCAAGCAA*TCCTCCCACCTTGGCTT
***CCCAATGTGCTAGG*TTTACAGGCGTGAAAACATTGTGTTGTGTGTGTGTGTTTTTTTTTTTTTT**
TTTTTTAGACAGAGTTTCGCTCTTGTTGTCCAGGCTGGAGTACAATGGCTTGATCTTGGCTCACTG
CAATCTCTGCCTCGCAGGTTCAAGCGATTCTCCTGCCTCAGCCTCTGGAGTGGCTGGGATTACAGG
CGCCCACCATCACGCCCGGCCAATTTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGGCC
AGGCTGGCCTCGAACTCTTGACCTCAGGTGATCCACCTGCCTTGGCCTCCCAATGTACTGAGATTA
CAGGCATGAGCCACCACGCCCGGCCAAAAACATTTAAAAAATGACTGTCCCTGCTCAAATACTGCA
GTAGGAAATGTAATTTGACATATATCACTTCCAGAAAAAAACTTTAAATCTTTCTATAAAATGAAT
TTGATACATCATCAGCATGAAGTGAAGTTAAAATCTCTTACAAAGTAAATTCAGGTATATCAACAA
TGAGATCCAAAAGTATCGGTTCAAGATCAAAGACTCATATGAGGTAAAGGCAAATAAAGCCCCTCA
TGCTTTCAGACATTTACTTGTTATCCTTAAAATGATTATTGTTCTAAGGTGCAAATCTTATCATTC
AGTGACCTATTTGCTATTTGGCTGTTTATCTCCTTTAAATAAAATACCATACAGGTTATAATGTAC
ATTTTAAAATCAAATATGAAAGTACACATATGTGAACAAGACATTAATTTAAATATCAATTATATT
CATAATACAGAAGTCTAAGAATGCTATTTGGTCATTTCAATTAGATGCTGATATATTTGGTCAAGA
AAACCCACATTCCTATCAGCCTAAGGCAAATAGTAAATGCCTCTATTTTTATTAAGTGGACTATAA
ACAGTAGACATTATTGTTTGATACTACTGGAATGCTCTCAATTAGAACGTACACTGATAACAAATA
TGTCTTTCTAATCTGGGTGATAACCTCAATGATTACATGGAAATCTACAAGTAACAGTCTATAACT
TAAAGCAGTAGAGCACCTGGTAATTGTCATACAGTGCCTCTCTATGAATTTAGAACATTCTACTTA
CATTGGCAGTGGAGAGAAAAGACCCCTTCACACTGAGACTTCCTGTGCACCGCGCCTGCCGCCTGC
CTCAGCTTAGGACATGGTACAGTCTTCTTTGCTTTTGCCCTTCTCATTCCCTCCTCTGGTATCTTC
TCTGCTTTCTGGATTGAAGTGCCCTGGCTCTCTTCCTGGTGCCGACAAGTCATTGTTCTCTAGGCT
ATTGCTGTCTAAAGCTTTCCTGACGGTCTGACTTGGATGTTCTGACATCTCACACCTTTCTGTCTT
CCTCTTCTCACCCTCCTCCCTCCCCTTAGCCATCACTTCTGGGAAGTAAAGAACTTGACTTAGTGC
CGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AA

Primers are indicated in boxes, lower primer shaded (ie 101,795; 798,1272)

AC012076 212275 bp; PRI 01-MAR-2002; Homo sapiens BAC clone RP11-497D24 from chr2, complete sequence. +/+ fit 118/118

GAGGTTATCACCCAGATTAGAAAGACATATTTGTTATCAGTGTACGTTCTAATTGAGAGCATTCCA
GTAGTATCAAACAATAATGTCTACTGTTTATAGTCCACTTAATAAAAATAGAGGCATTTACTATTT
GCCTTAGGCTGATAGGAATGTGGGTTTTCTTGACCAAATATATCAGCATCTAATTGAAATGACCAA
ATAGCATTCTTAGACTTCTGTATTATGAATATAATTGATATTTAAATTAATGTCTTGTTCACATAT
GTGTACTTTCATATTTGATTTTAAAATGTACATTATAACCTGTATGGTATTTTATTTAAAGGAGAT
AAACAGCCAAATAGCAAATAGGTCACTGAATGATAAGATTTGCACCTTAGAACAATAATCATTTTA

AGGATAACAAGTAAATGTCTGAAAGCATGAGGGGCTTTATTTGCCTTTACCTCATATGAGTCTTTG
ATCTTGAACCGATACTTTTGGATCTCATTGTTGATATACCTGAATTTACTTTGTAAGAGATTTTAA
CTTCACTTCATGCTGATGATGTATCAAATTCATTTTATAGAAAGATTTAAAGTTTTTTTCTGGAAG
TGATATATGTCAAATTACATTTCCTACTGCAGTATTTGAGCAGGGACAGTCATTTTTTAAATGTTT
TTGGCCGGGCGTGGTGGCTCATGCCTGTAATCTCAGTACATTGGGAGGCCAAGGCAGGTGGATCAC
CTGAGGTCAAGAGTTCGAGGCCAGCCTGGCCAACATGGTGAAACCCTGTCTCTACTAAAAATACAA
AAAATTGGCCGGGCGTGATGGTGGGCGCCTGTAATCCCAGCCACTCCAGAGGCTGAGGCAGGAGAA
TCGCTTGAACCTGCGAGGCAGAGATTGCAGTGAGCCAAGATCAAGCCATTGTACTCCAGCCTGGAC
AACAAGAGCGAAACTCTGTCTAAAAAAAAAAAAAAAAACACACACACACACAACACAATGTTTTCA
CGCCTGTAAA*CCTAGCACATTGGGAAGCCAAGGTGGGAGGATTGCTTGAGGCCAGGAGTTCAAGGC*
TGCAGTGAGCTATGATTGCACACTGTACTCTAGCCTGGGAGACAGAGTGAGACACTGTCTCTAAAA
***AAAAAAAAAAAAAAAAAAAAAG*TTTTTGAACCTTAAAATACTTTGTTTGAATTTCTAATCATCATT**
CAAAAGAGCAGTAAAAAATGGTTACTTGTTCTTGTACAAGCTACTAATTAGACTATAGTAGGATAT
TTTAAAGAGCTGAATCACTTTTGGTATTTTGGTATAAATATTTTCATTTGTTATGTCCCAGTATAT
TCTTACTGGAAAATTCTTGTTTTGATCTGCCTGAAGAAAATATCTGTTTTCTATATAAAAAAATTT
TTTAAAATAATTGTAAAGTTAGATTTAAAATTGTAAAATATAAAATCACAAAGGAATGTACCTTAT
GAATGTTGTTGACATTTTATGAAATTATGTGGATTCATATTACTGTTACAAGATAGAATTGAATGC
AAAAAGACCAAAACCTCAATAAAATTTGAGGAAAACGTGTTATTATGTAATTGAAATAAAAACATT
TTATAATTGTGCAAGCCTGTGGTTCCACTCTTTGGGCTGCTTGAAGTCTGAGTCTGACTACTGTTG
TATGTAATACCACCTTTGAGTAATATCTGCTTGGGGGTTGCTGATTCCTATTGGGGTTCCGTGGAA
GACCCACGCCTGATGCCCTCCCTCAGTAAGGAATGCACTTCCCTCCTGGCATCGAGCCTGGGGCTA
GCATGGGCATTACTGGGAGAACTGAGACGATTGTCACGCACCATCCCCTGTGATCTGTGGTTCTGA
TGTGCAGCCTCAGCTGAAAACGGTCTTCACCTGTGGCTGTGCTTACCTGCGCCACTACTTACAGTG
TTTTGAAGAGCAGGATGAAAACGGCAAAAACCTTGACTGTTTCTGCTCAAGTGTCCTGAAGTAGAG
GATGGTGGGGATGATAAATGCTTGCTAGGAAATAAGTCCAAGGGCTCTATAGGACTTCACAGGGTT
TTAGTTTATGTCTCTAACTTTAGCAAAGCTGCATTCCTATTGGAATGCATACTGGAAACAGCTCTC
ATTCCT

Insert fit is to nuc 87039 – 87156; underlined area is EST BE674143 match

```
NCBI notes
misc_feature 86584..87233/note="similar to EST BG286780 (NID:g13039960)"
repeat_region 86701..87000/rpt_family="Alu"
misc_feature 87005..87632/note="match to EST AW976721 (NID:g8167954)"
repeat_region 87027..87181/rpt_family="Alu"
misc_feature 87040..87638/note="match to EST BE674143 (NID:g10034684)"
misc_feature  87075..87633/note="match  to  EST  AI091818  (NID:g3430877)
qa58b09.s1"
```

Complementary sequence for 500 bp each side of insert – note sequence is the same as for AF130054 1653 bp mRNA for the first 540 bp of the mRNA (ie 3’ to fragment in this sequence). Italics = P4.4 insert, underlined = EST BE674143

ATGCCAGGAGGGAAGTGCATTCCTTACTGAGGGAGGGCATCAGGCGTGGGTCTTCCACGGAACCCC
AATAGGAATCAGCAACCCCCAAGCAGATATTACTCAAAGGTGGTATTACATACAACAGTAGTCAGA
CTCAGACTTCAAGCAGCCCAAAGAGTGGAACCACAGGCTTGCACAATTATAAAATGTTTTTATTTC
AATTACATAATAACACGTTTTCCTCAAATTTTATTGAGGTTTTGGTCTTTTTGCATTCAATTCTAT
CTTGTAACAGTAATATGAATCCACATAATTTCATAAAATGTCAACAACATTCATAAGGTACATTCC
TTTGTGATTTTATATTTTACAATTTTAAATCTAACTTTACAATTATTTTAAAAAATTTTTTTATAT
AGAAAACAGATATTTTCTTCAGGCAGATCAAAACAAGAATTTTCCAGTAAGAATATACTGGGACAT
AACAAATGAAAATATTTATACCAAAATACCAAAAGTGATTCAGCTCTTTAAAATATCCTACTATAG
TCTAATTAGTAGCTTGTACAAGAACAAGTAACCATTTTTTACTGCTCTTTTGAATGATGATTAGAA
ATTCAAACAAAGTATTTTAAGGTTCAAAAACTTTTTTTTTTTTTTTT*TTTTTTTTTTAGAGACAGTG*
TCTCACTCTGTCTCCCAGGCTAGAGTACAGTGTGCAATCATAGCTCACTGCAGCCTTGAACTCCTG
***GCCTCAAGCAATCCTCCCACCTTGGCTTCCCAATGTGCTAGG*TTTACAGGCGTGAAAACATTGTGT**

TGTGTGTGTGTGTTTTTTTTTTTTTTTTTAGACAGAGTTTCGCTCTTGTTGTCCAGGCTGGAGT
ACAATGGCTTGATCTTGGCTCACTGCAATCTCTGCCTCGCAGGTTCAAGCGATTCTCCTGCCTCAG
CCTCTGGAGTGGCTGGGATTACAGGCGCCCACCATCACGCCCGGCCAATTTTTTGTATTTTTAGTA
GAGACAGGGTTTCACCATGTTGGCCAGGCTGGCCTCGAACTCTTGACCTCAGGTGATCCACCTGCC
TTGGCCTCCCAATGTACTGAGATTACAGGCATGAGCCACCACGCCCGGCCAAAAACATTTAAAAAA
TGACTGTCCCTGCTCAAATACTGCAGTAGGAAATGTAATTTGACATATATCACTTCCAGAAAAAAA
CTTTAAATCTTTCTATAAAATGAATTTGATACATCATCAGCATGAAGTGAAGTTAAAATCTCTTAC
AAAGTAAATTCAGGTATATCAACAATGAGATCCAAAAGTATCGGTTCAAGATCAAAGACTCATATG
AGGTAAAGGCAAATAAAGCCCCTCATGCTTTCAGACATTTACTTGTTATCCTTAAAATGATTATTG
TTCTAAGGTGCAAATCTTATCATTCAGTGACCTATTTGCTATTTGGCTGTTTATCTCCTTTAAATA
AAATACCATACAGGTTATAATGTACATTTTAAAATCAAATATGAAAGTACACATATGTGAACAAGA
CATTAATTTAA

Compilation of AC012076 complementary sequence and AF130054 1653 bp mRNA to give the +/- strand to the fragment (+/+ wrt mRNA), providing the 5' sequence omitted from the mRNA which is an EST (underlined). This was used to design further primers .

