Polymorphisms in Natural Killer cell receptor protein 2D (NKG2D) as a risk factor for cholangiocarcinoma

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ABSTRACT

Background & Aims: Understanding of the significant genetic risk factors for cholangiocarcinoma (CC) remains limited. Polymorphisms in the natural killer cell receptor G2D (NKG2D) gene have been shown to increase risk of CC transformation in patients with primary sclerosing cholangitis (PSC). We present a validation study of NKG2D polymorphisms in CC patients without PSC. **Methods**: Seven common single nucleotide polymorphisms (SNPs) of the NKG2D gene were genotyped in 164 non-PSC related CC subjects and 257 controls with HaploView. The two SNPs that were positively identified in the previous Scandinavian study, rs11053781 and rs2617167, were included. **Results**: The seven genotyped SNPs were not associated with risk of CC. Furthermore, haplotype analysis revealed that there was no evidence to suggest that any haplotype differs in frequency between cases and controls ($p>0.1$). **Discussion**: The common genetic variation in NKG2D does not correlate significantly with sporadic CC risk. This is in contrast to the previous positive findings in the Scandinavian study with PSC-patients. The failure to reproduce the association may reflect an important difference between the pathogenesis of sporadic CC and that of PSC-related CC. Given that genetic susceptibility is likely to be multifaceted and complex, further validation studies that include both sporadic and PSC-related CC are required.

List of Abbreviations

CC – cholangiocarcinoma, PSC – primary sclerosing cholangitis, SNP - Single nucleotide polymorphism, NKG2D - natural killer cell receptor protein G2D, NK – natural killer, GWAS – genome wide association study

INTRODUCTION

Cholangiocarcinoma (CC) is an epithelial malignancy of the biliary tree and the second commonest primary hepatic cancer [1]. The notoriety of CC stems from its diagnostic difficulty and high mortality rate, as less than 5% of patients survive to 5 years [2]. Although CC is relatively rare worldwide, there has been a steadily increasing incidence of intrahepatic CC in Europe, North America, Japan and Australia [2-4]. Given that early surgical resection currently remains the only curative option, there is a need for timely identification of the premalignant and malignant stages of CC [5]. Studies, in response, have highlighted the importance of genetic alterations in early CC pathogenesis. Of note, genetic variations of natural killer cell receptor G2D (NKG2D) have been implicated in the malignant transformation of patients with primary sclerosing cholangitis (PSC) [6].

Natural Killer (NK) cells are a component of the innate immune system. They have an important role in early malignant transformation by mediating the lysis of target cells through specific surface receptor-ligand interaction [7]. NKG2D is a major activating receptor expressed on the surface of T cells and NK cells. It is encoded by a single gene (NKG2D) located on chromosome 12 and shows relatively little polymorphism [8]. NKG2D is activated by a diverse range of ligands, including MIC (A and B), ULBP (1, 2, 3 & 4), RAET1G and RAET1L. Eventually, tumours evade NK cell action and proliferate, due, in part, to high levels of cell bound NKG2D ligands leading to downregulation of receptor expression. Therefore, it is thought that NKG2D activity plays an important role in early tumour detection and control but with a diminishing role as the tumour progresses [9, 10].

Early mouse models of carcinogenesis have demonstrated reduced surveillance and increased tumour progression in NKG2D receptor knock out mice (Supplementary Figure 1). To quantify the importance of cytotoxic immunity in tumour surveillance, a

prospective Japanese cohort study was performed in 1986 [11]. Normal subjects with no known immunological defect were divided into low, medium and high activity tertiles to quantify circulating cytotoxic lymphocyte activity. At an 11-year follow up, subjects with low cytotoxic immunity had increased risk of cancer compared to those with medium or high cytotoxic immunity. Later, the same investigators explored genetic susceptibility factors in this cohort, and genotyped a 270kb region of natural killer complex gene region on chromosome 12, which includes CD94 and NKG2D genes [12]. The implication of NKG2D in CC was highlighted through a Norwegian cohort by Melum and colleagues [6]. The study selected 7 SNPs across *NKG2D* and compared the genotype frequencies of 46 subjects with PSC and CC with 319 control subjects with PSC and no CC. Two of these SNPs, rs11053781 and rs2617167, were associated with increased risk of CC with an OR of 1.95 (CI 1.23-3.07) and OR 2.20 (1.40-3.44), respectively [6].

