Microarray analysis of pediatric ependymoma identifies a cluster of 112 candidate genes including four transcripts at 22q12.1-q13.3[1,2]

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Ependymomas are glial cell–derived tumors characterized by varying degrees of chromosomal abnormalities and variability in clinical behavior. Cytogenetic analysis of pediatric ependymoma has failed to identify consistent patterns of abnormalities, with the exception of monosomy of 22 or structural abnormalities of 22q. In this study, a total of 19 pediatric ependymoma samples were used in a series of expression profiling, quantitative real-time PCR (Q-PCR), and loss of heterozygosity experiments to identify candidate genes involved in the development of this type of pediatric malignancy. Of the 12,627 genes analyzed, a subset of 112 genes emerged as being abnormally expressed when compared to three normal brain controls. Genes with increased expression included the oncogene *WNT5A*; the *p53* homologue *p63*; and several cell cycle, cell adhesion, and proliferation genes. Underexpressed genes comprised the *NF2* interacting gene *SCHIP-1* and the *adenomatous polyposis coli (APC)*-associated gene *EB1* among others. We validated the abnormal expression of six of these genes by Q-PCR. The subset of differentially expressed genes also included four underexpressed transcripts mapping to 22q12.3-13.3. By Q-PCR we show that one of these genes, *CBX7* (22q13.1), was deleted in 55% of cases. Other genes mapping to cytogenetic hot spots included two overexpressed and three underexpressed genes mapping to 1q31-41 and 6q21-q24.3, respectively. These genes represent candidate genes involved in ependymoma tumorigenesis. To the authors' knowledge, this is the first time microarray analysis and Q-PCR have been linked to identify heterozygous/homozygous deletions. *Neuro-Oncology 7, 20–31, 2005 (Posted to Neuro-Oncology [serial online], Doc. 04-059, December 6, 2004. URL http://neuro-oncology .mc.duke.edu; DOI: 10.1215/S1152851704000596)*

Ependymomas are glial cell–derived tumors that arise from the ependymal lining of the ventricular system of the central nervous system and manifest preferentially in childhood. They are the third most com-

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[4] Abbreviations used are as follows: $\Delta\Delta C_T$ method, comparative C_T method; *APC, adenomatous polyposis coli*; CGH, comparative genomic hybridization; LOH, loss of heterozygosity; Q-PCR, quantitative real-time polymerase chain reaction analysis; SDM, standard deviation from the mean.

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mon primary brain tumor in childhood (following low-grade astrocytomas and medulloblastomas) and account for 6% to 12% of all intracranial neoplasms in the pediatric population (Heideman, 1979). These tumors may occur at any site in the ventricular system, although they most commonly develop in the posterior fossa (Pollack et al., 1995). There are four variants of ependymoma in the WHO classification, myxopapillary (WHO grade I), subependymoma (WHO grade I), classic ependymoma (WHO grade II), and anaplastic ependymoma (WHO grade III) (Kleihues et al., 2002). Ependymomas have a propensity to recur. The five-year survival rates in children are 34% to 45%, with local relapse being the major source of therapeutic failure (Pollack et al., 1995).

At present, the genetic events that contribute to the pathogenesis of pediatric ependymoma are essentially unknown. Comparatively few cytogenetic analyses have been carried out, and many of these have been single case reports or small series of tumors (Bhattacharjee et al., 1997; Bigner et al., 1997). Furthermore, up to 50% of tumors appear karyotypically normal. To date, the most common chromosomal aberrations reported are deletions or rearrangements of 6q, 17, and 22, although all are present in only <30% of cases (Kramer et al., 1998; Mazewski et al., 1999; Reardon et al., 1999). Recent comparative genomic hybridization (CGH)[4] studies have also demonstrated gain of 1q to be present in a subset of ependymomas, and our laboratory has also reported high-copy-number amplification at 1q24-31 in three cases (Carter et al., 2002; Dyer et al., 2002; Ward et al., 2001). However, candidate genes mapping to these areas have not yet been identified. Although the *NF2* gene maps to 22q12, and individuals affected by *NF2* have increased susceptibility to ependymomas, *NF2* is rarely mutated in sporadic tumors (Rubio et al., 1994; Slavc et al., 1995). Similarly, mutations of *p53* are extremely rare in ependymoma (Tong et al., 1999).

At the molecular level, a small number of studies have linked ependymoma with abnormal expression of the *ERBB2* and *ERBB4* receptors (Gilbertson et al., 2002), the vascular endothelial growth factor protein (*VEGF*) (Korshunov et al., 2002), *p73* (Kamiya and Nakazato, 2002), and *MDM2* (Suzuki and Iwaki, 2000) in a subset of samples. However, to date, no consistent molecular alteration has been found in these tumors.

In recent years, global expression technologies such as oligonucleotide microarrays have been successfully used in the development of statistical algorithm-based classifications in several types of tumors (Dyrskjot et al., 2003; Luo, 2002; Perou et al., 2000; Watson et al., 2002). In this study we have used the Affymetrix GeneChip system U95Av2 (Affymetrix, Santa Clara, Calif.) validated by quantitative real-time polymerase chain reaction analysis (Q-PCR) to investigate abnormally expressed genes in ependymoma. We have identified 112 abnormally expressed genes, 10 of which map to regions of genomic imbalance identified by CGH at 1q, 6q, and 22q. We also report deletion of *CBX7* at 22q13 in more than 50% of ependymomas as validated by Q-PCR. Real-time PCR provides a means for continuous detection of product throughout the amplification process, and this technique has been used to detect deletions and duplications in cancer (M'soka et al., 2000; Senchenko et al., 2003). Unlike loss of heterozygosity (LOH) analysis, it is not necessary to identify polymorphic markers. The subset of genes identified in this study may represent candidate genes involved in the genesis and development of intracranial pediatric ependymoma.

Materials and Methods

Tumor Samples and RNA Preparation

Tumor specimens were obtained with informed consent from 19 patients (Table 1). The mean age was 7.8 years (range, 6 months to 15 years). All tumors were graded according to WHO criteria and comprised two subependymomas, 13 ependymomas, and four anaplastic ependymomas (Kleihues et al., 2002). Twelve samples were fresh-frozen biopsies collected directly from the operating theatre and stored in liquid nitrogen until ready for use. In our validation experiments, we included seven short-term cell cultures, which were prepared as described previously (Lewandowicz et al., 2000). Samples were directly adjacent to tumor tissue processed for routine histologic evaluation and were first examined macroscopically to ensure that no frankly normal tissue was included in either culture preparation or DNA extraction.