ATGCCAGGAGGGAAGTGCATTCCTTACTGAGGGAGGGCATCAGGCGTGGGTCTTCCACGGAACCCC
AATAGGAATCAGCAACCCCCAAGCAGATATTACTCAAAGGTGGTATTACATACAACAGTAGTCAGA
CTCAGACTTCAAGCAGCCCAAAGAGTGGAACCACAGGCTTGCACAATTATAAAATGTTTTTATTTC
AATTACATAATAACACGTTTTCCTCAAATTTTATTGAGGTTTTGGTCTTTTTGCATTCAATTCTAT
CTTGTAACAGTAATATGAATCCACATAATTTCATAAAATGTCAACAACATTCATAAGGTACATTCC
TTTGTGATTTTATATTTTACAATTTTAAATCTAACTTTACAATTATTTTAAAAAATTTTTTTATAT
AGAAAACAG[405]ATATTTTCTTCAGGCAGATCAAAACAAGAATTTTCCAGTAAGAATATACT[455]GGG
ACATAACAAATGAAAATATTTATACCAAAATACCAAAAGTGATTCAGCTCTTTAAAATATCCTAC
TATAGTCTAATTAGTAGCTTGTACAAGAACAAGTAACCATTTTTTACTGCTCTTTTGAATGATGAT
TAGAAATTCAAACAAAGTATTTTAAGGTTCAAAA(startAF130054)ACTTTTTTTTTTTTTTTTT*T*
TTTTTTTTTAGAGACAGTGTCTCACTCTGTCTCCCAGGCTAGAGTACAGTGTGCAATCATAGCTCA
***CTGCAGCCTTG[719]AACTC[101]CTGGCCTCAAGCAATCCTCCCACCTTGGCTTCCCAATGTGCTAGG*T**
TTACAGGCGTGAAAACATTGTGTTGTGTGTGTGTGTTTTTTTTTTTTTTTTTTAGACAGAGT
TTCGCTCTTGTTGTCCAGGCTGGAGTACAATGGCTTGATCTTGGCTCACTGCAATCTCTGCCTCGC
AGGTTCAAGCGATTCTCCTGCCTCAGCCTCTGGAGTGGCTGGGATTACAGGCGCCCACCATCACGC
CCGGCCAATTTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGGCCAGGCTGGCCTCGAAC
TCTTGACCTCAGGTGATCCACCTGCCTTGGCCTCCCAATGTACTGAGATTACAGGCATGAGCCACC
ACGCCCGGCCAAAAACATTTAAAAAATGACTGTCCCTGCTCAAATACTGCAGTAGGAAATGTAATT
TGACATATATCACTTCCAGAAAAAAACTTTAAATCTTTCTATAAAATGAATTTGATACATCATCAG
CATGAAGTGAAGTTAAAATCTCTTACAAAGTAAATTCAGGTATATCAACAATGAGATCCAAAAGTA
TCGGTTCAAGATCAAAGACTCATATGAGGTAAAGGCAAATAAAGCCCCTCATGCTTTCAGACATTT
ACTTGTTATCCTTAAAATGATTATTGTTCTAAGGTGCAAATCTTATCATTCAGT[795]GACC[799]TATT
TGCTATTTGGCTGTTTATCTCCTTTAAATAAAATACCATACAGGTTATAATGTACATTTTAAAATC
AAATATGAAAGTACACATATGTGAACAAGACATTAATTTAAATATCAATTATATTCATAATACAGA
AGTCTAAGAATGCTATTTGGTCATTTCAATTAGATGCTGATATATTTGGTCAAGAAAACCCACATT
CCTATCAGCCTAAGGCAAATAGTAAATGCCTCTATTTTTATTAAGTGGACTATAAACAGTAGACAT
TATTGTTTGATACTACTGGAATGCTCTCAATTAGAACGTACACTGATAACAAATATGTCTTTCTAA
TCTGGGTGATAACCTCAATGATTACATGGAAATCTACAAGTAACAGTCTATAACTTAAAGCAGTAG
AGCACCTGGTAATTGTCATACAGTGCCTCTCTATGAATTTAGAACATTCTACTTACATTGGCAGTG
GAGAGAA[1272]AAGACCCCTTCACACTGAGACTTCCTGTGCACCGCGCCTGCCGCCTGCCTCAGCTT
AGGACATGGTACAGTCTTCTTTGCTTTTGCCCTTCTCATTCCCTCCTCTGGTATCTTCTCTGCTTT
CTGGATTGAAGTGCCCTGGCTCTCTTCCTGGTGCCGACAAGTCATTGTTCTCTAGGCTATTGCTGT
CTAAAGCTTTCCTGACGGTCTGACTTGGATGTTCTGACATCTCACACCTTTCTGTCTTCCTCTTCT

CACCCTCCTCCCTCCCCTTAGCCATCACTTCTGGGAAGTAAAGAACTTGACTTAGTGCCGGAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

New primers (U405, L719; U455, L719) on this compiled sequence (shadowed boxes), old primers (ie 101,795; 799,1272) in normal boxes. Lps are shaded, hatched areas are shared between Lps and Ups

Up: CCTGGCCTCAAGCAA (15mer, 101-115) 714 bp 59C
Lp: GCCAAATAGCAAATAGGTCA (20mer, 795-814)
10/6/3: P pool, 30,35,40,45, then further 20,30,40,50 cyc @53C. No products. Sigma said primers dodgy
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @57C. No products, though 18S and a2 actin are lower than normal
17/9/03: N and P pools, 40,45,50,55cyc @57C. No products, a2a/18S OK
No Products with these primers

Up: CTATTTGCTATTTGGCTGTT (21mer, 799-819) 493 bp 59C
Lp: TCAGTGTGAAGGGGTCTTT (19mer, 1272-1290)
10/6/3: P pool, 30,35,40,45, then further 20,30,40,50 cyc @53C. No products. Sigma said primers dodgy
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @57C. No products, though 18S and a2 actin are lower than normal
17/9/03: N and P pools, 40,45,50,55cyc @57C. From 40 cyc 470bp product.
Recommend 42 cyc,59C for x10-x80 cDNA dilutions

Second phase
P4-4**: Compilation of AC012076 genomic complementary sequence and AF130054 1653 bp PRO1252 mRNA to give the +/- strand to the fragment (as used for primers previously) but including the 5' sequence omitted from the mRNA which is an EST.**

Lp: CTTGAGGCCAGGAGTT (16mer, 719-734)

Up: GATATTTTCTTCAGGCAGAT (20mer, 405-424) 330 bp 55C
16/6/3: 40,50 cyc @55C, N and P pools. Equal product at 50cyc- a2 actin was low
4/7/3: N/P pool, 30-55 cyc @57C. Products from 35 cyc onwards, tends to doublet
17/9/03: N and P pools, 32,35,39,41 cyc@57C. products at 35 cyc weak, P>N, a2a/18S OK
Recommend 38 cyc (check intensity),55C for x10-x80 cDNA dilutions

Up: TGGGACATAACAAATGAAAA (20mer, 455-474) 281 bp 58C
16/6/3: 40,50 cyc @55C, N and P pools. Equal product at 50cyc- a2 actin was low
4/7/3: N/P pool, 30-55 cyc @57C. Products from 45 cyc onwards, tends to doublet
17/9/03: 41,44,47,50 cyc@57C. products at 41 cyc weak, P>N, a2a/18S OK; doublet in some 370bp/440bp
Recommend 42 cyc (check intensity),57C for x10-x80 cDNA dilutions

Analysis of P5 clones and P7-1 clone insert sequence. Homology detected with BAC chromosome 15 clones listed below.

P7-1

GAATTCACACGACTGT**GAAAATGCCCATTTCATTGCACGCTTTCTAACATTTATACCAATCTGAT**
AAATAAAAGCTGGTACCTAGAAAAAAAAAAAACTCGAG
P5-1
GAATTCACACGACTGT**GAAAATGCCCATTTCATTGCACGCTTTCTAACATTTATACCAATCTGAT**
AAATAAAAGCTGGTACCTAGAAAAAAAAAAAACTCGAG
P5-3
GAATTCACACGACTGT**GAAAATGCCCATTTCATTGCACGCTTTCTAACATTTATACCAATCTGAT**
AAATAAAAGCTGGTACCTAGAAAAAAAAAAAACTCGAG
P5-5
GAATTCACACGACTGT**GAAAATGCCCATTTCATTGCACGCTTTCTAACATTTATACCAATCTGAT**
AAATAAAAGCTGGTACCTAGAAAAAAAAAAAACTCGAG

Summary: The sequence AC126339.6 is probably most accurate and is exactly the same as AC136698.6 and AC 005630.1 except that: 1) it is sequenced in the opposite direction (no problem) 2) a TTTTTTT insertion in AC126339.6, though this is reflected as a run of 22Ts in AC126339 and 15Ts in AC136698.6 and AC 005630.1.

Small differences exist between AC126339.6 and AC139425.3, noted in bold type below, which probably represent sequence errors as the sequences were derived from the same group and, presumably, sampled individual. Alternatively it is possible each sequence is derived from a different chromasome from the diploid chr15 pair.

Even more significant is that AC 005630.1 quotes the region of the insert homologous sequence as being similar to EST AA443771 and ESTAI338526, suggesting that this sequence is at least expressed at the RNA level.

AC126339.6+/-, sub 8/4/03
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATTTTTTTTCTACCTTAACTAATGCTTTGGACA
TCATCAACAGGTAGAGCTAAATCCTTGAAACCTTCCAAGTGGTGGCTTTCAGTTATTGCTGAAT
TGGTTTTTAGAGATGGAACAAATTATATTGTATGGAAACTTTTTTTTTTTTTTTTTTTTGAG
ACAAAGTCTCAGCTTGTCACCCAGGCTGGAGGGCAATGGCATGATCTTGGCTCACTAAAACCTCC
ACCTCCCAGGTTCAAGTGATCCTCTTGCCTCAGCCTCCCGAGTAGTTGGGATTACAGGGGCCTGC
CACCATGCCCGGCTAATGTTTGTGTTTTTACTAGAGATGGGGTTTCATCATGTTGGCCAGGCTTG
TCTCGAACTCCTGACCTCAGGTGATCCACTCACCTTGGTCTCCCAAAGTACTGGGATTACAGGCA
TGAGCCACCATACCCAGCCTTTTTTTC*TTCTAGGTACCAGCTTTTATTTATCAGATTGGTATAAA*
***TGTTAGAAAGCGTGCAATGAAATGGGCATTTTCACAGTCGTGG*GCAGAAAGTATAATTATCTTTGA**
CTTTCTAGAAAGCAGTCTGGCATTCTAGAAACTTGCCTAACCTCTTCCCATTTAGACAAGATGAA
TTCTGACAGGGCCAGCCTTGTCCCTGTGATTCCGTATCTCCCAGAAAGAGAGGTCTAGTCTCAGG
GAAAACCCAGATTTTCTTGGCTTAGCCCACCTGACAGCTAATCACTGGAAATGGGGCGGGCCGGT
AGAGTCCTTTGGTCAGGTTTTGTGTCAAGAGAGGGATGTGGAAAGATGGGAGAGAGGTAGCAAAA
CTGGCCTCAATGGAACTGTGTAAGTTAACATAGAATGGCAAAGGAATGTTTCTTCCAAGGAAGAA

ATTCTAGGGAAGGAAT**AAAGT**A**GAGGGGAAGGCAGCAGTTCTCAAAGTTTTGGGGTCAGGATTCC**
TTTACACTCTTAAAAGTATATTGAGGGCCCAAGGAGCTTTTGTGTATGTGGGTTATATCTATTGG
TA

Insert comprises nuc 84817 - 84896 of this clone (RP13-98N21) in +/- fit. Sequence here includes 480 bp each side of insert. Note excerpts from NCBI report regarding this region

repeat_region 84331..84368

/rpt_family="(TG)n"

repeat_region complement(84505..84808)

/rpt_family="AluSx"

repeat_region complement(85277..85421)

/rpt_family="Charlie1"

P5: AC139425.3+/-, sub 21/2/03
AGAGAGAGAGAGAGTGTGTGTGTGTGTGTGTGTGTGTGTATTTTTTTTCTACCTTAACTAATG
CTTTGGACATCAACAGGTAGAGCTAAATCCTTGAAACCTTCCAAGTGGTGGCTTTCAGTTATTGC
TGAATTGGTTTTTAGAGATGGAACAAATTATATTGTATGGAAACTTTTTTTTTTTTTTTTGAGAC
AAAGTCTCACCTTGTCGCCCAGGCTGGAGAGCAATGGCATGATCTTGGCTCACTG**C**A**A**T**CTC**T**G**C**
CTCCCAGGTTCAAGTGATT**CTCTTGCCTCAGCCTCCCGA**A**TAGTTGGGATTACAGG**T**GC**T**TGCCA**
CCATGCCCGGCTAATT**TTTGT**C**TTTTTACTAGAGATGGGGTTTCATCATGTTGGCCAGGCTTGTC**
TCGAACTCCTGACCTCAGGTGATCCACTCACCTTGGC**CTCCCAAA**C**TACTGGGATTACAGGCAT**A
AGCCACCATACCCAGCCTTTTTTTC*TTCTAGGTACCAGCTTTTATTTATCAGATTGGTATAAATG*
***TTAGAAAGCGTGCAATGAAATGGGCATTTTCACAGTCGTG*GCAGAAAGTATAATTATCTTTGACT**
TTCTAGAAAGCAGTCTGGCATTCTAGAAACTTGCCTAACCTCTTCCCATTTAGGCAAGATGAATT
CTC**AC**TACCCGTAGGT**GGCCA**A**CCTTGTCC**T**TGTGATTCCGTATCT**T**CCAGAAAGAGAGGTCTAG**
TCTCAGGGAAAACCCAGATTTTCTTGGCTTAGCCCACCTGACAGCTAATCACTGGAAATGA**GG**T**G**
GGCCGGTAGAGTCCTTTGGTCAGGTTTTGTGTCAAGAGAGGA**ATGTGGAAAGATGGGAG**G**GAGGT**
AGCAAAACTGGCCTCAATGGAACTA**TGTAAGTTAACATAGAATG**A**CAAAGGAATGTTTCTTCCA**G
GGAAGAAATTCTAGGGAAGGAAG**AAAGT**G**GAGGGGAAGGCAGCAGTTCTCAAAGTTTTGGGGTCA**
GGATTCCTTTACACTCTTAAAAGTATATTGAGGGCCCAAGGAGCTTTTCTGTATATAGGTTATAT