Aims and Hypothesis

Genetic variation in the NKG2D receptor has been associated with reduced receptor function and impaired NK cell activation, and with increased risk of a number of malignancies, including PSC related CC. The same genetic variation may reduce tumour immunosurveillance in non-PSC patients, permitting survival and proliferation of transformed cholangiocytes and so progression to advanced malignancy. In view of the association of NKG2D with PSC-associated CC, we hypothesized that a similar variation in the gene encoding NKG2D is associated with altered susceptibility to sporadic CC.

METHODS

Blood samples were collected from 164 CC subjects with median age 66.1 (range 55-80). Sample collection was comprised of 44 prospective, consenting patients and 120 from the hepatobiliary biobank archives of Imperial College Healthcare NHS Trust and University College Hospitals NHS Foundation Trust). Cases were collected from Caucasian patients without PSC, and the diagnosis of CC was confirmed by a) pre- or post-operative histology or b) multidisciplinary team consensus on the basis of $\geq$2 imaging modalities, clinical course and serum markers. 257 control samples (median age 68, range 30-90) were collected from Caucasian patients to form a gender and age matched cohort (Table 2). The study was adequately powered to detect a difference of the magnitude found in the Norwegian study. The study protocol received ethical approval from the local Research Ethics Committee (Ref 09/H0712/82).

SNP selection

HaploView (V 4.2, Broad Institute) was used to search HapMap (V3 Build R2, NCBI) data from genomic regions of interest within, and 5KB up and down stream of, *NKG2D*. The polymorphisms selected were relatively common with a minimum mean allele frequency (MAF) of >5%. Markers with a MAF of less than 5% were excluded. The SNPs that captured the maximum genetic variation in NKG2D were selected, with the two SNPs identified to be of interest in the Norwegian study being force included. Pairwise comparisons only were used with an R^2 cut-off of >0.8, a measure of linkage disequilibrium (LD) between two SNPs. This resulted in a total of 7 SNPs to be genotyped in *NKG2D*. These SNPs are listed in Table 1. Due to LD, the SNPs selected represent far more variation around the candidate gene than the absolute number of single nucleotide polymorphisms genotyped.

Primer design and genotyping

Primer design was performed by collating the corresponding DNA sequence from the NCBI dbSNP database for each SNP shortlisted (Supplementary Table 1). The DNA primer sequences were reverse checked by searching the NCBI basic local alignment search tool (BLAST). These sequences were then input into 'PrimerPicker' (KBioscience).

Statistical analysis

The raw genotyping data were managed and manipulated with MS Excel (Microsoft). Differences were considered significant if $p < 0.05$.

Hardy-Weiberg Equilibrium (HWE)

HWE in all 7 genotyped SNPs using Pearson's χ^2 test in PLINK (V1.07) were confirmed. We used a p-value threshold of 0.001, in line with standard practice and the HWE p-value criteria set in the tagger algorithm during SNP selection. We determined that any SNPs that breached this HWE threshold in the control cohort would be excluded from further analysis.

RESULTS

All samples were successfully genotyped and HWE was confirmed in all genotyped SNPs in case, control and combined groups. HWE results from the control group, for each SNP genotyped, are presented in Table 3. In particular, the two SNPs that were previously significant in the Norwegian study (rs11053781 and rs2617167) were negative for correlation, with p-values of 0.7968 and 0.5102, respectively. As none of these SNPs breached the defined p-value threshold of <0.001, all genotyped SNPs were included in subsequent analyses.