Two normal brain postmortem samples derived from the ventricular region of the corpus callosum were obtained from the Queen Square Brain Bank, Institute of Neurology. A third sample of normal corpus callosum (pooled RNA enriched for glial cells from tissues of 70 individuals) was purchased from Clontech (Clontech Laboratories Inc., Palo Alto, Calif.) as previously described (Ljubimova et al., 2001).

Total RNA was isolated from 12 biopsy samples by guanidine isothiocyanate buffer extraction (Life Technologies Inc., Rockville, Md.), phenol/chloroform extraction, and ethanol precipitation, followed by a second cleanup step (Qiagen Ltd., Crawley, UK). RNA was extracted from seven short-term cultures by using an anion exchange method on a resin column in the presence of a highly denaturing cell-lysis buffer containing guanidine isothiocyanate (Qiagen). Each RNA sample was quantified and quality assessed with an Agilent 2100 bioanalyzer machine (Agilent Technologies UK Ltd., West Lothian, Scotland).

cRNA Synthesis, Microarray Hybridization, and Data Collection

Total RNA from six biopsy samples and three normal brain controls was used to prepare biotinylated target RNA, with minor modifications from the manufacturer's recommendations (Affymetrix, 2004). Briefly, 10 µg of total RNA was used to generate first-strand cDNA by using a T7-linked oligo(dT) primer. After second-strand synthesis, in vitro transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics Inc., Farmingdale, N.Y.), resulting in approximately 100-fold

Table 1. Ependymoma case information. Summary of clinical information of tumor samples used in the (A) microarray and (B) Q-PCR sections of this study

Sample	Age[a]	Sex	Histology	Location	Source[b]	LOH[c]	Sequencing analysis[d]
A. Microarray samples							
IN2931	1.3	F	E	Posterior fossa	FF	yes	yes
IN2935	9.3	M	E	Posterior fossa	FF	yes	no
IN2939	0.6	F	E	Posterior fossa	FF	yes	no
IN3087	3.8	M	E	Posterior fossa	FF	yes	yes
IN3037	2	M	AE	Posterior fossa	FF	no	no
IN3108	4	M	AE	Posterior fossa	FF	yes	yes
B. Q-PCR samples							
IN772	4	M	SE	Supratentorial	CC	no	no
IN2242	2	F	E	Posterior fossa	CC	no	no
IN2376	15	M	E	Posterior fossa	FF	yes	no
IN2767	1.8	F	AE	Posterior fossa	FF	no	no
IN3008	10.5	M	E	Posterior fossa	FF	no	no
IN3071	4	M	E	Posterior fossa	FF	no	no
IN3121	8	M	E	Posterior fossa	FF	yes	no
IN3125	6	F	E	Posterior fossa	FF	yes	yes
IN1134	5.5	M	SE	Supratentorial	CC	yes	yes
IN1231	7	F	E	Supratentorial	CC	no	no
IN1258	2.5	F	E	Posterior fossa	CC	yes	yes
IN1759	10	M	E	Posterior fossa	CC	yes	yes
IN2443	4.5	M	AE	Posterior fossa	CC	no	no

Abbreviations used: AE; anaplastic ependymoma; CC, short-term cultures, E, benign ependymoma; F, female; FF, fresh-frozen material; M, male; Q-PCR, quantitative real-time PCR; SE, subependymoma

[a]Age at diagnosis in years

[b]Source

[c]Samples used in LOH based on Q-PCR

[d]Samples used in sequencing analysis of the *CBX7* gene

amplification of RNA. Target cDNA generated from each sample was then processed according to manufacturer's recommendation using an Affymetrix GeneChip Instrument System (Affymetrix, 2004). Briefly, spike controls were added to 10 μg of fragmented cRNA before overnight hybridization to U95Av2 GeneChip arrays. Arrays were then washed and stained with streptavidin-phycoerythrin (Molecular Probes Europe BV, Leiden, The Netherlands), before being scanned on an Affymetrix GeneChip scanner. After scanning, array images were assessed to confirm scanner alignment and the absence of significant bubbles or scratches. BioB spike controls were found to be present on all chips, with BioC, BioD, and CreX also present in increasing intensity. When scaled to a target intensity of 600 (with Affymetrix MAS 5.0 array analysis software), scaling factors for all arrays were within acceptable limits (0.3–1.5), as were background, Q values, and mean intensities.

All data and details of quality control measures were stored in a format compliant with MIAME (Minimum Information About a Microarray Experiment; Brazma et al., 2001) standards in an ArrayExpress database at the BioMap consortium at University College London and can be made available from the UCL ArrayExpress on request to the authors.

Data Analysis

The U95Av2 chip, which contains 12,627 transcripts including bacterial control spikes, was used in this study. To obtain a list of informative genes we used the GeneSpring software version 4.2.1 (Silicon Genetics, Redwood City, Calif.). The global error model option of GeneSpring based on replicates was applied (which allows for standard deviation values and *P* values to be computed). Genes whose signal did not significantly exceed background strength (control signal filter was set at a minimum of 17) and genes whose expression did not reach a threshold value for reliable detection (based on Affymetrix MAS 5.0 software) were filtered out. The remaining genes were considered informative and were subjected to a *t* test between two conditions (samples and controls), with the variance statistic derived from replicates. Finally, to remove false differential gene expression, a Benjamini-Hochberg multiple correction test (Benjamini and Hochberg, 1995) was applied to the list of genes generated above.