Italics show the –ve complement to P5 inserts, bolds the differences and shadow boxes the insertions, between AC139425.3 and AC126339.6. Insert comprises nuc 114595 - 114674 of this clone (RP13-996F3) in +/- fit. Sequence here includes 480 bp each side of insert. Note excerpts from NCBI report regarding this region.

repeat_region 114084..114128/rpt_family="(GA)n"

repeat_region 114128..114155/rpt_family="(TG)n"

repeat_region complement(114289..114586)/rpt_family="AluSx"

AC136698.6+/+, sub 8/2/02

TACCAATAGATATAACCTACATACACAAAAGCTCCTTGGGCCCTCAATATACTTTTAAGAGTGTA
AAGGAATCCTGACCCCAAAACTTTGAGAACTGCTGCCTTCCCCTCCACTTTATTCCTTCCCTAGA
ATTTCTTCCTTGGAAGAAACATTCCTTTGCCATTCTATGTTAACTTACATAGTTCCATTGAGGCC
AGTTTTGCTACCTCTCTCCCATCTTTCCACATCCCTCTCTTGACACAAAACCTGACCAAAGGACT
CTACCGGCCCACCCCATTTCCAGTGATTAGCTGTCAGGTGGGCTAAGCCAAGAAAATCTGGGTTT
TCCCTGACACTAGACCTCTCTTTCTGGGAGATACGGAATCACAGGGACAAGGCTGGCCCTGTCAG
AACTCATCTTGTCTAAATGGGAAGAGGTTAGGCAAGTTTCTAGAATGCCAGACTGCTTTCTAGAA
AGTCAAAGATAATTATACTTTCTGC*CACGACTGTGAAAATGCCCATTTCATTGCACGCTTTCTAA*
***CATTTATACCAATCTGATAAATAAAAGCTGGTACCTAGAA*GAAAAAAAGGCTGGGTATGGTGGCT**
CATGCCTGTAATCCCAGTACTTTGGGAGACCAAGGTGAGTGGATCACCTGAGGTCAGGAGTTCGA
GACAAGCCTGGCCAACATGATGAAACCCCATCTCTAGTAAAAACACAAACATTAGCCGGGCATGG
TGGCAGGCCCCTGTAATCCCAACTACTCGGGAGGCTGAGGCAAGAGAATCACTTGAACCTGGGAG
GTGGAGGTTTTAGTGAGCCAAGATCATGCCATTGCCCTCCAGCCTGGGTGACAAGGTGAGACTTT
GTCTCAAAAAAAAAAAAAAAGTTTCCATACAATATAATTTGTTCCATCTCTAAAAACCAATTCAG
CAATAACTGAAAGCCACCACTTGGAAGGTTTCAAGGATTTAGCTCTACCTGTTGATGATGTCCAA
AGCATTAGTTAAGGTAGAAAAAAAATATACACACACACACACACACACACACACACACACACACA

Insert comprises nuc 110098 - 110177 of this clone (RP13-262C2). Sequence here includes 480 bp each side. Note excerpts from NCBI report regarding this region. Repeat_region 110186..110482/rpt_family="AluSx"

repeat_region 110621..110664/rpt_family="(CA)n"

AC136698.6 Complementary strand

TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATATTTTTTTTCTACCTTAACTAATGC
TTTGGACATCATCAACAGGTAGAGCTAAATCCTTGAAACCTTCCAAGTGGTGGCTTTCAGTTATT
GCTGAATTGGTTTTTAGAGATGGAACAAATTATATTGTATGGAAACTTTTTTTTTTTTTTT(T7m
issing)**GAGACAAAGTCTCACCTTGTCACCCAGGCTGGAGGGCAATGGCATGATCTTGGCTCAC**
TAAAACCTCCACCTCCCAGGTTCAAGTGATTCTCTTGCCTCAGCCTCCCGAGTAGTTGGGATTAC
AGGGGCCTGCCACCATGCCCGGCTAATGTTTGTGTTTTTACTAGAGATGGGGTTTCATCATGTTG
GCCAGGCTTGTCTCGAACTCCTGACCTCAGGTGATCCACTCACCTTGGTCTCCCAAAGTACTGGG
ATTACAGGCATGAGCCACCATACCCAGCCTTTTTTTC*TTCTAGGTACCAGCTTTTATTTATCAGA*
***TTGGTATAAATGTTAGAAAGCGTGCAATGAAATGGGCATTTTCACAGTCGTGG*CAGAAAGTATAA**
TTATCTTTGACTTTCTAGAAAGCAGTCTGGCATTCTAGAAACTTGCCTAACCTCTTCCCATTTAG
ACAAGATGAGTTCTGACAGGGCCAGCCTTGTCCCTGTGATTCCGTATCTCCCAGAAAGAGAGGTC
TAGTGTCAGGGAAAACCCAGATTTTCTTGGCTTAGCCCACCTGACAGCTAATCACTGGAAATGGG
GTGGGCCGGTAGAGTCCTTTGGTCAGGTTTTGTGTCAAGAGAGGGATGTGGAAAGATGGGAGAGA
GGTAGCAAAACTGGCCTCAATGGAACTATGTAAGTTAACATAGAATGGCAAAGGAATGTTTCTTC
CAAGGAAGAAATTCTAGGGAAGGAATAAAGTGGAGGGGAAGGCAGCAGTTCTCAAAGTTTTGGGG
TCAGGATTCCTTTACACTCTTAAAAGTATATTGAGGGCCCAAGGAGCTTTTGTGTATGTAGGTTA
TATCTATTGGTA

Comparison of AC136698.6 complementary strand with AC126339.6. Italics show the –ve complement to P5 inserts, bolds the differences and underlined the insertions, between the two sequences above

AC005630.1, sub 21/2/03, +/+ fit (match to AC 136698.6)
TACCAATAGATATAACCTACATACACAAAAGCTCCTTGGGCCCTCAATATACTTTTAAGAGTGTA
AAGGAATCCTGACCCCAAAACTTTGAGAACTGCTGCCTTCCCCTCCACTTTATTCCTTCCCTAGA
ATTTCTTCCTTGGAAGAAACATTCCTTTGCCATTCTATGTTAACTTACATAGTTCCATTGAGGC
CAGTTTTGCTACCTCTCTCCCATCTTTCCACATCCCTCTCTTGACACAAAACCTGACCAAAGGA
CTCTACCGGCCCACCCCATTTCCAGTGATTAGCTGTCAGGTGGGCTAAGCCAAGAAAATCTGGGT
TTTCCCTGACACTAGACCTCTCTTTCTGGGAGATACGGAATCACAGGGACAAGGCTGGCCCTGTC
AGAACTCATCTTGTCTAAATGGGAAGAGGTTAGGCAAGTTTCTAGAATGCCAGACTGCTTTCTAG
AAAGTCAAAGATAATTATACTTTCTGCC*ACGACTGTGAAAATGCCCA*TTTCATTGCACGCTTTC
***TA*ACATTTATACCAATCTGATAAATAAAAGCTGGTACCTAGAA**GAAAAAAAGGCTGGGTATGGTG
GCTCATGCCTGTAATCCCAGTACTTTGGGAGACCAAGGTGAGTGGATCACCTGAGGTCAGGAGTT
CGAGACAAGCCTGGCCAACATGATGAAACCCCATCTCTAGTAAAAACACAAACATTAGCCGGGCA
TGGTGGCAGGCCCCTGTAATCCCAACTACTCGGGAGGCTGAGGCAAGAGAATCACTTGAACCTGG
GAGGTGGAGGTTTTAGTGAGCCAAGATCATGCCATTGCCCTCCAGCCTGGGTGACAAGGTGAGAC
TTTGTCTCAAAAAAAAAAAAAAAGTTTCCATACAATATAATTTGTTCCATCTCTAAAAACCAATT
CAGCAATAACTGAAAGCCACCACTTGGAAGGTTTCAAGGATTTAGCTCTACCTGTTGATGATGTC
CAAAGCATTAGTTAAGGTAGAAAAAAAATATACACACACACACACACACACACACACACACACAC
ACA

Insert comprises nuc 85207-85286 (ITALICS) of this clone (RP5-1129D5). Sequence here includes 480 bp each side. Note excerpts from NCBI report regarding this region misc_feature 85103.85583 /note="similar to EST AA443771 (NID: g2156446) zw95f08.s1" UNDERLINED

Primers are indicated in boxes (first design 98, 790; 499, 790), or shadowed boxes (second design 191,481, successful) lower primers shaded

AC136698.6+/+, sub 8/2/02; Expanded coverage
AACTGGCAGAGGCTCTAAGAGTGGGAGGTGAGTTGTTTTCTCCTTCATGTAAATATAT
TTACCTTTTAAACACTAGGCCCAATTTTATATCCTATTTCATTTAACTTTATGAACATAT
TTATGTATGTATGCATGTATGTATGTATCTCATGTGATGTTTTAGACACTGAAAAATAA
CTCATTTCTATTATAAAACTGATATCTTTAGATGTTCAGAAGCAACTTCCTAAAAGGAG
GTAGCAGTAATGGAGCTATGTCTATCATTCTTTCCCATCAACCCCCTTGATGGAGATG
TAAACATGTGTCCATCAAGCCTTTAATTTTTACCTCTTATCTTCATGGCTCTCCATACA
AAACTTAACTCTTTTTTTTTTCTATTTGTATACGTATATTTATATGTATATCTATATCGAG
AGAGAGAGAGAGAGAAAGAGTCTTGCTGTGTTGCCCAGGCTGATCTCAAACTCCTGG
GCTCAAGCAATCCTCCCACCTTGGCCTCGCAAAGTGCTGGGATTACAGGCGTGAAC
CACTGTGCCCAGCCTCAGCCTTAACTCTTAAAATATCTTCAAACCAATATTCTTCTGTT
CTAATTTTTAAGAATAGATGTGTTTAAACCAACTAACTTATTTTGACAAAAATTGGAGTT
AAGACTCAGACTTCCTCAAATAGTTCTCCTAAAACCATTTACAGAATAATCTATCTTTT