Alellic and Cochran-Armitage trend testing

Allele frequency and Cochran-Armitage trend testing results for each SNP are listed in Supplementary Table 2. None of the SNPs genotyped were associated with altered susceptibility to CC. Dominant and recessive models were also tested, with no significant difference between groups.

Haplotype analysis

Haplotype analysis was performed to detect association between different combinations of SNPs in *NKG2D* and altered susceptibility to CC (Table 4). There was no evidence to suggest any haplotype differs in frequency between cases and controls ($p > 0.1$). Given the lack of association of the SNPs to altered susceptibility to CC, HapMap and NCBI dbSNP interrogation for associated SNPs was not performed.

DISCUSSION

The significant role of NK cells in differential tumour surveillance has become increasingly evident. Of note, varying NKG2D gene expression has been shown to correlate with the level of cytotoxicity in peripheral blood. Polymorphisms in the NKG2D gene, therefore, may be key in the malignant transformation of CC.

In the Norwegian study by Melum and colleagues, polymorphisms in the gene encoding NKG2D identified two SNPs that were associated with altered susceptibility to CC in patients with PSC [6]. The same genetic variation may reduce tumour immunosurveillance in non-PSC patients, permitting survival and proliferation of transformed cholangiocytes and so progression to advanced malignancy.

This is the first study to examine *NKG2D* polymorphisms in *sporadic* CC. The study had adequate *a priori* power, but no relationship was found between common genetic variation in *NKG2D* and susceptibility to CC. This is in contrast to the prior finding of the study by Melum and colleagues of an association between rs11053781 and rs2617167 and CC, in their study. The SNPs tested, and those associated in the Norwegian study, are illustrated in the LD plot in Supplementary Figure 2. Although this proved to be a clear negative study, the findings are of importance nonetheless.

The failure to reproduce the association may reflect an important difference between the pathogenesis of sporadic CC and that of PSC-related CC. PSC, unlike risk factors such as cholelithiasis and hepatitis C, is a *strong* risk factor for CC - with a lifetime incidence of CC of around 15% in PSC patients. PSC is an autoimmune disease that remains poorly understood, but is associated with other autoimmune diseases. There are clear genetic associations between PSC and variation in the HLA genetic region [13]. PSC-related CC has significant clinical differences to sporadic CC, including a much earlier

age of onset, frequent multifocal high-grade dysplasia and a particularly poor prognosis [14-17]. It is therefore conceivable that NK cell killing plays a more important role in PSC than it does in CC patients with otherwise normal bile ducts.

The populations of the Norwegian study were recruited from Norway and Sweden, which differs from the cohort of this study, which were Caucasians residing in the UK. It is possible that a genetic influence in the Scandinavian population may not be present in the UK.

Although this study was well powered to detect differences of the magnitude observed in the Norwegian study, we cannot exclude the possibility of smaller effects in non-PSC-related CC. Confidence intervals from this study suggest any such effects must have OR <1.5 and considerably larger studies would be needed to detect, or exclude, effects of this magnitude. Finally, although executed with statistical rigor and with strong positive results, the Norwegian study may have reported a false positive in PSC-related CC.

In conclusion, common genetic variation in NKG2D does not contribute substantially to *sporadic* cholangiocarcinoma risk. The findings here cannot refute those of Melum and colleagues, as patients with PSC-related CC were excluded. This could be elucidated in an additional candidate-gene validation study in further cohorts of patients with sporadic CC and PSC-related CC, along with appropriate control groups. However, as genetic susceptibility to CC is likely to be highly complex and involve many genes, a genome wide association study (GWAS) would offer the advantage of being an unbiased screen for associated genes. With increasing availability and affordability, a GWAS may also prove a more cost-effective method for further exploring such genetic factors. CC is a relatively rare disease and such a study would require a multi-centre, international collaboration to collate adequate numbers of well-characterised cases and control.

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Tables and Figures

Table 1: SNPs in *NKG2D* selected for genotyping

Legend: By gene, RS number and location on chromosome. SNPs force added as associated in Norwegian PSC/CC study in bold.