Q-PCR

A total of 13 RNA samples were incubated for 30 min at 37°C with 2 units of RNase-free DNase (Ambion,

Table 2. Fold change expression ratios derived from microarray analysis and Q-PCR of six genes found to be abnormally expressed by microarray analysis[a]

Gene Sample	*p63*	*FN1*	*WNT5A*	*SCHIP-1*	*EB1*	*CBX7*
A. Microarray Analysis						
IN2931	4.3	5.2	4.9	–5.9	–8.6	–7.8
IN2935	4.2	3.43	9.4	–4.4	–4.7	–3.7
IN2939	2.5	3.2	4.2	–3.9	–7.2	–2.7
IN3037	3.8	14.0	7.0	–6.0	–8.7	–6.4
IN3087	2.1	4.5	8.3	–3.7	–5.1	–2.9
IN3108	4.8	7.1	13.9	–7.7	–9.9	–10.5
B. Q-PCR Analysis						
IN772	37.1	30.2	345.9	–15.2	–4.0	–19.3
IN2242	21.3	27.7	262.0	–15.3	–6.3	–37.6
IN2376	17.0	14.5	6.5	–14.4	–2.8	–4.4
IN2767	8.6	23.3	24.8	–14.7	–3.2	–56.0
IN3008	12.7	43.4	5.4	–26.3	–4.6	–5.6
IN3071	13.1	40.5	14.6	–23.2	–2.4	–22.0
IN3121	24.5	46.3	20.1	–7.1	–2.0	–5.8
IN3125	4.3	2.0	69.0	–8.0	–3.1	–35.2
IN1134	ND	ND	ND	ND	ND	–11.0
IN1231	ND	ND	ND	ND	ND	–4.0
IN1258	ND	ND	ND	ND	ND	–20.4
IN1759	ND	ND	ND	ND	ND	–3.7
IN2443	ND	ND	ND	ND	ND	–17.0

Abbreviations: ND, reaction not done; Q-PCR, quantitative real-time polymerase reaction analysis.

[a]Fold change expression ratios were obtained as follows: (A) By microarray analysis. Hybridization intensities normalized by GeneSpring v. 4.2.1 (Silicon Genetics) from each tumor sample were divided by the mean normalized hybridization intensity of all three controls. (B) By Q-PCR on a different set of ependymoma samples. Each Q-PCR value obtained per gene per neoplastic sample was first normalized with the corresponding mean (*ß-actin* value obtained per sample and subsequently divided by the mean value obtained from the normalized control brain Q-PCR reaction for each of the six genes listed).

Austin, Tex.) to allow for DNA degradation. Total RNA (1 µg) was processed to cDNA by reverse transcription with Superscript II (Invitrogen Ltd., Paisley, UK) following manufacturer's instructions in a total volume of 20 µl. Q-PCR was performed by using an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, Calif.). We performed Q-PCR of *p63*, *FN1*, *WNT5A*, *SCHIP-1,* and *EB1* in eight samples (Table 2). Gene-specific oligonucleotide probes with 5' fluorescent and 3' quencher dyes (TaqMan probes, Applied Biosystems), and primers were obtained from the Assays on Demand choice of Applied Biosystems (www.appliedbiosystems.com). The TaqMan Universal PCR Master Mix with AmpErase UNG was used in a 20-µl reaction volume following cycling conditions recommended by the manufacturer (Applied Biosystems). Each reaction was carried out in duplicate, together with a negative (H_2O as template) and a reverse-transcriptase control (each cDNA synthesis reaction was carried out in the absence of reverse transcriptase). To compensate for RNA degradation and variability in the starting amounts of RNA, the *ß-actin* gene was used as an endogenous control (Applied Biosystems; www.appliedbiosystems.com). The amplification values obtained from each of the five genes used in this analysis were divided by the corresponding amplification values for *ß-actin* to produce an expression index.

Expression analysis of the *CBX7* gene was carried out for 13 samples in multiplex reactions. Selection of PCR primers and MGB probe sequences was performed with the ABI Primer Express software (version 5.1, Applied Biosystems). Primers (MWG-Biotech UK Ltd., Milton Keynes, UK) were as follows: *CBX7* forward 5'-CCG ACC CCT CCC AGA TAC A-3', *CBX7* reverse 5'-CTT CCT TTG CAC AGA ATG AGC TT-3', and the MGB probe 5'-AGT CTG AAC AAA GCT C-3', and they were labeled with FAM. The sequences of primers and the MGB probe for the *ß-actin* gene were as follows: *ß-actin* forward 5'-ACG AGG CCC AGA GCA AGA G-3', *ß-actin* reverse 5'-GAC GAT GCC GTG CTC GAT-3' and the MGB probe 5'-CAC CCT GAA GTA CCC-3' and was labeled with VIC. Q-PCR reactions were carried out in triplicate in 25-µl volumes consisting of 1× Quantitect probe PCR mix (Qiagen), 75 nM *ß-actin* primers, 900 nM *CBX7* primers, 25 ng DNA, and 200 nM MGB probe. The thermal cycling conditions were as follows: 15 min at 95°C, followed by 40 cycles at two temperatures, 95°C for 10 s and 60°C for 1 min. Prior to expression quantification, primer limitation experiments were carried out in a matrix of forward and reverse primer concentrations (12.5–100 nM) to check that the two independent reactions did not compete. To determine changes in expression levels of *CBX7* in relation to *ß-actin*, we used the comparative C_T method ($\Delta\Delta C_T$ method), where target sequence copy number of the *CBX7* gene (target) in the tumor samples is compared to the relative amount of *CBX7* in normal cDNA from pooled corpus callosum samples (calibrator) and relative to expression of the *ß-actin* gene, which was used as an endogenous control (reference). Relative copy number of *CBX7* in tumor and the corpus callosum samples is given by $2^{\Delta\Delta C_T}$, where

$$\Delta\Delta C_T = \Delta C_T^{tumor} - \Delta C_T^{calibrator}$$

and each $\Delta C_T = C_T^{target} - C_T^{reference}$ (Applied Biosystems, 1997).

LOH Analysis by Q-PCR

High-molecular-weight DNA was extracted from a total of eight biopsies, three short-term cell cultures, and 20 blood samples with the Qiagen genomic DNA kit following manufacturer's instructions (Qiagen).

LOH analysis of the *CBX7* gene was performed with the ABI Prism 7000 Sequence Detector System (Applied Biosystems). Reactions were carried out in quadruplets following the $\Delta\Delta C_T$ method (see Q-PCR section, above) to determine *CBX7* copy number in 11 neoplastic samples relative to normal DNA obtained from 20 pooled blood samples. Prior to carrying out this Q-PCR experiment, DNA quality was assessed by analyzing the PCR amplification in 2.5% agarose gel electrophoresis stained with ethidium bromide (results not shown).