CAGTATTAAGTTAAAATACCACCTTTTCCTTATACTAAATTCTCGTTTGCATGACTCTG
GTTCTAAACTTCCATTGCCTTTATCTGTCTGGCCCAGGGATAGTCCACAATATTTTATT
TACTATCTGGTTGAAAGAGTCTATACTTTATTACTTTTTATTACTTATTCTTCTAAACAA
ATTTTAGAGTCGTTTTGTCAAGTGTCAAAAATAAATCTGCCAGAATTTGTACTGAAATT
TGTGTGTGTGTGTATATATATAATACACACACACTATATATAAAATATAAAATGTATATA
TACAATTTATATATATAAATATTTATAAAATATGAAATATATATATATATACACACACACA
CTTTTCTAGTTCTTTTTTTTTTTTTTGAGACAGGGTCTCACTCTGTCACCTAGGCTGGA
GTTCAGAGGCATGATCTCGGCTCACTGCAACCTCTGCCTCCCAGGCTCAAGTGCTCC
TCCCACCTCAGCCTCAGAAGTAGTTGGAAATACAAGTGTGTGCCACAGACACCCAGC
TAATTGTCATCTACCCGCCTCAGCTTCCCAAACTGTTTGGATTACAGGTATGAGCCAC
TGTGCCCAGCAGAAATTACATTTACAAATTAATATGAAGACATGGTGATAACTAACATA
TTTATAACATGAAATCTGCTCATCCAGGAACATAGAATGCAAATCTTTCATTCCACTCA
GCAAAATTTTGTCCTGTCCTTGATAAAAGTCCTGCACATCTAAGTTTATTCCTAGGTAT
TTAATTTTTGCTGAAATACCTGAAAAAATACTTCATCACTATATCTTCTACGTGATTATA
GCTAACACTGGGGAAGGCTATTGATTTTTATATAAAAGAACTTTTAACCAGTAATCTTA
AAAATTGTTTTTCTCAGTTGGTTCCTTTGGATATTTTTAGGTAAACAATCATGTCAACT
GAAAATAATGATAAATTTTCTATAAAGACTATGACATCACAGGAAAATACAGTAAATAC
TTTTTAAAAGA[1720]ATATAAAAGGGCCAGGCACAGTGGCTCACGCCTGTAATCCCAGC
ACTTTGGGAGGCCAAGGTGGGCAGACCATGAGGTCAGGAGATCGAGACCATCCTGG
CTAACACGGTGAAACCCCATCTCTACTAAAAAATACAAAAAATTAGCCGGGAATGGTG
GCGGGCGCCTGTAGTCCCAGCTACTGGGGAGGCTGAGGCAGGAGAATGGTGGGAA
CCCAGGAGGTGGAGCTTGCAGTGAGCCGAGATCACGGCACTGCACTCCAGCCTGG
GTGACAGAGCAAGACTCTGTCTCAAAAAAAAAAAAAAAAAAAAAAGAATATAAAACT
ATAGAGAATATGACCTCAACTATTAAGCATATGTGTAAGGGTTATGTATTTTAATAGCA
AAGAAAAACTATATACTGGTAGAAAATGACCATCATGTCAACAGTCAATAGTGGTTAT
ATTAGATAGAGAAATTATGGGAGACTTTAATTTTTTTCTTTTATCTTTTCTGTACTTTAC
CAATTTTCTCAACAATGGTTGCTTATGAGTTTTAAAATTAAAAAAAGGTTTTAAAAATTT
TTCCAACATGGAAAGTTATATTTCTTTATATACTAAAACAAAAACAAAACTTTCTATTTG
AATACCTATGGCAAAACCCTATCTCTACAAAAAATACAAAAAATTAGCAAGGTGGGGT
AGTACACACCTGTAGTCCCAGCTACTCTGGAGGCTGAGGTGGGAGGATC[2449]ACCTG
AGTCCCCAGAGAATGAGGCTCCAGTGAGCCGTGATCATAGCACTGCATTCCAGCCT
GGGAGACAGAGAAAGACCCCATCTCAAAAAAAAAAAAAAGAAAGAAAGCAAAAAGAA
ATATCCATAATGATCTGAAATGCCTATCTGTATGAGACTGTCCTTTGTTCACATTTTTT
CAAGCAAATATCACACAATAAATTGAATGCAGGTACAAATGACATATCAAACATCAAA
GAAATTTGCAAAGGACGTAAGACTGTACTACCTTGGGTTTAGAAATTTTCTTATCATAA
AAGCATTTATAACAATATTTTGTGAGCTTTTAAGGAATATTTTAAGTATTTCTGATTTAA
TTTCTAGTGATAAATACCAATAGATATAACCTACATACACAAAAGCTCCTTGGGCCCT
CAATATACTTTTAAGAGTGTAAAGGAATCCTGACCCCAAAACTTTGAGAACTGC[98]CCT
TCCCCTCCACTTTATTCCTTCCCTAGAATTTCTTCCTTGGAAGAAACATTCCTTTGCCA
TTCTATGTTAACTTACATAGTTCCATTGA[191]GGCCAGTTTTGCTACCTCTCTCCCATCT
TTCCACATCCCTCTCTTGACACAAAACCTGACCAAAGGACTCTACCGGCCCACCCCA
TTTCCAGTGATTAGCTGTCAGGTGGGCTAAGCCAAGAAAATCTGGGTTTTCCCTGAC
ACTAGACCTCTCTTTCTGGGAGATACGGAATCACAGGGACAAGGCTGGCCCTGTCAG
AACTCATCTTGTCTAAATGGGAAGAGGTTAGGCAAGTTTCTAGAATGCCAGACTGCTT
TCTAGAAAGTCAAAGATAATTATACTTTCTGCC*A[482]CGACTGTGAAAATGCCC[499]ATTT*
*CATTGCACGCTTTCTA*ACATTTATACCAATCTGATAAATAAAAGCTGGTACCTAGAAG
AAAAAAAGGCTGGGTATGGTGGCTCATGCCTGTAATCCCAGTACTTTGGGAGACCAA
GGTGAGTGGATCACCTGAGGTCAGGAGTTCGAGACAAGCCTGGCCAACATGATGAA
ACCCCATCTCTAGTAAAAACACAAACATTAGCCGGGCATGGTGGCAGGCCCCTGTAA
TCCCAACTACTCGGGAGGCTGAGGCAAGAGAATCACTTGAACCTGGGAGGTGGAGG
TTT[790]TGAGCCAAGATCATGCCATTGCCCTCCAGCCTGGGTGACAAGGTGAGACTTT
GTCTCAAAAAAAAAAAAAAAGTTTCCATACAATATAATTTGTTCCATCTCTAAAAACCA
ATTCAGCAATAACTGAAAGCCACCACTTGGAAGGTTTCAAGGATTTAGCTCTACCTGT
TGATGATGTCCAAAGCATTAGTTAAGGTAGAAAAAAAATATACACACACACACACACA
CACACACACACACACACACACACACTCACCCCTATGTAGTCAGTACCAGGAAACACGAA
AGACTAGATGGTACAGTCATCCAACACAAAGCACACAATAACTGAAGGCACTGTAGA
GGAGTAACTTATGACACAGATCTACAATATTGAGTGAAAATGCAGATTA

Primers are indicated in boxes (first design 98, 790; 499, 790; second design 1720,482 (aka3299); 2449,482; 98,482; 191,482) lower primers shaded.

Up: CTGCCTTCCCCTCCACTT (18mer, 98-115) 713 bp 63C
Lp: GGCATGATCTTGGCTCACTAA (21mer, 790-810)
22/5/3: N and P pools, 32-50 cyc @60C. No products. Sigma said primers dodgy
10/6/3: N pool, 30,35,40,45, then further 20,30,40,50 cyc @53C. No products. Sigma said primers dodgy
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @61C. No products
3/10/03: N/P pool, 48,50,52 @59C. Doublet at 750bp, weak at 48 building up.
Recommend 48 cyc (needs more optimisation – doublet??, temp),59C for x10-x80 cDNA dilutions

Up: CATTTCATTGCACGCTTTCTA (21mer, 499-520) 312 bp 62C
Lp: GGCATGATCTTGGCTCACTAA (21mer, 790-810)
22/5/3: N and P pools, 32-50 cyc @60C. No products. Sigma said primers dodgy
27/5/3: N and P pools, 32-50 cyc @60C. No products even with further sub-amplification
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @61C. Products 30cyc onwards
Recommend 32 cyc (?? Check intensity),60C for x10-x80 cDNA dilutions

Second phase
Lp: TGGGCATTTTCACAGTCGT (19mer, 482-500, aka 3299)

Up: ATATAAAAGGGCCAGGCACA (20mer, 1720-1739) 1598 bp 63C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/03: 30-55 cyc@61C, NP pool, No product
No products

Up: CACCTGAGTCCCCAGAGAAT (20mer, 2449-2468) 869 bp 63C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/03: 30-55 cyc@61C, NP pool. Product 40cyc onwards
10/7/3: 40,42,44 cycles @59C, N and P pools, N>P but a2actin failed in P
15/7/3: 40,42,44 cycles @59C, N and P pools N>P, a2actin P>>N, 18S N just above P as expected
Recommend 38 (check intensity, should do one more optimisation) cyc,59C for x10-x80 cDNA dilutions

Up: CTGCCTTCCCCTCCACTT (18mer, 98-115, aka 2915) 403 bp 63C
16/6/3: 40,50 cyc @55C, N and P pools. No products, though a2 actin was low
4/7/03: 30-55 cyc@61C, NP pool. Product 35 cyc onwards
10/7/3: 32,34,36 cycles @59C, N and P pools, N>P but a2actin failed in P
15/7/3: 32,34,36 cycles @59C, N and P pools, N>P , 18S N just above P as expected
Recommend 30 cyc (check intensity, should do one more optimisation) cyc,59C for x10-x80 cDNA dilutions

Up: AGGCCAGTTTTGCTACCTCT (20mer, 191-210, aka 3008) 310 bp 63C
27/5/3: 32-50 cyc @60C. N and P pools, Products from 32 cyc, N = P? - a2actin-N failed
29/5/3: No apparent difference in C,N,P expression in full quant approach
16/6/3: 40,50 cyc @55C, N and P pools. Product at 40 cyc, P>>N
4/7/03: 30-55 cyc@61C, NP pool. Product 30 cyc onwards

Recommend 30 cyc (check intensity, should do one more optimisation),59C for x10-x80 cDNA dilutions

Analysis of P6 clones and P7-2/7-3 clones insert sequence. Homology detected with chromosome 22g11 and expressed gene listed below.

P6-1
GAATTCACACGACTGT**ACTCCAGCCTGGTGACAGAGCCAGACTGTTTTAAAATAAATAAAAATAA**
AAAATTGGTTTTGGAAAAAAAAAAAACTCGAG
P6-2
GAATTCACACGACTGT**ACTCCAGCCTGGTGACAGAGCCAGACTGTTTTAAAATAAATAAAAATAA**
AAAATTGGTTTTGGAAAAAAAAAAAACTCGAG
P6-3
GAATTCACACGACTGT**ACTCCAGCCTGGTGACAGAGCCAGACTGTTTTAAAATAAATAAAAATAA**
AAAATTGGTTTTGGAAAAAAAAAAAACTCGAG
P7-2
GAATTCACACGACTGT**ACTCCAGCCTGGTGACAGAGCCAGACTGTTTTAAAATAAATAAAAATAA**
AAAATTGGTTTTGGAAAAAAAAAAAACTCGAG
P7-3
GAATTCACACGACTGT**ACTCCAGCCTGGTGACAGAACCAGACTCTGTTTTAAAATAAATAAAAAT**
AAAAAATTGGTTTTGGAAAAAAAAAAAACTCGAG

AP000344.1; +/-
TCACGCCTGTAATCCCAGCACTTTGAGACGCAGAGGCAGGCAGATCACGAGGTCAGGAGATCGAG
ACCATCCTGGCTAATGCGGTGAAACCCCATCTCTACTAAAAATACAAAAAAATTAGCCGGGCGCG
GTGGCGGGCGCCTGTAGTCCCGGCTGCTCAGGAGGCTGAGGCAGGAGAATGGCGTGAACCCGGGA
GGCGGAGCTTGCAGTGAGCCGAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCAAGACCC
CATCTCAAAAAAAAGAAAAAAAAGAAAGAAAAGCCAGACATTTGAGCACTGAGTCTCATGGGAAG
TTCCAAGACTAAATAAACAAAAAAGAACCATGAGAGAAAATGCAGAAAGCAAAAGATTCGCCTTC
CTCCAAACCCACTTTATGCCTAAGAGCAGAACTGAATTCTCTGGAACAAGAAGAGGGGAGCTCAG
GGAATGATAGAGATGTCTTGGAAATGAAAAACAGGACTGCTAAAA*TCCAAAACCAATTTTTTATT*
***TTTATTTATTTTAAAACAGTCTGGCTCTGTCACCAGGCTGGAGTACAGTGGTGTG*ATTTCGGCTC**
ACTGCAACCTCTGCCACCCGGGTTCAAGCCAGTCTCCTGCCTCAGCCTCCTGAGTAGCTGGGATT
ACAGGTGCACACCACCACATTCAGCTACTTTTTGTATTTTTAGTAGAGACGGGGTTTCACCATGT
TGGCCAGGCTGGTCTGGAACTCCTGACCTCAAGTGATCTGCCTGCCTCGGCCTCCCAAAGTGCCT
GGGACTACAGGTGTAAGCCACTACATCCAGCCCCAAAACTAATTTTTTAAAAAGCAGAGAGAGTG
TGTGTGTTTGGATTCAAGGAAAATCTTATCTACAAGCAAAGGTAGAAAGAAGGACTCACAGAATC
TATCCAAAAGGCCCAACATCTGACTGACAGGAGTTCCAGAATGAGCAGGAAACGAGAAGAAATGT
TATCAAAGAAATGCCAGGTCTCAAGGCAGAACGTGCTCACCAAGCGCCCAGCGTGGTGAGGAAAC
ACCCGCAGTTGGTGTGACCCCCCAGAGGTGCAGGG

Insert comprises nuc 67478 - 67553 of this clone (KB1269D1 on chromosome 22q11.2). Sequence here includes 480 bp each side. Insert homologous region in italics.

Complementary strand of AP000344 with insert in italics, now +/+.