Ref	RS number	Chromosome	BP location
1	rs7397310	12	10412260
2	rs10772271	12	10415387
3	rs1049172	12	10417007
4	**rs11053781**	**12**	**10428536**
5	rs12819494	12	10442808
6	rs2617165	12	10445197
7	**rs2617167**	**12**	**10450498**

Table 2: Demographics of case and control groups

Legend: n – number in group

	n	Female (%)	Male (%)	Median age (range)
Controls	257	121 (47%)	136 (53%)	66.1 (55-80)
Cases	164	71 (43.4%)	93 (56.7)%	68 (30-92)

Table 3: Hardy-Weinberg equilibrium results for SNPs tested in *NKG2D*

Legend: Using Pearson's $\chi 2$ test. P-value threshold for non-conformity to HWE set at 0.001. Abbreviations: SNP, single nucleotide polymorphism; A1, allele 1; A2, allele 2; GENO, genotype distribution; ObHet, observed heterozygosity; ExpHet, expected heterozygosity; p, p-value. Results from control cohort only shown.

SNP	A1	A2	GENO	ObHet	ExpHet	P
rs7397310	T	C	12/71/167	0.284	0.3078	0.2191
rs10772271	G	A	33/129/85	0.5223	0.4778	0.1826
rs1049172	G	A	22/99/129	0.396	0.4084	0.6428
rs11053781	T	C	53/119/73	0.4857	0.4967	0.7968
rs12819494	T	C	2/60/190	0.2381	0.2217	0.3909
rs2617165	A	G	6/63/175	0.2582	0.2601	0.8091
rs2617167	A	G	19/92/140	0.3665	0.3838	0.5102

Table 4: Summary haplotype results in NKG2D

Legend: Hap Ref - allocated haplotype reference, Hap-Score - score statistic for association of haplotype with the binary trait, p-val - p-value for the haplotype score statistic (based on a chi-square distribution with 1 degree of freedom), control hf - estimated haplotype frequency for control group subjects, case hf - estimated haplotype frequency for case group subjects, glm.eff - the haplo.glm function modeled haplotype effects as: baseline (Base) or additive haplotype effect (Eff), OR. lower - lower limit of the Odds Ratio 95% Confidence Interval, OR - Odds Ratio based on haplo.glm model estimated coefficient for the haplotype, OR upper - Upper limit of the 95% odds ratio confidence interval.

Hap ref	Genotyped alleles contributing to haplotype							Hap score	p-val	Control hf	Case hf	glm. eff	OR lower	OR	OR upper
	rs7397310	rs10772271	rs1049172	rs11053781	rs12819494	rs2617165	rs2617167								
17	C	G	G	**C**	T	G	**G**	-1.15	0.25	0.0981	0.0706	Eff	0.43	0.76	1.3
2	C	A	A	**C**	C	G	**A**	-1.05	0.29	0.0651	0.0507	Eff	0.43	0.84	1.7
1	C	A	A	**C**	C	A	**A**	-0.93	0.35	0.1399	0.1215	Eff	0.53	0.85	1.4
10	C	G	A	**C**	C	G	**A**	-0.83	0.41	0.0349	0.0226	Eff	0.23	0.63	1.7
20	T	G	G	**C**	C	G	**G**	-0.37	0.71	0.1823	0.1739	Eff	0.64	0.96	1.4
5	C	A	A	**T**	C	G	**G**	-0.18	0.86	0.3533	0.3434	Base	NA	1	NA
7	C	A	A	**T**	T	G	**G**	1.4	0.16	0.0238	0.0386	Eff	0.81	1.87	4.3
13	C	G	A	**T**	C	G	**G**	1.61	0.11	0.0595	0.0901	Eff	0.85	1.6	3
3	C	A	A	**C**	C	G	**G**	NA	NA	0.0055	0.0034	R	1.09	1.98	3.6
4	C	A	A	**T**	C	A	**A**	NA	NA	0.012	0.0142	R	1.09	1.98	3.6

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