Mutation Screening

The *CBX7* gene was sequenced in seven samples previously shown to have both copies or LOH for the *CBX7* gene (Table 1). Three pairs of primers were designed to amplify the coding region of *CBX7* using the Primer3 program developed by the Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 3). Amplifications were performed in a 20-μl reaction volume containing 20 ng cDNA (see above), 10 mM each of dNTPs, 10-pmol primers, 2 μl 10× Qiagen PCR buffer containing 15 mM $MgCl_2$ and 1 unit of Qiagen HotStarTaq DNA polymerase (Qiagen). An initial denaturation step of 15 min at 95°C was followed by a sequence of 15 s at 95°C, 30 s at 56°C/58°C/60°C (see Table 3), and 45 s at 72°C for 35 cycles, with a final extension step at 72°C for 7 min. Amplification products were run in 1.5% agarose gels and purified with the QIAquick PCR purification kit. Sequencing reactions were performed with the Bigdye terminator v1.1 cycle sequencing kit following manufacturer's instructions (PE Applied Biosystems, Foster City, Calif.). Reactions were run on a 377 ABI Prism bioanalyzer (PE Applied Biosystems), and sequences were analyzed with Sequencher 3.0 sequence analysis (Gene Codes Corporation, Ann Arbor, Mich.).

Table 3. PCR primers used in mutation screening of the CBX7 gene (gi: 46852393) coding region

Primer	Sequence	3′ end of primer position	PCR fragment length	Ann. temp. (°C)
CBX7F1	cccgcatggagctgtca	–5 (upstream)	413	56
CBX7R1	agggcagggtgggcac	+393		56
CBX7F2	gaagctctgcttctccctgac	+296	412	60
CBX7R2	atggagttggcggtgatgt	+688		60
CBX7F3	ccctgaagaggaggcagat	+584	249	58
CBX7R3	cccccaacccatccctat	+833		58

Abbreviation: Ann. temp., annealing temperature.

Results

Gene Expression Profiles in Ependymoma

Using the GeneChip Affymetrix U95Av2 array technology, we studied differences in expression of >12,000 genes from six pediatric ependymoma samples and three samples derived from normal brain. Filtering was performed to remove genes whose expression in ependymoma samples did not differ from that in controls and genes whose hybridization signals fell below the threshold for reliable detection using GeneSpring v. 4.2.1. (Silicon Genetics). Further analysis was performed using a *t* test with *P* value cutoff at 0.05. Finally, to reduce false positives, a Benjamini-Hochberg multiple correction test (Benjamini and Hochberg, 1995) was applied to generate a final list of genes consisting of 112 transcripts. To establish fold change in expression levels, we compared the mean normalized value of each gene in the ependymoma cohort to the mean normalized value of each gene in the control cohort. A total of 26 genes were overexpressed ⩾3-fold ($P < 0.05$) in ependymoma. Genes with increased expression included many encoding adhesion and extracellular matrix proteins such as *FN1* and *p63* (Korshunov et al., 2003) and the transcription factors *Zic1*. Overexpressed genes previously associated with brain tumors included the angiogenesis factor *VEGF* (Korshunov et al., 2002) and the oncogene *WNT5A* (Howng et al., 2002).

A total of 84 genes were underexpressed ⩾2-fold in the neoplastic group ($P < 0.05$). These comprised the *NF2* interacting protein coding gene *SCHIP-1* and several genes involved in vesicle trafficking and recycling, such as *NPC1*, *RAB40B*, *TJ2*, *SH3GL3*, and *EB1*. A full list of the genes described above with associated statistical values is available (http://www.ion.ucl.ac.uk/molpat/neuro-oncology/secured/microarray.html). To visualize the relationship between gene expression and sample identity we performed unsupervised hierarchical clustering per gene and per experiment with the 112 genes described above using GeneSpring v.4.2.1 (Silicon Genetics). Ependymoma and control groups clustered into two different branches of the tree, suggesting the data set identified in this study is likely to be involved in ependymoma genesis and progression (Fig. 1).

Identification of Differentially Expressed Genes Located in CGH Hot Spots

By CGH analysis, we and others have identified gains at 1q and losses at 6q and 22q as the most common genomic imbalances in ependymoma (Mazewski et al., 1999; Reardon et al., 1999; Ward et al., 2001). Our subset of 112 genes included a group of four underexpressed genes mapping to 22q12.3-22q13.3, which comprised the F-box protein coding gene *FBX7*, the uncharacterized transcript *C22orf2*, the chromobox protein coding gene *CBX7*, and the SET domain-binding protein coding gene *SBF1*. Expression analysis of other genes mapping to 22q12.3-22q13.3 was, in most cases, uninformative, as transcripts were either not present in U95Av2 or not expressed in neoplastic and control samples. However, we observed normal expression of a group of five genes flanking the set *FBX7-C22orf2-CBX7-SBF1*, suggesting the presence of a microdeletion (Fig. 2). Within the 112 subset, we also identified three underexpressed genes mapping to 6q, namely, the polyamine biosynthesis gene *AMD1* (6q21), the cyclin-dependent kinase *CDK11* (6q21), and the tumor suppressor gene *SASH1* (6q24.3), and two overexpressed genes mapping to 1q, namely, *laminin* (1q31) and the glioma amplified gene *GAC1*, mapping to the 1q32-q41 amplicon (Bhattacharjee et al., 1997; Kramer et al., 1998; Ward et al., 2001).