CTTTCCCACTTCTATCTCCTGTCCCTGCAGCCAGGAGCCGCCTCTTTAAGCTCCTCCTGGAGGTG
GGCTGCCGTCCTCTGCCCCTCGTCCCCACGGAAGAGGTCATTCTGTAGCCCAGCTTTTATTTTCG
CCTAATGTATCTCTGAGGTTTTAAAACATTCTTTTCTAGCTATCTATTGAACATTTACTTTATGC
CAGATCCAGTACATGCCATTGTTATCCTCTGTTTACAGGTGGGGAAGCTGAGGCAGGAAGCCCTT
TAGTCACTTGCCGAAGGCCACGCTGTTACCCATGGGACCGGTTTTGGGCGGCCGAAGAGCACTCA

TGGGGCCGGATTCACGCCCCAGGCCCGTTCCCTCCTGCTCTCTGGTGCTCCTCACGCCATTGGCC
CCACTGCCTCTCACTGCCCGTGAGTCCCTGTGCCCGTGTCCTCCTTCTTGAACCCCTCAGCCCTC
AGTTAACCCTCAGAAAGCTGGCTCGGAGAAGTCCTTGTGTGGTATCTGGGAGGCAGAGTTTGCCG
TGAGCCGAGATTGTGCCACTGCACGCACTCCAGCCTGGGCGACAGAGCGAGACCCCATCTCAAAA
AAAAAAAAAAAAAGGCCTCACGGGACTAGAAGCGTCTTACCCTGACCTCACACCTGCTGGACATC
AGGCTGGGGAGAGAATCGGGGAAGAGGGCATTTTCCAACAGGACTGGAAGGCCAGTCGTGGTCCT
TGGTGGTGAGAGCTAGCCAGGCCGCCGGCCTTCTCCCTGGAAGCCTGCAGGGTCCCCTCTGATCC
CTGCACCTCTGGGGGGTCACACCAACTGCGGGTGTTTCCTCACCACGCTGGGCGCTTGGTGAGCA
CGTTCTGCCTTG[1881]AGACCTGGCATTTCTTTGAT AACATTTCTTCTCGTTTCCTGCTCATTCTG
GAACTCCTGTCAGTCAGATGTTGGGCCTTTTGGATAGATTCTGTGAGTCCTTCTTTCTACCTTTG
CTTGTAGATAAGATTTTCCTTGAATCCAAACACACACACTCTCTCTGCTTTTTAAAAAATTAGTT
TTGGGGCTGGATGTAGTGGCTTACACCTGTAGTCCCAGGCACTTTGGGAGGCCGAGGCAGGCAGA
TCACTTGAGGTCAGGAGTTCCAGACCAGCCTGGCCAACATGGTGAAACCCCGTCTCTACTAAAAA
TACAAAAAGTAGCTGAATGTGGTGGTGTGCACCTGTAATCCCAGCTACTCAGGAGGCTGAGGCAG
GAGACTGGCTTGAACCCGGGTGGCAGAGGTTGCAGTGAGCCGAAA*TCACACCACTGT*[2309]*ACT*[15]*C*
*CAGCCTGGGTGACAGA*GCCAGACTGTTTTAAAATAAATAAAAATAAAAAATTGGTTTTGGATTTT
AGCAGTCCTGTTTTTCATTTCCAAGACATCTCTATCATTCCCTGAGCTCCCCTCTTCTTGTTCCA
GAGAATTCAGTTCTGCTCTTAGGCATAAAGTGGGTT[181]TGGAGGAAGGCGAATC TTTTGCTTTCT
GCATTTTCTCTCATGGTTCTTTTTTGTTTATTTAGTCTTGGAACTTCCCATGAGACTCAGTGCTC
AAATGTCTGGCTTTTCTTTCTTTTTTTTCTTTTTTTTGAGATGGGGTCTTGCTCTGTCGCCCAGG
CTGGAGTGCAGTGGCGCGATCTCGGCTCACTGCAAGCTCCGCCTCCCGGGTTCACGCCATTCTCC
TGCCTCAGCCTCC[416]TGAGCAGCCGGGAC TACAGGCGCCCGCCACCGCGCCCGGCTAATTTTTTT
GTATTTTTAGTAGAGATGGGGTTTCACCGCATTAGCCAGGATGGTCTCGATCTCCTGACCTCGTG
ATCTGCCTGCCTCTGCGTCTCAAAGTGCTGGGATTACAGGCGTGAGCCACTGTGCCCAGCCTTTT
CTTTCTTTTTGATCCCTGTTTATAGGGGAGGCAGGCAGAGCTGCCCGGCACTCCTCTCTCCTTGT
TAACTTGAGAAAGCCGGACACCCAGGTCTTGGAAGGGAGGCCTCCTGCCTCCAGGCAGCATCTTG
GGCTGCAGGGCTGCAGTCTGGAGTGGGGACAGTCCCGGCTCCCACTCGTCTCGGGCTGCAGGGCT
GCGGTCTGGAGTGGGGGCAGTACTGGCTCCCACTCGTCTCGGGCTGCAGGGCTGCGGTCTGGAGT
GGGGGCAGTACCGGCTCCCACTCGTCTCGGGCTGCAGGGCTGCGGTCTGGAGTGGGGACAGTACC
AGCTCCCGCTGCTGTCCTGCCATGCTTGTTTGCAGCACACGTCTCCTCATACCAGTGACGAGGCT
ACTGCTTGCCAGCTGCTTGCCTTCTTCCTGCCAGGGTGTCCTGCTATGTCAGCCTCTGCTCTCCA
CTTCAAGAAGTGTCTTGAAAGATCTTGTCTGCAGAAGACCTCTCTCCTGTTCTCAGTGCCACGTG
GGGCCTCCCCAGCAGAGGCGGCTGGATACCTGGCCCTGGCTACTGGAGGCTGTCTGTTAGCTGCC
AGAGGGCGGCGGCAGCCTTCAGGAACCTCCCCTGGCCTCTGCCCTGTTCATTCCTTGCTGGAATG
TCATGGGTCTCCGGATTTGCTGAGCTCAGAGACCCCATGGGGCAACTTGGGATGGGGGCTAAGGA
AGGCCTCTTGGAGGAACCCTGAGGTGAGGTGGCCCTGGGGTGGGGTGGAGGAGGCCTTGAGCAGC
AGGGGAGGGGGCTGCCCCCTGCTCTCTGCATCCCTGAGTGAGTGGGGCGGGGCCTGCCCCCTGCT
CTCTGTGTCCCTGAGTGAGTGGGGCGGGAGGATGAACCCTGCTCTCTGTCCCTGAGTGCATGGGG
CGGGGCCTGCCCCCTGCTCTCTGCGTCCCTGAGTGAGAGGGGCGGGGGGCTGCACCCTGCTCTCT
GCGTCCCTGAGTGAGTGGGGTGGGGGGCTGCACCCTGCTCTCTGTGTCCCTGAGTGAGTGGGGCG
GGGGCTGGCCCCTGCTCTCTGCGTCCCTGAGTGAGTGGGGCGGGGGCTGGCCCCTGCTCTCTGCG
TCCCTGAGTGAGTGGGGCGGGGGGCTGCACCCTGCTCTCTGTGTCCCTGAGTGAGTGGGGCGGGG
GCTGCCTCCTGCTCTCTGTGTCCCTGAGTGGGGCGGGGCCTGCCCCCTGCTCTCTGTGTCCCTGA
GTGAGTGGGGTGGGGGCTGCAC

The insert (italics) and 5’ sequence (underlined) matches NM-152509.1 and AK092530.1 +/+,(see below), and primers were originally designed to this region. This sequence also includes 3’ sequence allowing design of primers U15,L181 and U15,L416 numbering relative to start of fragment.

NM-152509.1, +/+

AAGACATTCATCCTCCCGGAGGCCCGCGCCGCCCTCGCCCTGCACGCCTAGCGTTGTCCCGCGGC
CAGGCCCGGCCGGAGCGGCGGCGCCTGGATGTGGACCTGGCTGCGGGGAGACGGGCGCCGGCCCC
AAAGCGACTTTCGGTCCTCGACGCGCCCCGCCCCACCCCTGCGATGAAGAGGGCGTCTGCTGGAG
GTTCCAGGCCCCGATTCCCTTATCCTGGGGGACGACAGCATCCATAGCCTGGACTTTGTGTCGAG
CCGAGCCTGGACTTCCCTGACTATGGGCCCGGGGGCCTGCACGCAGCCTACCTGCCATCCCCACC
GCTCAGCGCCTCTGATGCCTTCTCATTGCTTTGTGCTCCCTGAGCCTCAAGGCCTCAAGCCGGCA
GGGCGGGGACTACG**TGGCCCTGCAGCCCCTGTGCTCCGAGGGTGGGCCTCCCATGCCCCACCGTA**
GCATCTTTGCCCCCCCATGCACTGCCCAACCACAATCGCAGCCTGTCCTACAATAATGTGCTCAA
CCCTGGCTCGCCTGGTGGCCACGCCTGCCTCGCCCATCCAGCAGTTGGCGTGGCCGGATACCACT
CACCCTACCTGCACCCTGGGGCAACGGGCGACCCACCATGGCCCCTGCCCCACAGCTTCAGTCCC
GTGCTGGGCCCCCGGCCCCAGGAGCCCTCGCCTGTGCTCTACGACATCCTGTCCAGGACCATCAT
GGCATCCATCCAGGAGCGCAAGGACAGGGAGGAGCGTGAGTGCCTGCTGCGCTCCCAGGCCGACT
CACTCTTCGGCGACTCAGGCGTCTATGATGCTCCCAGCTCCTACAGCCTGCAGCAGGCCAGTGTG
CTGTCTGAGGGCTTCT**GAGGTCCTACGCTGTGCTACAGCTCTACAGATGACCTTGTGGCCAGGCC**
CGGCTTCGGCGGCGCCTGCAACCCTGTCCTGCAGACATCATTGTCCTCGCTTTCCAGCTCCGTGA
GCCGTGCACTGCGGACGTCGTCCTCCTCCCTGCAGGCTGATCAGGTGGGGAAGCTGAGGCAGGAA
GCCCTTTAGTCACTTGCCGAAGGCCACGCTGTTACCCATGGGACCGGTTTTGGGCGGCCGAAGAG
CACTCATGGGGCCGGATTCACGCCCCGGGCCCGTTCCCTCCTGCTCTCTGGTGCTCCTCACGCCA
TTGGCCCCACTGCCTCTCACTGCCCGTGAGTCCCTGTGCCCGTGTCCTCCTTCTTGAACCCCTCA
GCCCTCAGTTAACCCTCAGAAAGCTGGCTCGGAGAAGTCCTTGTGTGGTATCTGGGAGGCAGAGT
TTGCCGTG**AGC**T**GAGATTGTGCCACT**GCAC**GCACTCCAGCCTGGGCGACAGAGCGAGACCCCAT**
CTCAAAAAAAAAAAAAAAGGC**CTCACGGGACTAGAAGCGTCTTACCCTGACCTCACACCTGCTGG**
ACATCAGGCTGGGGAGAGAATCGGGGAAGAGGGCATTTTCCAACAGGACTGGAAGGCCAGTCGTG
GTCCTTGGTGGTGAGAGC**TAGCCAGGCC**G**CCGGCCTTCTCCCCG**G**AAGCCTGCAGGGTCCCCTCT**
GATCCCTGCACCTCTG**GGGGGTCACACCAACTGCGGGTGTTTCCTCACCACGCTGGGCGCTTGGT**
GAGCACGTTCTGCCTTGAGACCTGGCATTTCTTTGATAACATTTCTTCTCGTTTCCTGCTCATTC
TGGAACTCCTGTCAGTCA**GATGTTGGGCCTTTTGGATAGATTCTGTGAGTCCTTCTTTCTACCT**
TTGCTTGTAGATAAGAT**TTTCCTTGAATCCAAACACACACACTCTCTCTGCTTTTTAAAAAATTA**
GTTTTGGGGCTGGATGTAGTGGCTTACACCTGTAGTCCCAGGCACTTTGGGAGGCCGAGGCAGGC
AGATCACTTGAGGTCAGGAGTTCCAGACCAGCCTGGCCAACATGGTGAAACCCC**GTCTCTACTAA**
AAATACAAAAAGTAGCTGAATGTGGTGGTGTGCACCTGTAATCCCAGCTACTCAGGAGGCTGAGG
CAGGAGACTGGCTTGAACCCGGGTGGCAGAGGTTGCAGTGAGCCGAAA*TCACACCACTGTACTCC*
AGCCTGGTGACAGAGCCAGACTGTTTTAAAATAAATAAAAATAAAAAATTGGTTTTGG

This is a hypothetical mRNA-protein sequence of 2136 bp, insert homology is to 2062 – 2136. Comparison of AK092530.1 and NM-152509.1. Italics show the homologous sequence to clone inserts, bolds the differences and shadowed boxes the insertions, relative to other sequence. Underlined area shows the cds as predicted from comments regarding sequence AK092581.1 below

AK092581.1, +/+: (exact match to NM-152509.1 above)
Also says.