Corroboration of Gene Expression by Q-PCR

To validate the data obtained by microarray profiling, and to extend the analysis to a larger set of clinical sam-

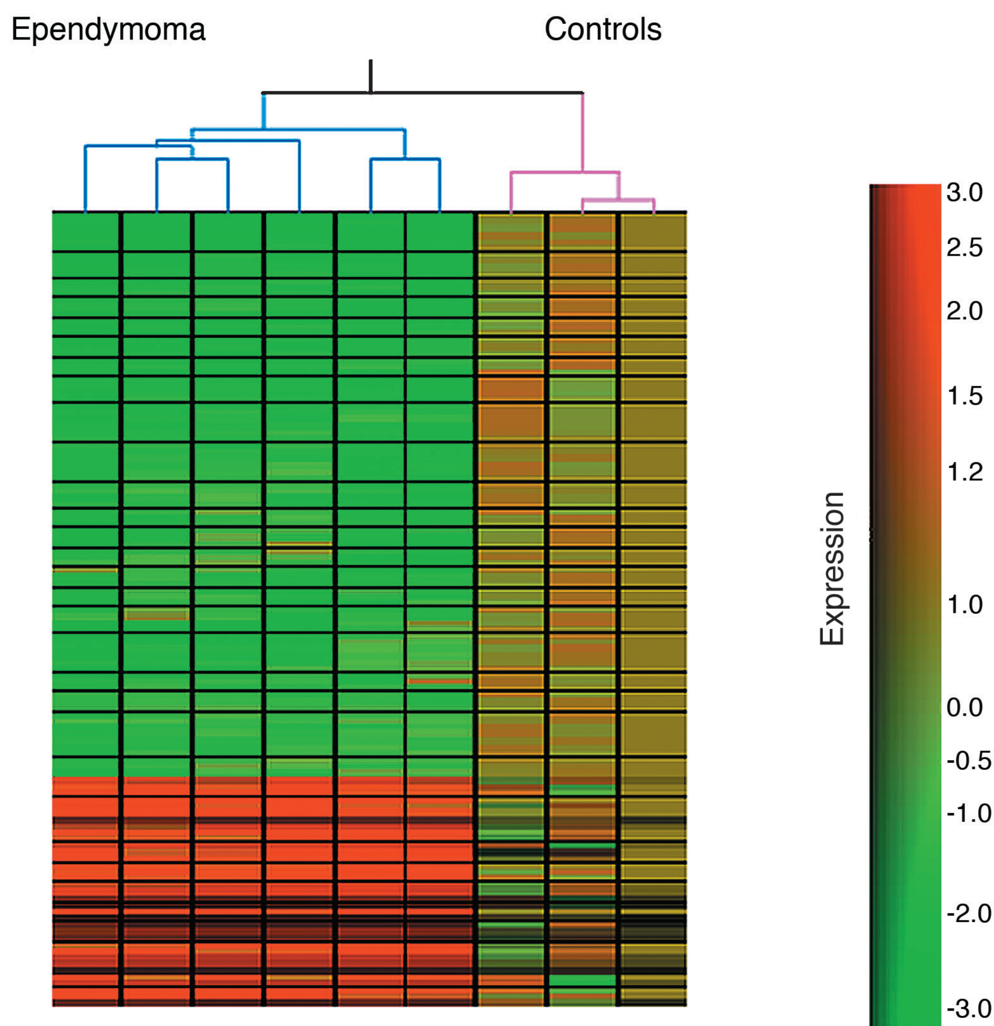
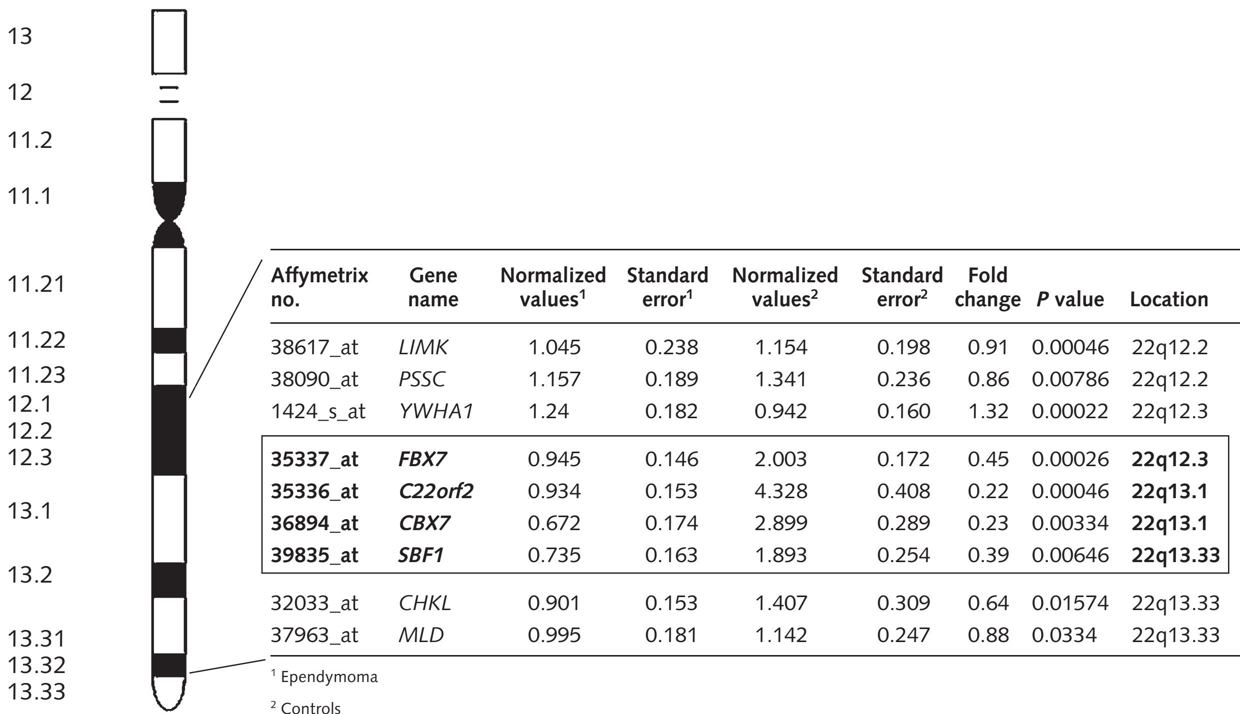


Fig. 1. Unsupervised hierarchical clustering of six ependymoma samples (indicated with blue brackets at top of figure)and three controls (indicated with pink brackets). Cluster analysis was performed with the 112 genes that comprised the group of differentially expressed genes in neoplastic and nonneoplastic samples as computed by the predictive software. Color saturation is proportional to magnitude of the difference from the mean, and ranging from green (underexpressed) to red (overexpressed). See bar at right of figure.

ples, we have chosen five genes from our 112 gene list to evaluate in a further set of eight ependymoma samples (six biopsies and two short-term cell cultures) by Q-PCR (Table 2). These genes were chosen by biological relevance and chromosomal location and included the oncogene *WNT5A*; *p63*, a *p53* homologue; *FN1*, which has been previously associated with overexpression of *ERBB2* (Mackay et al., 2003); the *adenomatous polyposis coli* (*APC*)-associated gene *EB1*; and the *NF2* interacting gene *SCHIP-1*. The expression of a sixth gene, *CBX7*, which maps to 22q13.1, was studied in an additional 13 samples. To establish the concordance between microarray and Q-PCR we produced tumor to normal ratios of the values obtained by Q-PCR for *WNT5A*, *FN1*, *p63*, *EB1*, and *SCHIP-1*. When these ratios were depicted together with ratios calculated from the fold changes by the GeneSpring software (Silicon Genetics), the results that were obtained followed the same trend observed in our microarray data in five out of five cases. A comparison of fold change values collected from microarray analysis and Q-PCR is shown in Table 2.

We were able to perform multiplex reactions with the *CBX7* and *ß-actin* genes and chose to carry out the expression analysis using the $\Delta\Delta C_T$ method. First, to check that the two independent reactions do not compete, primer limitation experiments were performed using a matrix of forward and reverse primer concentrations (12.5–100 nM) for the *ß-actin* gene. The chosen concentration was 75 nM, which produced the lowest ΔR_n (magnitude of the signal generated by the given set of PCR conditions), but had little effect on C_T (results not shown). Second, for the $\Delta\Delta C_T$ calculation to be valid, both reactions should have equal efficiencies. We therefore performed a validation experiment in which PCR reactions were carried out in quadruplets for both reference (*ß-actin*) and target (*CBX7*) genes in a series of seven dilutions. For both primers to be compatible, the absolute value of the slope of log input amount versus



Affymetrix no.	Gene name	Normalized values[1]	Standard error[1]	Normalized values[2]	Standard error[2]	Fold change	*P* value	Location
38617_at	*LIMK*	1.045	0.238	1.154	0.198	0.91	0.00046	22q12.2
38090_at	*PSSC*	1.157	0.189	1.341	0.236	0.86	0.00786	22q12.2
1424_s_at	*YWHA1*	1.24	0.182	0.942	0.160	1.32	0.00022	22q12.3
35337_at	***FBX7***	0.945	0.146	2.003	0.172	0.45	0.00026	**22q12.3**
35336_at	***C22orf2***	0.934	0.153	4.328	0.408	0.22	0.00046	**22q13.1**
36894_at	***CBX7***	0.672	0.174	2.899	0.289	0.23	0.00334	**22q13.1**
39835_at	***SBF1***	0.735	0.163	1.893	0.254	0.39	0.00646	**22q13.33**
32033_at	*CHKL*	0.901	0.153	1.407	0.309	0.64	0.01574	22q13.33
37963_at	*MLD*	0.995	0.181	1.142	0.247	0.88	0.0334	22q13.33

[1] Ependymoma

[2] Controls

Fig. 2. Expression pattern of a subset of four genes found to be underexpressed in ependymoma as compared to normal brain (shown in bold) next to a group of five flanking genes. Values are based on hybridization signals normalized as explained in Materials and Methods. Normalized values are shown as an average of [1]ependymoma and [2]controls. Standard error values refer to those obtained from normalized values from [1]ependymoma and [2]controls. Detected *P* values shown refer to those computed by the Affymetrix MAS 5.0 array analysis software on chip intensities. *P* values are represented as an average of all samples and controls. The cutoff *P* value to reliably detect a transcript was set at <0.05 per individual sample. Other genes mapping to 22q12.2-q13.33 were either not present in U95Av2 or had *P* values >0.05 by MAS 5.0 in all control and neoplastic samples and were flagged absent by the computer program. The distance of the potential microdeletion was obtained from Project Ensembl on the Internet (http://www.ensembl.org) and is shown above the chromosome figure.

C_T should be <0.1 (see the Q-PCR section in Materials and Methods). In all 13 cases, *CBX7* expression in the tumors was at least 3 times lower than in the normal corpus callosum control (Table 2).

LOH Analysis by Q-PCR and Mutation Analysis

Subsequently, we investigated the copy number status of the *CBX7* gene in 11 ependymoma samples, all of which were previously used either in the microarray or in the Q-PCR sections (see Table 1). The *ß-actin* gene was used as a reference, and as a control we used DNA obtained from blood samples from 20 individuals. Results from normal DNA samples were subsequently analyzed on the ABI sequence analyzer 7000 and averaged before they were used to calculate copy number changes in the tumor samples. (For methodology, see Senchenko et al. [2003]; http://www. appliedbiosystems.com.)

From our data, the mean value from the pooled blood samples was 1 (95% reference range, 1 ± 0.16). Taking account that normal tissue contamination could reach up to 40%, one could expect values less than 0.5 and 0.9 to account for homozygous and hemizygous deletions, respectively (Senchenko et al., 2003). The Mann-Whitney nonparametric *t* test was applied to both groups (blood and tumor), producing highly significant results ($P = 0.003$). Samples with values $\leqslant 0.84$ (mean value of normal samples minus 95% reference range) were considered hemizygously deleted. Alleles were considered as homozygously deleted if their highest SDM value was <0.56 (0.4 plus $2 \times$ SDM of controls). Our Q-PCR results suggest that 1/11 (9%) samples was homozygously deleted and 5/11 (46%) were hemizygously deleted (Table 4). These data show allelic loss at 22q13.1 in 55% of the cases, which is significantly higher than previously reported in pediatric intracranial ependymoma (Bhattacharjee et al., 1997; Carter et al., 2002; Ward et al., 2001). Mutation analysis of *CBX7* in seven of the above samples which had retained one or two copies of the gene did not reveal any sequence alterations in the coding region.