CDS 472..975 /note="unnamed protein product" /codon_start=1 /protein_id="BAC03919.1" /db_xref="GI:21751207"

MHCPTTIAACPTIMCSTLARLVATPASPIQQLAWPDTTHPTCTLGQRATHHGPCPTASVPC
WAPGPRSPRLCSTTSCPGPSWHPSRSARTGRSVSACCAPRPTHSSATQASMMLPAPTACSR
PVCCLRASEVLRCATALQMTLWPGPASAAPATLSCRHHCPRFPAP

AK092530.1, +/+

ACCTGATTCCAT**AAGACATTCATCCTCCCGGAGGCCCGCGCCGCCCTCGCCCTGCACGCCTAGC**
GTTGTCCCGCGGCCAGGCCCGGCCGGAGCGGCGGCGCCTGGATGTGGACCTGGCTGCGGGGAGAC
GGGCGCCGGCCCCAAAGCGACTTTCGGTCCTCGACGCGCCCCGCC[175]**CCACCCCTGCGATGAAGA**
GGGCGTCTGCTGGAGTCCAAGGGCAGCCTGGACCCGCTAGATAAGCCGCTGGACTTGGGGCCGG
CGCTGCCCCCCCAAGATGGAGGCTGGCACGTTCAGCAGTGACCTGCAGACCCCACGCCCAGGCA
GTGTTGAGAGTGCCCTGTCGGTGCAGAGGACCAGCCTCCGCCACCCCCCGACGCCTGCCATGTA
CAAGTTTAGGCCGGCTCACGGGTCCTAAGGCACCTTCTGTGGATGAGGCGAGCAG**GTTCCAGGC**
CCCGATTCCCTTATCCTGGGGGACGACAGCATCCATAGCCTGGACTTTGTGTCGAGCCGAGCCTG

GACTTCCCTGACTATGGGCCCGGGGGCCTGCACGCAGCCTACCTGCCATCCCCACCGCTCAGCGC
CTCTGATGCCTTCTCATTGCTTTGTGCTCCCTGAGCCTCAAGGCCTCAAGCCGGCAGGGCGGGGA
CTAC**C**TGGCCCTGCAGCCCCTGTGCTCCGAGGGTGGGCCTCCCATGCCCCACCGTAGCATCTTTG
CCCCCCCATGCACTGCCCAACC[731]ACAATCGCAGCCTGTCCTACAATAATGTGCTCAACCCTGGC
TCGCCTGGTGGCCACGCCTGCCTCGCCCATCCAGCAGTTGGCGTGGCCGGATACCACTCACCCTA
CCTGCACCCTGGGGCAACGGGCGACCCACCATGGCCCCTGCCCCACAGCTTCAGTCCCGTGCTGG
GCCCCCGGCCCCAGGAGCCCTCGCCTGTGCTCTACGACATCCTGTCCAGGACCATCATGGCATCC
ATCCAGGAGCGCAAGGACAGGGAGGAGCGTGAGTGCCTGCTGCGCTCCCAGGCCGACTCACTCTT
CGGCGACTCAGGCGTCTATGATGCTCCCAGCTCCTACAGCCTGCAGCAGGCCAGTGTGCTGTCTG
AGGGCTTC**C**GAGGTCCTACGCTGTGCTACAGCTCTACAGATGACCTTGTGGCCAGGCCCGGCTTC
GGCGGCGCCTGCAACCCTGTCCTGCAGACATCATTGTCCTCGCTTTCCAGCTCCGTGAGCCGTGC
ACTGCGGACGTCGTCCTCCTCCCTGCAGGCTGATCAGGTGGGGAAGCTGAGGCAGGAAGCCCTTT
AGTCACTTGCCAAAGGCCACGCTGTTACCCATGGGACCGGTTTTGGGCGGCCGAAGAGCACTCAT
CCGACAGTGGGGCCGGATTCACGCCCCGGGCCCGTTCCCTCCTGCTCTCTGGTGCTCCTCACGC
CATTGGCCCCACTGCCTCTCACTGCCCGTGAGTCCCTGTGCCCGTGTCCTCCTTCTTGAACCCCT
CAGCCCTCAGTTAACCCTCAGAAAGCTGGCTCGGAGAAGTCCTTGTGTGGTATCTGGGAGGCAGA
GTTTGCCGT**A**AGC**C**GAGATTGTGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACCCCATCTC
AAAAAA**GACG**CTCACGGGACTAGAAGCGTCTTACCCTGACCTCACACCTGCTGGACATCAGGCTG
GGGAGAGAATCGGGGAAGAGGGCATTTTCCAACAGGACTGGAAGGCCAGTCGTGGTCCTTGGTGG
TGAGAG**T**TAGCCAGGCC**A**CCGGCCTTCTCCCCG**T**AAGCCTGCAGGGTCCCCTCTGATCCCTGCAC
CTCT**A**GGGGGTCACACCAACTGCGGGTGTTTCCTCACCACGCTGGGCGCTTGGTGAGCACGTTCT
GCCTTG[1881]AGACCTGGCATTTCTTTGATAACATTTCTTCTCGTTTCCTGCTCATTCTGGAACTC
CTGTCAGATGTTGGGCCTTTTGGATAGATTCTGTGAGTCCTTCTTTCTACCTTTGCTTGTAGATA
AGA**C**TTTCCTTGAATCCAAACACACACACTCTCTCTGCTTTTTAAAAAATTAGTTTTGGGGCTGG
ATGTAGTGGCTTACACCTGTAGTCCCAGGCACTTTGGGAGGCCGAGGCAGGCAGATCACTTGAGG
TCAGGAGTTCCAGACCAGCCTGGCCAACATGGTGAAACCC**T**GTCTCTACTAAAAATACAAAAAGT
AGCTGAATGTGGTGGTGTGCACCTGTAATCCCAGCTACTCAGGAGGCTGAGGCAGGAGACTGGCT
TGAACCCGGGTGGCAGAGGTTGCAGTGAGCCGAAA*TCACACCACTGT*[2309]*ACTCCAGCCTGGTGA*
*CAG*AGCCAGA*CT*CTGTTTTAAAATAAATAAAAATAAAAAATTGGTTTTGG……..ATTTTAGCAG
TCCTGTTTTTCATTTCCAAGACATCTCTATCATTCCCTGAGCTCCCCTCTTCTTGTTCCAGAGAA
TTCAGTTCTGCTCTTAGGCATAAAGTGGGTT[181]TGGAGGAAGGCGAATCTTTTGCTTTCTGCATT
TTCTCTCATGGTTCTTTTTTGTTTATTTAGTCTTGGAACTTCCCATGAGACTCAGTGCTCAAATG
TCTGGCTTTTCTTTCTTTTTTTTCTTTTTTTTGAGATGGGGTCTTGCTCTGTCGCCCAGGCTGGA
GTGCAGTGGCGCGATCTCGGCTCACTGCAAGCTCCGCCTCCCGGGTTCACGCCATTCTCCTGCCT
CAGCCTCC[416]TGAGCAGCCGGGACTACAGGCGCCCGCCACCGCGCCCGGCTAATTTTTTTGTATT
TTTAGTAGAGATGGGGTTTCACCGCATTAGCCAGGATGGTCTCGATCTCCTGACCTCGTGATCTG
CCTGCCTCTGCGTCTCAAAGTGCTGGGATTACAGGCGTGAGCCACTGTGCCCAGCCTTTTCTTTC
TTTTTGATCCCTGTTTATAGGGGAGGCAGGCAGAGCTGCCCGGCACTCCTCTCTCCTTGTTAACT
TGAGAAAGCCGGACACCCAGGTCTTGGAAGGGAGGCCTCCTGCCTCCAGGCAGCATCTTGGGCTG
CAGGGCTGCAGTCTGGAGTGGGGACAGTCCCGGCTCCCACTCGTCTCGGGCTGCAGGGCTGCGGT
CTGGAGTGGGGGCAGTACTGGCTCCCACTCGTCTCGGGCTGCAGGGCTGCGGTCTGGAGTGGGGG
CAGTACCGGCTCCCACTCGTCTCGGGCTGCAGGGCTGCGGTCTGGAGTGGGGACAGTACCAGCTC
CCGCTGCTGTCCTGCCATGCTTGTTTGCAGCACACGTCTCCTCATACCAGTGACGAGGCTACTGC
TTGCCAGCTGCTTGCCTTCTTCCTGCCAGGGTGTCCTGCTATGTCAGCCTCTGCTCTCCACTTCA
AGAAGTGTCTTGAAAGATCTTGTCTGCAGAAGACCTCTCTCCTGTTCTCAGTGCCACGTGGGGCC
TCCCCAGCAGAGGCGGCTGGATACCTGGCCCTGGCTACTGGAGGCTGTCTGTTAGCTGCCAGAGG
GCGGCGGCAGCCTTCAGGAACCTCCCCTGGCCTCTGCCCTGTTCATTCCTTGCTGGAATGTCATG
GGTCTCCGGATTTGCTGAGCTCAGAGACCCCATGGGGCAACTTGGGATGGGGGCTAAGGAAGGCC
TCTTGGAGGAACCCTGAGGTGAGGTGGCCCTGGGGTGGGGTGGAGGAGGCCTTGAGCAGCAGGGG
AGGGGGCTGCCCCCTGCTCTCTGCATCCCTGAGTGAGTGGGGCGGGGCCTGCCCCCTGCTCTCTG
TGTCCCTGAGTGAGTGGGGCGGGAGGATGAACCCTGCTCTCTGTCCCTGAGTGCATGGGGCGGGG
CCTGCCCCCTGCTCTCTGCGTCCCTGAGTGAGAGGGGCGGGGGGCTGCACCCTGCTCTCTGCGTC
CCTGAGTGAGTGGGGTGGGGGGCTGCACCCTGCTCTCTGTGTCCCTGAGTGAGTGGGGCGGGGGC
TGGCCCCTGCTCTCTGCGTCCCTGAGTGAGTGGGGCGGGGGCTGGCCCCTGCTCTCTGCGTCCCT
GAGTGAGTGGGGCGGGGGGCTGCACCCTGCTCTCTGTGTCCCTGAGTGAGTGGGGCGGGGGCTGC
CTCCTGCTCTCTGTGTCCCTGAGTGGGGCGGGGCCTGCCCCCTGCTCTCTGTGTCCCTGAGTGAG
TGGGGTGGGGGCTGCAC

This is composite sequence consisting of a full insert sequence generated from an expressed mRNA in prostate gland of 2374 bp, insert homology is to nuc 2298 – 2374. Then separated by comes the complementary strand of AP000344, thus allowing all primers to be shown. Italics show the homologous sequence to clone inserts, bolds the differences, shadowed boxing the insertions, relative to NM-152509.1 sequence. Underlining indicates the cds based on comments for AK092581.1 and boxes the primer selections (lower shaded; 175,731; 1881,2309)

Up: CCACCCCTGCGATGAAGA (18mer, 175-192) 577 bp 66C
Lp: TGTAGGACAGGCTGCGATTGT (21mer, 731-751)
22/5/3: N and P pools, 32-50 cyc @60C. No products. Sigma said primers dodgy
10/6/3: N pool, 30,35,40,45, then further 20,30,40,50 cyc @53C. No products. Sigma said primers dodgy
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @61C. No products
3/10/03: N/P pool, 48,50,52 cyc @59C. Lots of bands in all 48>52
No reaction – needs more optimisation, temp – 66C should stop non specific amplification

Up: GAGACCTGGCATTTCTTTGAT (21mer, 1881-1901) 447 bp 61C
Lp: CTGTCACCAGGCTGGAGTA (19mer, 2309-2327)
22/5/3: N and P pools, 32-50 cyc @60C. No products. Sigma said primers dodgy
27/5/3: N and P pools, 32-50 cyc @60C. No products even with further sub-amplification
24/6/3: N/P pool, 24-50 cyc @55C. No products. 18S and a2actin very poor. Fresh primers though
4/7/3: N/P pool, 30-55 cyc @61C. Products 40 cyc onwards
3/10/03: N/P pool, 40,42,44 @59C. High in all, 1000 bp and 450 bp at 40, 450 bp only at 42,44 cyc.
Recommend 38 cyc (optimise for cyc number, also doublet),60C for x10-x80 cDNA dilutions

Second phase
Up: TCCAGCCTGGTGACAG (18mer, 15-32)

Lp: GATTCGCCTTCCTCCAA (17mer, 181-197) 183 bp 62C
16/6/3: 40,50 cyc @55C, N and P pools. Product at 50 cyc in N alone
4/7/03: 30-55 cyc@61C, NP pool. Product 45 cyc onwards
10/7/3: 46,48,50 cycles @59C, N and P pools, N>P but a2actin failed in P
15/7/3: 46,49,50 cycles @59C, N and P pools, numerous products, problems somewhere
Needs further optimising, multiple products

Lp: GTCCCGGCTGCTCAG (15mer, 416-431) 416 bp
62C
16/6/3: 40,50 cyc @55C, N and P pools. Product at 50 cyc in P alone
4/7/03: 30-55 cyc@61C, NP pool. Product 50 cyc onwards, but doublet and odd sizes
10/7/3: 46,48,50 cycles @59C, N and P pools, N>P but a2actin failed in P. Variable length of product
15/7/3: 46,49,50 cycles @59C, N and P pools, N>>P , 18S N just above P as expected, also product length varies (416,516,280 bp)
Needs further optimising, multiple products

Analysis of P8 clones

All P8 clones (P8.2, .3, .4) contained a unique sequence analysis of P8.2 clone insert. Single 106/106 hit with clone carrying human DNA (clone RP1-34B21) from chr 6p12.1-21.1.