Discussion

Microarray technology allows the generation of multiple gene expression profiles in a single experiment, with the potential to accelerate the identification of prognostic markers and improve diagnosis and choice of therapy. Several studies have used microarray technology to iden-

Table 4. Deletion analysis results for the *CBX7* gene by Q-PCR on 11 ependymoma samples

Sample ID	ΔCT	SDM ΔC_T	ΔΔCT	SDM $\Delta\Delta C_T$	Copy no. $2^{-\Delta\Delta CT}$	SEM $2^{-\Delta\Delta CT}$	No. of Alleles
Controls[a]	1.23	0.03	0	0.13	1.01	0.02	2
IN1134	1.32	0.06	0.08	0.06	0.94	0.01	2
IN1258	1.55	0.09	0.32	0.09	0.81	0.01	1
IN1759	1.99	0.01	0.76	0.01	0.59	0.01	1
IN2931	1.28	0.04	0.05	0.15	0.97	0.02	2
IN2935	1.99	0.06	0.76	0.06	0.59	0.01	1
IN2939	2.24	0.08	1.01	0.08	0.5	0.01	0
IN3087	1.29	0.1	0.06	0.08	0.96	0.01	2
IN3108	1.43	0.12	0.19	0.1	0.88	0.01	2
IN2376	1.61	0.06	0.37	0.12	0.77	0.01	1
IN3121	1.85	0.00	0.61	0.02	0.62	0.00	1
IN3125	1.01	0.00	–0.22	0.09	1.17	0.00	2

Abbreviations: SDM, standard deviation from the mean; SEM, standard error of the mean. The terms C_T, ΔC_T, $\Delta\Delta C_T$, and $2^{-\Delta\Delta C_T}$ are defined elsewhere (Applied Biosystems, 1997).

[a]Control values were derived from normal DNA obtained from blood from 20 individuals.

[b]Number of alleles was estimated as heterozygously deleted $\leq 2^{-\Delta\Delta C_T}$ of controls minus 95% reference range of controls (or 2 × SDM of controls), homozygously deleted ≤0.4 + 2 × 0.08 (SDM of controls). See "LOH Analysis by Q-PCR and Mutation Analysis" in the Results section.

tify patterns of gene expression in time course experiments (Arbeitman et al., 2002; Mandel et al., 2002), classify tumor samples (Perou et al., 2000; van 't Veer et al., 2003), and reliably map novel genes to molecular pathways (Voehringer et al., 2000; Zhao et al., 2000), giving an unprecedented insight into the function of unknown genes and their interactions with other genes in different cellular pathways.

At the molecular level very little is known regarding the etiology of ependymoma, and to date, most studies have employed cytogenetic and CGH analysis to elucidate the genetic makeup of these tumors. The aim of the present study was to identify candidate genes responsible for pediatric ependymoma development by using a microarray-based approach, validated by Q-PCR followed by LOH analysis of a candidate gene mapping to 22q13.1. We report a group of 112 abnormally expressed genes, four of which mapping to 22q12-13, which constitutes the most recurrent region of loss in ependymoma. We have also shown that deletions in 22q in pediatric ependymoma are more common than previously reported.

Abnormally Expressed Genes in Pediatric Ependymoma

Some of the genes identified in this study have been associated with other types of cancer, particularly brain tumors, and are likely to play an important role in ependymoma development. These include *COL4A1* (>29.5-fold change), *IBP2* (>13.5-fold change), *HOX7* (>11.5-fold change), *Wee1* (>11-fold change), *GAC1* (>7-fold change), *WNT5A* (>5.5-fold change), and *FN1* (>5.5-fold change) (Almeida et al., 1998; Howng et al., 2002; Masaki et al., 2003; Wang et al., 2003; Wasenius et al., 2003). We also showed underexpression of *EB1* and *SCHIP-1* by both expression profiling and Q-PCR. These genes interact with the tumor suppressors *APC* and *NF2*, respectively (Renner et al., 1997; Rouleau et al., 1993). *NF2* is a tumor suppressor gene responsible for an autosomal dominant disease that predisposes to the development of central nervous system tumors. Mutations in this gene have been found in intraspinal ependymoma (Alonso et al., 2002; Rubio et al., 1994) but are rarely observed in intracranial ependymomas. Although the biological function of *SCHIP-1* remains unknown, our results suggest that it may play an important role in ependymoma development and should be further investigated.

One previous study by Korshunov et al. (2003) investigated gene expression in ependymoma by comparing a heterogeneous mix of spinal and intracranial biopsy samples to a pooled control comprising 10 different cell lines. The authors were able to differentiate between spinal/intracranial, adult/pediatric, and benign/anaplastic samples and concluded that subgroups of ependymoma patients may be suffering from molecularly distinct diseases (Carter et al., 2002; Korshunov et al., 2002, 2003). In the present study we have produced a molecular signature of pediatric intracranial ependymoma compared to three biopsy controls to identify genes involved in tumor development. We did not observe abnormal expression of other genes found to be abnormally expressed in other types of brain tumors such as *EGFR*, *PDGF*, *PDGFR*, *CDK2*, *Rb*, *MDM2,* and *PTEN*, and we therefore postulate that development of ependymoma in pediatric patients may depend on alternative molecular pathways.

Expression of Genes Located in Common Regions of Loss or Gain

By cytogenetic analysis, we and others have identified gains and amplifications at 1q and losses at 6q and 22q as

the most common genomic imbalances in ependymoma (Mazewski et al., 1999; Reardon et al., 1999; Ward et al., 2001). Chromosome 22q has also been involved in translocations and interstitial deletions in ependymoma, both in sporadic and familial cases, supporting the presence of a tumor suppressor gene at this location (Nijssen et al., 1994; von Haken et al., 1996). By cytogenetic analysis, monosomy 22 has been reported in up to 31% of pediatric samples (Mazewski et al., 1999), compared to an incidence of 56% in adult samples (Kramer et al., 1998).