P8-2
CCCCCGGGCTGCAGGAATTCACACGACTGT**AATCCCAGCACTTTGGGAAGCCGAAGTGGGAGAATC**
CTTTGAGCTCAGGAGTTTGAGACCAGCCTGGGTAGCATAGGGAGACCCCCTCTCTTAAAAGAAAAA
AAAAAAACTCGAGGG**GGGGCCCGGTACCC**

AL031778.1, +/+ fit, Submitted 4/3/3
GAGCTTAAGTAGTCTAAAAAACATGTATATTTGCCTTTGGAACGTTTTTTTGTTTGTTTTTGGAT
ACAGGGCTTTCCTCTGTCATCCAGGCTGGAGTACAGTGGCGCAATCGTAGCTCACTAACCTGGAA
CTCCTGGGCTCAAGCCATCCTCCCTTCTTGTTCTCCCAAAACGTTGGGATTACAGGCATGAGCCA
CTGCGTCCTGACCTGAAATAGATGTTTTAACCTTAGTATTTAAAAATAGGTATGTTCTGGTCACT
TCAAAATCACTAAATACTTTTGCTTGGCATCTTTCTAAAAGGCATTATAATCTGGTAACTTTTTT
GCTAAATACCTTACAAGCTTCCACCAGTCTGCAGAAGAATTTCTTTGTCTGACATTTTGAGAGAA
AGTAGTGTGGCCCCAAGCTTTTTTTCCTGTTTAGTCCACTGTTCACATGCTGAAATATTTCTGTT
CTTGTTTTTATGCTCTCTTGATTTGTAGTACTTCACCTTCCTCCACATCATCACATAGCTAAATC
TTTCTCACACTTAAGACCAGTTCATCCTGCAGGCCTCTGATAGCAAAGAAAAAAAAAGTTCAAGT
AGTACCTTTTTTTTTTTTAAGCCTTGTTGATTTATCCCCAGCTGGAACAAATCTCTGTCCTCCAAA
TTCCCATATCACTTTATCTCTTAGGATTCCAGTATAGGTTTTTAGTCCATTACTAAACTTAAAGA
CTCTTTGAAGTTTGAATCTTCGTCAGAGATTTCTTTTTATTTTTAAAACCTTCAGAACAGTGTCT
GGCACATGGTACATAATTAGTGGAGAGAAGTGCATTAGAAAGCTCCAATCCAGAAATCTGTAGGA
TGATGGGGAAGCTGGACAGCCCTTTGTTGTAGTCTAGTCTTTCATCAAGCGTCTATCAGGACCCA
CTTGTTGAGTTGTGAAAGAAATTGAGTGAGCCAATGCTGGGAATTTTTAAATAATAGAATAGAAA
ATATCTGCCAGGCATGATGATGGCT*CACGACTGTAATCCCAGCACTTTGGGAAGCCGAAGTGGGA*
GAATCCTTTGAGCTCAGGAGTTTGAGACCAGCCTGGGTAGCATAGGGAGACCCCCTCTCTTAAAA
***G*AAAAAAAAGAATAGAAAATACCAAAGTGCATCTTGTGTTGTATAAAGGTAAATGCTGTTTCATG**
AAACTTTCATTTCAGTTACATGCTTTATACATGTGTGCACTGAGTTGTGATAATGAAATGTGTTT
CTTACTAAGGGTTGTGGTCAAAAAAGTTAAAAAGCTACTGTTCTAGGCTTTGCCTGGTAGTGATT
ATCAACTGTGATAATCCTATGGCTTTTCATATGCTTACACTAGTTGAGTTTCTGAAGAGGTTTTA
GCTTTGGAGTTTGTGTAGGTCCACATAGAATGGGTTCCTAGTAAGTGGTTGGCTAAGTCTCAGCT
TTTGCACCTTGGAATAGTGGGGCAAAGAGAATTGCACTGAGAAGAGAATGCACTAGGAAGAGAAT
TTTCATGCTAGCCAGAATAAACACTTATTATTTGTTTCTCTCTCTTTGAGGAAAAACCATTCAAA
ACTTTGTGTTAAGAGCAAGAGCTAGACAGACTAAATCTGGCTTAGTTTGTCATAATCTTGGGCAA
AGAGGCACTTTGGGAAAGACTATCGAATTGTATTGCAGTCTTGCTAACTGAAGCTCACAGTAGTA
GGAACATATGACTTTACTGAAGCCAGTTGCTTATAGCATTAACCATTAACAGTATCAATATCATG
ATACAGGCTAAATCACTCTGTCATTAATGTGAGAGAGAATTGCTGCTAGATTTGAAGCTGCTCTT
CAAATCTTGATACTCTTCAAAGTATAAATTTAAATATTGCTAAATTGGTTCATTTGTATTACCAG
AATAAACTAGTTAAGAAGAAAATCTGTACGTTACATATATTAGTCATTATGTGCTTCAAGTTATA
TTTAGTCTTGTAAAGATGTAAACTTCTGGATATTAGTCAAGGAAAAAATCTCATATAAGAAGGAA
ATTTGGAGTTGGGGAGGCGTTGAAACCTAAAAGACATTCCTTTCATGATCCTTATTTTTTAAAAT

CAATTTATTGAGAGATACTTTAGAAACAAAGTATAATAATTTTAAATGTGTATTTTGATGAGTTT TGACAAATTTGTACA

Insert homology to nuc 121751 - 121856

Analysis of P8.3 clone insert.

P8-3
CCCCCGGGCTGCAGGAATTCACACGACTGT**AGTCCTAGCTACTCAGGAAGCTGAGTTGGGAGGATT GGCAGACTCTACAGCTTATACTGAAGAAGTCAGTACCAGTATAATCTGTTAGGTCTATTTAGG**GAA AAAAAAAAAACTCGAGG**GGGGGCCCGGTACCC**

AC025594.5, +/- fit, Submitted 28/11/00.120/121 hit with clone carrying human male DNA (clone RP11-309L24) from chr 7.

GCGGGGGTGGGTGCAGTTTGAAAACCACCAGACTAAATCATGACTAAGGTTGTGTG
GAGATACAAAATACCATGACTCAAACACAATACTAACATTAAAATTAAGAGAACAGCA
CGTGGGATTTAATTGTAAGCAATAAAACTTACTGCAGGTCCTAGATTAAAGGACGGAT
TCAACCAACCAACAGGATGAAATCGATGTGGCAAAATTTGAAGAGACTAAGCATGAT
GTTTGAAGAGTTTTCTAATAAACTAATAATTTTCTAGTATTTTTTTCTAAGCAAATAGAT
TTCTATATTCTTTTAAACTACAAAATCAGTATAAATAAGATTTAAAAAATGTGTATCTTC
ATGGGGCAAAATCATACACACAAATATTTCACTTTTGTATTAGTTTTCATCTAACCACT
TATTTTCTACTATTTAATAAGGTACAAAATTTTGGCCAGGGGCGGTGGCTCACACCTG
TAATCTCAGCACTTTGGGAGGCTGAGGTGGGTGGATCATGAGGTCAGGAGATCGAG
ACCATCCTGGCTAACACGGTGAAACCCCATCTCTACTAAATATACAAATAATTAGCCT
GGCATGGTGGCGGGTGCCTGTAATCCCAGCTACTCGGGAGGCTGAGACAGGAGAAT
GGCGTGAACCCAGGAGGCAGAGTTTGCAGTGAGCAGAGATCACGCCACTGCACTCC
AGCCTGGGTGACAGAGCGAGACTCCGTCTCAAAAAAAAAAAAAAAAAAAAAAAAAG
AGTACATAATTTTACATTCTGATTTTTCTCTTACCATATCATCATTTCACTACCTTTTAA
TTGCTGCATCAAGTTGCTAAATCATAACTCTTCTTATATAATTCATATAACTCTTGTTAT
ACTGCTGTACATGGGTTTATAATTTTTACTTTTACAGATAACATTTTGTATATAAAATAG
CTGTTTTTTTCTTCTGCAGATTTAAGATATACTCCCAGGAAAAGAATTACTGGTTTAGC
TGGAGTGTTTTGCTT*TTTTTTTTCCCTAAATAGACCTAACAGATTATACTGGTACTGAC*
TTCTTCAGTATAAGCTGTAGAGTCTGCCAATCCTCCCAACTCAGCTTCCTGAGTAGCT
*AGGACTACAGGCGTGTG*CCACCAGGCCCAGCTAATTTTCTTTTATTTCTTTTTGTAGA
GACAGGGGATTCCCTATGTTGCCCAGGCTGGTCTTGAATTCCTGGGCTCTAACGATT
CATCTGCCTTGGCCTCCCAAAGTGCTGGACTACAGGCATGAGCCAGGGTGCCCAGC
CAGGTTTATTATCTATCTTCTCAGTTCCTTTTCAAGTTGGCACTGACAAGAGGAAGAA
ATTAAGATTTGAAGAGGTAGAGAAGGGAACTTACATGGTCTGGTTCCAGCTGGCAGT
GTCGAGCCAAGGCGTGAAGCAGCCTCTGAATGTAAGCTTTGAAGATGCCATGAATAA
CTTCATCGTTAGTTTTGTACAAATGTTCCCCCAGTCGGTACCAAAAGTTAAATGAAATT
TCTACTACCTGTTAGGTTAAAAGAGATGATTTATTTGAATGGGAAAGGAATAGAATAA
GAAGACATTTTATTATCACCACGACCATATAGGAAGAAAGATATATTTTTAAATAATAT
GAGTGGAGCCAGTTAAGAAGTCACCTTTTCTCTAGCAGATTTACTATTATCTCCCCAA
ATTGTCTTCCCACGTTAGGCCCAAATCTCCATTTAGAATGACTGGCTTTACATTCCATT
TAGTCCTGAGGTAATGCCTCCAAAAGTATACTGAATTTTGAATTTAGAGAAAACAATAA
CAGACATGGAAGTCTTTATTTTTATTTATTTTTTAGAGACGGAGTTTCATTCTGTTGCC
CCAGCTAGAGTGCAGTGGAATGAACACAGTTCACTGCTGCCTCAAACTCCTGAGCTC
AAGTGATCCTCCTGCCTCAGCCTCCCAAGTAGCTGGGACTACAGGTACACACCACCA
AGCTCAGCTAATTTTTTTTTTTTTTTTTTTTTTTAGTAGAAACAGGGCCTTGCCATCTTG
CTCAGGATGGTTTTGAACTCCTGCCCTCAAGTAATCCTCCCACCTAGGTCTCCCAAA
GTGCTGGGACTACAGGCATGAGCCACCACATAGTTTTATTTTATTTAAAACAAATTTC
AGCCAGGCGCGAAACACCATCTCTAAAAAAATACAAAAATTAGCCAGGCATGTAGGT
GGCACATGCCTGTATTCCCAGCTACTCAGGAGGCTGAGGTTGGAGGATCACCTGAG
CCCAGGAGGCTGAGGCTACAGTGAGCTGTGATTGCACCACAGCATTCCAGCCTAGG
CAACAGAGTGAGACCCTGTCTCAAAAGATATATATATTTTCTTTAGAGACAGGGTCGC
CTAGGCTGGAGCACAGTGGCACAATCATGGCTCACTGCAGCCTTGAACTTCTGGGCT

CAAGCAATCCTTCCACACAGCCTCCTGAGTAGCTAGGAGGACTACAGGCGCTTGCCA
CCATGTCTGGCTAATTTTTAAAATTTTCTGTAGCGACAGGG

Insert comprises nuc 186172 - 186292 of this clone (RP13-262C2). Sequence here includes 1100 bp each side. No details in NCBI report regarding this region.

COMPLEMENTARY STRAND

TATTATTTAAAAATATATCTTTCTTCCTATATGGTCGTGGTGATAATAAAATGTCTTCTTATTCT
ATTCCTTTCCCATTCAAATAAATCATCTCTTTTAACCTAACAGGTAGTAGAAATTTCATTTAACT
TTTGGTACCGACTGGGGGAACATTTGTACAAAACTAACGATGAAGTTATTCATGGCATCTTCAAA
GCTTACATTCAGAGGCTGCTTCACGCCTTGGCTCGACACTGCCAGCTGGAACCAGACCATGTAAG
TTCCCTTCTCTACCTCTTCAAATCTTAATTTCTTCCTCTTGTCAGTGCCAACTTGAAAAGGAACT
GAGAAGATAGATAATAAACCTGGCTGGGCACCCTGGCTCATGCCTGTAGTCCAGCACTTTGGGAG
GCCAAGGCAGATGAATCGTTAGAGCCCAGGAATTCAAGACCAGCCTGGGCAACATAGGGAATCCC
CTGTCTCTACAAAAAGAAATAAAAGAAAATTAGCTGGGCCTGGTGG*CACACGCCTGTAGTCCTAG*
CTACTCAGGAAGCTGAGTTGGGAGGATTGGCAGACTCTACAGCTTATACTGAAGAAGTCAGTACC
AGTATAATCTGTTAGGTCTATTTAGGGAAAAAAAAAAGCAAAACACTCCAGCTAAACCAGTAATT
CTTTTCCTGGGAGTATATCTTAAATCTGCAGAAGAAAAAAACAGCTATTTTATATACAAAATGTT
ATCTGTAAAAGTAAAAATTATAAACCCATGTACAGCAGTATAACAAGAGTTATATGAATTATATA
AGAAGAGTTATGATTTAGCAACTTGATGCAGCAATTAAAAGGTAGTGAAATGATGATATGGTAAG
AGAAAAATCAGAATGTAAAATTATGTACTCTTTTTTTTTTTTTTTTTTTTTTTTGAGACGGAG
TCTCGCTCTGTCACCCAGGCTGGAGTGCAGTGGCGTGATCTCTGCTCACTGCAAACTCTGCCTCC
TGGGTTCACGCCATTCTCCTGTCTCAGCCTCCCGAGTAGCTGGGATTACAGGCACCCGCCACCAT
GCCAGGCTAATTATTTGTATATTTAGTAGAGATGGGGTTTCACCGTGTTAGCCAGGATGGTCTCG
ATCTCCTGACCTCATG

AC018639 +/- FIT. SUBMITTED 7/2000 .120/121 hit with clone carrying human male DNA (clone RP11-128A6) from chr 7.This sequence is identical to AC025594.5 above in the insert area and for 500 bp in each direction.

CCGGTCTCAAATTCCTGACCTCAAGTGATCCACCCGCCTCGGCCTCCCAAAGTGCTGGGATTACA
GGCATGAGCCACCACGCCCAGTTGGATTCTTCACTTTAAAAAAATAGCGGGGGTGGGTGCAGTTTG
AAAACCACCAGACTAAATCATGACTAAGGTTGTGTGGAGATACAAAATACCATGACTCAAACACA
ATACTAACATTAAAATTAAGAGAACAGCACGTGGGATTTAATTGTAAGCAATAAAACTTACTGCA
GGTCCTAGATTAAAGGACGGATTCAACCAACCAACAGGATGAAATCGATGTGGCAAAATTTGAAG
AGACTAAGCATGATGTTTGAAGAGTTTTCTAATAAACTAATAATTTTCTAGTATTTTTTTCTAAG
CAAATAGATTTCTATATTCTTTTAAACTACAAAATCAGTATAAATAAGATTTAAAAAATGTGTAT
CTTCATGGGGCAAAATCATACACACAAATATTTCACTTTTGTATTAGTTTTCATCTAACCACTTA
TTTTCTACTATTTAATAAGGTACAAAATTTTGGCCAGGGGCGGTGGCTCACACCTGTAATCTCAG
CACTTTGGGAGGCTGAGGTGGGTGGATCATGAGGTCAGGAGATCGAGACCATCCTGGCTAACACG
GTGAAACCCCATCTCTACTAAAATATACAAATAATTAGCCTGGCATGGTGGCGGGTGCCTGTAATC
CCAGCTACTCGGGAGGCTGAGACAGGAGAATGGCGTGAACCCAGGAGGCAGAGTTTGCAGTGAGC
AGAGATCACGCCACTGCACTCCAGCCTGGGTGACAGAGCGAGACTCCGTCTCAAAAAAAAAAAAAA
AAAAAAAAAAAAAAGAGTACATAATTTTACATTCTGATTTTTCTCTTACCATATCATCATTTCACT
ACCTTTTAATTGCTGCATCAAGTTGCTAAATCATAACTCTTCTTATATAATTCATATAACTCTTG
TTATACTGCTGTACATGGGTTTATAATTTTTACTTTTACAGATAACATTTTGTATATAAAATAGC
TGTTTTTTTCTTCTGCAGATTTAAGATATACTCCCAGGAAAAGAATTACTGGTTTAGCTGGAGTG
TTTTGC*TTTTTTTTTTCCCTAAATAGACCTAACAGATTATACTGGTACTGACTTCTTCAGTATAA*
***GCTGTAGAGTCTGCCAATCCTCCCAACTCAGCTTCCTGAGTAGCTAGGACTACAGGCGTGTG*CCA**
CCAGGCCCAGCTAATTTTCTTTTATTTCTTTTTGTAGAGACAGGGGATTCCCTATGTTGCCCAGG
CTGGTCTTGAATTCCTGGGCTCTAACGATTCATCTGCCTTGGCCTCCCAAAGTGCTGGACTACAG
GCATGAGCCAGGGTGCCCAGCCAGGTTTATTATCTATCTTCTCAGTTCCTTTTCAAGTTGGCACT
GACAAGAGGAAGAAATTAAGATTTGAAGAGGTAGAGAAGGGAACTTACATGGTCTGGTTCCAGCT

GGCAGTGTCGAGCCAAGGCGTGAAGCAGCCTCTGAATGTAAGCTTTGAAGATGCCATGAATAACT
TCATCGTTAGTTTTGTACAAATGTTCCCCCAGTCGGTACCAAAAGTTAAATGAAATTTCTACTAC
CTGTTAGGTTAAAAGAGATGATTTATTTGAATGGGAAAGGAATAGAATAAGAAGACATTTTATTA
TCACCACGACCATATAGGAAGAAAGATATATTTTTAAATAATATGAGTGGAGCCAGTTAAGAAGT
CACCTTTTCTCTAGCAGATTTACTATTATCTCCCCAAATTGTCTTCCCACGTTAGGCCCAAATCT
CCATTTAGAATGACTGGCTTTACATTCCATTTAGTCCTGAGGTAATGCCTCCAAAAGTATACTGA
ATTTTGAATTTAGAGAAAACAATAACAGACATGGAAGTCTTTATTTTTATTTATTTTTTAGAGAC
GGAGTTTCATTCTGTTGCCCCAGCTAGAGTGCAGTGGAATGAACACAGTTCACTGCTGCCTCAAA
CTCCTGAGCTCAAGTGATCCTCCTGCCTCAGCCTCCCAAGTAGCTGGGACTACAGGTACACACCA
CCAAGCTCAGCTAATTTTTTTTTTTTTTTTTTTTTTTTAGTAGAAACAGGGCCTTGCCATCTTGCT
CAGGATGGTTTTGAACTCCTGCCCTCAAGTAATCCTCCCACCTAGGTCTCCCAAAGTGCTGGGAC
T

Insert comprises nuc 14062 - 14182 of this clone (RP13-262C2). Sequence here includes CA1100 bp each side. No details in NCBI report regarding this region

Analysis of P8.4 clone insert.

P8-4

CCCCCGGGCTGCAGGAATTCACACGACTGT**ACTGGAGCCTGGATGACAGCAAGACCCTGTCTCCAA**
AAAAATGTCTAAGACATTGCTGGAGCCTTGAAAAAAAAAAAACTCGAGGG**GGGGCCCGGTACCC**

AL355385 +/+ FIT. SUBMITTED 30/9/2000

88/90 hit with clone carrying human male DNA (clone RP11-387M24) from chr 6.

GGCCGTGATTTGGGTGGGAATCACAGCACATGTGTAACGAGTGCTCAGGACACTCACAGTGCAGG
TGCAAAGGCAAGATGCACAGTCAAGCCTTCCTCCAGTTGAGGTCTGAGAATCACCTGCTTTCATT
AGGGATACTTTTTCTTCTTTTGTTTGAGACGGAGTCTCACTCTGTCACCCAGGCTGGAGTGTAGT
GGTGTGATCTTGGCTCACTGCAACCTGCGCCTCCCAGGTTCAAGTGATTCTCGTGCCTCAGCCTC
CCAAGTAGCTGGGTTTAAAGGTGTGTGCCACCACACCTGGCTAATTTTTGTATTTTTAGTAGAGG
CGATGTTTTACCATGTTGGCCAGGCTAGTCTCAAACTCTTGACCTCAAGTGATCCACCTGCCTCG
GCCTCCTAAAGTGCTGGGATTATGGGTGTGAGCTGCCATGCCCGGCCTCATTAGGGATACTTTTT
AAAACACAGGCCAGGCGTGGTGGTTCACACCTATAATCCCAGTGCTTTGGGAGGCCGAGATAGGA
AGATCTCTTGAGCCCAGGAGTTTGAGACCAGGCTGGACAAAATAGTGAGACCTTGTGTCTACAGA
AATTTAAAAAGTTAGCTGGGCGCGGTGGCTCACGCTTGTAATCCCAGCACTTTGGGAGGCCGAGG
TGGGTGGATCACGAGGTCAGGAGTTCGAGATCAGCCTGACCAACATGGTGAAACCCCGTCTCTAC
TAAAAATACAAAAATTAGCTGGGCATGTTGGCACTCACCTGTAATCCCAGCTACTTGGGAGGCTG
GGGCAGGAGAATTGCTTGAATCCAGGAGGCAGAGGTTGCAGTGAGTCGAGATCGTGCCATTGCAC
TCCAGCCTGGGTGACAGAGCGAGACTTTGTCTCAGGAAAAAAAAAAAAAAAAATTTAGCCAGGTA
TGGTGATGCACACCTATAATCCCAACTATTTGGGAGGCTGAGGCTGGAGGATCATTTGAGCTCGG
GAGCTGGAGGTTCCAGTGAGCTATGA*TCACACTACTGTACTGGAGCCTGGATGACAGCAAGACCC*
***TGTCTCCAAAAAAAATGTCTAAGACATTGCTGGAGCCTTGAAAAAAAG*AAA**AAAAATGCAGAATTC**
GGGGCCCCACTCCACAGCTTCCAACCCAGGCTCTAGGGGTGGGACCCAGGTAGATGTGTATTTAA
CAAGTGCTCTGGGTGATACTTCATGTGTGTTCAAGTTGGGGAACTCTGGCTTTGATCAGTCATTC
CACAGATGCTGACTGAGGCTGACACTGAGCCATGCCAGTGTCCCTTGCTGTGCTCTGAGAAGGAG
GCCTGTGGCATTTGAGAGTGTGTGATGCAAACAGGGCCAGATCAGGTGCTGGGTGTTAGAGGGTA
GAGCCCAGAAGACGGAGAGCTCTGGGAATGCATTCAGTCAACAGAGATTTGTCGAGCACCTTGTG
CTAGCTCTTCCCAGTACTGGATGCAACTGTGAACAAAGCAGGCAGAAGCCCCTCTCCTTGTGGAG
TTCATATTCTAACAGCGGGGACAAAGCCAGTAAGAAAAACTATCAAATGAACAAATATAATTTTA

GGTGCCAGTAGAACAAGGGAGAACAATGAAATAGGGCAAGTGATAGAGAAAGGGCAGGGCCAGTG
AGAGTTTAGGGAAGACTTCTGTAAAAGGTGCATTGGAGTTGGTATTTAAATTAAGAAGGGAGCCA
GCCATGTGATAGGCCCTGAGATGGAAATGGGCTTGTATGTTGGAGGAACAGTAGTCGCCTGTGTG
GCTTGAGTTGAATAAACGATGGTGAGAGGGGTTGAAGTTGCCTGAGAGGTGGAAACTAAATCCCA
TAGGCCTTGTAAAAGCATAGTAAGGACTTTGGCTTCGGCATCAGTTATCCATTGCTACATAGCAA
ACCACTTCAAAACGTGGTGGCACTTTGGGAGGCCGAGGCAGGTGGATCACTTGAGGTCAGGAGTT
TGAGACCAGCCTGGCCAACATGGTGAAACCCCATCTCTACTAAAAATACAAAAATGGCTGGGTGC
AGTGGCTCACGCCTTGTAATCCCAGCACTTTGGGAGGCCGAGGCAGGTGGATCACGAGGTCAGGA
ATTCGAGACCAG

Insert comprises nuc 86974 - 87063 of this clone (RP11-387M24). Sequence includes CA1100 bp each side. No details in NCBI report regarding this region.

According to the sequence primers were designed using Oligo 5.0 software and synthesized by Sigma Genosys Ltd. NCBI Genbank accession numbers for target gene sequences have been indicated, as have the sequence for upper (U) and lower (L) primers.