Four candidate genes have previously been proposed as potential tumor suppressors mapping to 22q. One such gene, *NF2*, is mutated in sporadic cases of *NF2*-associated schwannomas and meningiomas but has been rarely reported in astrocytic tumors and ependymomas (Rubio et al., 1994). Another candidate gene is the tumor suppressor *hSNF5/INI1*, found to be mutated in pediatric rhabdoid tumors, atypical teratoid and rhabdoid tumors of the brain, renal and extrarenal rhabdoid tumors, choroid plexus tumors, and primitive neuroectodermal tumors (Biegel et al., 1999; Sevenet et al., 1999; Versteege et al., 1998). However, screening of the *hSNF5/INI* gene in 52 ependymomas failed to identify any mutations (Sevenet et al., 1999). *Chk2* and *EP300*, also mapping to 22q, have been associated with various types of cancers, such as gastric carcinoma and colorectal and breast cancer, but so far no association between *Chk2* and *EP300* and brain tumors has been found (Bryan et al., 2002; Hartmann et al., 2004; Muraoka et al., 1996; Vahteristo et al., 2002). Recently, by using high-resolution mapping analysis of 22q, two groups have shown 22q12.3-22q13.2 to be a critical region associated with astrocytoma grade and progression (Ino et al., 1999; Oskam et al., 2000).

In this study we have identified a group of four underexpressed genes mapping to 22q12-q13.1, suggesting the presence of a microdeletion at this site of 22q. By Q-PCR we have shown that *CBX7* is also underexpressed in 13 further ependymoma samples as compared to a commercially available control from normal human brain.

The use of Q-PCR in the detection of genomic deletions has been applied in a number of cancer studies (Braga et al., 2002; M'soka et al., 2000; Senchenko et al., 2003). Recently, Senchenko et al. (2003) showed that this technique is sensitive enough to distinguish between one and two alleles by examining differences in gene dosage between the X and Y chromosomes. We used Q-PCR to show that abnormal expression of *CBX7* was due to allelic loss in 55% of cases (6/11), where underexpression was due to loss of one allele in 46% of cases and both alleles in 9% of cases. Subsequently, we performed mutation analysis of the coding region of the *CBX7* gene in seven samples, showing the presence of one or two copies by Q-PCR. We were unable to identify any sequence alteration of the *CBX7* coding region, which suggests that other mechanisms, such as promoter methylation or histone deacetylation, may be responsible for the silencing of this gene in our ependymoma samples. Extensive methylation of promoters and regulatory sequences that control gene activity have previously been reported near tumor suppressor genes (Esteller et al., 2000a, b; Herman and Baylin, 2000). Histone acetylation and deacetylation are major regulatory mechanisms of transcription that function by modulating the accessibility of transcription factors to their binding site on DNA. An example of such a mechanism is performed by the Rb gene, which transmits active repression to E2F-responsive genes by modifying chromatin architecture (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998).

CBX7 belongs to a family of protein coding genes containing the chromodomain motif, which are involved in gene silencing by mediating changes in higher order chromatin structure (Paro and Hogness, 1991). *CBX7* is highly expressed in a number of different normal tissue types, including brain, kidney, heart, and skeletal muscle, although it has not previously been investigated in tumor cells (Gil et al., 2004). A recent study has demonstrated that *CBX7* expression is associated with extension of cellular life span in mouse embryonic fibroblasts and human prostate primary epithelial cells by downregulating expression of the Ink4a/Arf locus (Gil et al., 2004). The role of the $p16^{Ink4a}$/Rb and Arf/p53 pathways in ependymoma is unclear. Although gene mutations are rare, hypermethylation of $p16^{Ink4a}$, Rb, and p14ARF has been reported in a subgroup of tumors (4%–32%) (Alonso et al., 2003, 2004; Sato et al., 1996; Tong et al., 1999). However, in view of our findings that *CBX7* was consistently underexpressed in 19 ependymoma samples and that at least one copy is deleted in 55% of cases examined, further work is required to discern an alternative mechanism for *CBX7* function in these tumors.

Loss of 6q was the most common genomic imbalance in one study, present in 23% of ependymoma (Reardon et al., 1999). In our CGH analysis, loss of 6q was the sole abnormality in one case (Ward et al., 2001). We have found underexpression of three genes located on 6q by microarray analysis; two such genes, *ADM1* and *CDK11*, map at 6q21 ~200 kb away from each other (http://www.ensembl.org). Statistical analysis of microarray data did not detect any further underexpressed genes mapping to 6q21. A third gene, namely *SASH1* (6q24), also underexpressed in our ependymoma cohort, has recently been reported to be downregulated in breast cancer (Zeller et al., 2003).

Gains of 1q constitute the most common region of gain in ependymoma (Carter et al., 2002; Ward et al., 2001) and have also been involved with drug resistance (Muleris et al., 1994; Schrock et al., 1994) and poor prognosis in other types of solid tumors (Gronwald et al., 1997; Nishida et al., 2003; Petersen et al., 2000). We identified two overexpressed genes mapping to 1q, namely *laminin* and *GAC1*, which represent potential oncogenes mapping to the 1q32 amplicon.

Our knowledge regarding the molecular pathways leading to ependymoma development is scarce. In this study, we present a group of 112 genes that are abnormally expressed in ependymoma and that may contribute to the development of this pediatric brain tumor. We have also validated the expression of five genes, namely *WNT5A*, *FN1*, *p63*, *SCHIP-1*, and *EB1*, in a further eight samples. These genes have potential roles in cancer

development and have not been previously involved in ependymoma. The expression of a sixth gene, *CBX7*, which maps to the 22q13.1 CGH hot spot, was further analyzed in 13 samples by Q-PCR. We have identified a cluster of abnormally expressed genes mapping to 1q21-q31, 6q21-24, and 22q12-q13 that constitute the most common regions of genomic imbalance identified by CGH. We also provide evidence that underexpression of one gene, *CBX7*, was due to homozygous allele loss in 9% of cases and heterozygous loss in 45%, an overall incidence of 55%, higher than previously reported following other methodologies (Table 4). Q-PCR is emerging as an alternative method to microsatellite deletion mapping and CGH to detect copy number changes (Braga et al., 2002; Senchenko et al., 2003). CGH is limited by poor resolution of 20 Mb for losses, and microsatellite mapping requires the identification of polymorphic markers and paired normal somatic tissue. Homozygous deletions are excellent indicators of the location of tumor suppressor genes, and therefore, the genes identified in this study mapping to hot spot CGH regions should be further investigated, as they constitute potential tumor suppressors responsible for the neoplastic phenotype in pediatric ependymoma patients.

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