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RESEARCH ARTICLE

Low Frequency of Chromosomal Imbalances in Anaplastic Ependymomas as Detected by Comparative Genomic Hybridization

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We screened 26 ependymomas in 22 patients (7 WHO grade I, myxopapillary, myE; 6 WHO grade II, E; 13 WHO grade III, anaplastic, aE) using comparative genomic hybridization (CGH) and fluorescence *in situ* hybridization (FISH). 25 out of 26 tumors showed chromosomal imbalances on CGH analysis. The chromosomal region most frequently affected by losses of genomic material clustered on 13q (9/26). 6/7 myE showed a loss on 13q14-q31. Other chromosomes affected by genomic losses were 6q (5/26), 4q (5/26), 10 (5/26), and 2q (4/26). The most consistent chromosomal abnormality in ependymomas so far reported, is monosomy 22 or structural abnormality 22q, identified in approximately one third of Giemsa-banded cases with abnormal karyotypes. Using FISH, loss or monosomy 22q was detected in small subpopulations of tumor cells in 36% of cases. The most frequent gains involved chromosome arms 17 (8/26), 9q (7/26), 20q (7/26), and 22q (6/26). Gains on 1q were found exclusively in pediatric ependymomas (5/10). Using FISH, MYCN proto-oncogene DNA amplifications mapped to 2p23-p24 were found in 2 spinal ependymomas of adults. On average, myE demonstrated 9.14, E 5.33, and aE 1.77 gains and/or losses on different chromosomes per tumor using CGH. Thus, and quite paradoxically, in ependymomas, a high frequency of imbalanced chromosomal regions as revealed by CGH does not indicate a high WHO grade of the tumor but is more frequent in grade I tumors.

Introduction

Ependymomas arise throughout the neuraxis in intimate relationship to the ependyma or its remnants. They account for 3-9% of intracranial brain tumors and about 60% of spinal tumors. Nevertheless, they are the third most common group of primary brain tumors in childhood. The World Health Organization (WHO) classification of central nervous system tumors defines various ependymal tumors (22): Myxopapillary ependymomas (grade I, myE) are tumors of young adults. They differ from other ependymomas. In their localization at the cauda equina and in their histomorphology and biological behavior. These tumors do not undergo malignant transformation. In general, intracranial or spinal ependymomas grade II and III are well-circumscribed masses, which occur in children and adults. Histologic hallmarks include perivascular pseudorosettes and/or ependymal rosettes. They are classified as grade II ependymomas (E), if they are characterized by moderate cellularity and exhibit low or no mitotic activity. Foci of necrosis may be seen, but are not necessarily indicative of malignancy. Anaplastic (malignant) ependymomas (grade III, aE) are characterized by high cellularity, variable nuclear atypia, brisk mitotic activity and often prominent vascular proliferation. Necrosis may be widespread. The histologic classification of grade II and III ependymomas continues to be a contentious issue and little consensus has been reached on the prognostic value of specific histologic features (7, 22). Several studies have focused on the molecular biology and cytogenetics of ependymal neoplasms (17, 29). The most consistent chromosome abnormality in ependymomas reported so far is monosomy 22 or structural abnormality 22q, identified in approximately one third of Giemsa-banded cases with abnormal karyotypes. Chromosome 22 abnormalities, however, are also seen in a number of other brain tumors (4). In the

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Case	Diagnosis; Grade	Sex	Age (years)	Ki-67 %	Localization	Tissue
1	myE; I	m	39	5	Cauda equina	P
2	myE; I	m	27	2	Cauda equina	F/P/N
3	myE; I	f	50	2	Cauda equina	F/P
4	myE; I	m	40	5	Cauda equina	P
5	myE; I	m	47	<1	Cauda equina	P
6	myE; I	m	41	4	Cauda equina	P
7	myE; I	m	14	<1	Cauda equina	F
8	E; II	m	13	13	Spinal	F/P
9	E; II	f	3	2	Ventricle II	F/P
10	E; II	m	13	2.5	Ventricle II	F/P
11	E; II	f	70	3	Ventricle IV	P
12	E; II	f	33	<1	Spinal	F
13	E; II	f	40	7	Spinal	P
13R	aE; III	f	41	10	Spinal	P
13M	aE; III	f	41	15	Temporal	F/P
14	aE; III	m	0.5	ND	Ventricle IV	F
15	aE; III	m	8	49	Ventricle IV	P
15R	aE; III	m	8	ND	Ventricle IV	P
16	aE; III	f	31	19	Ventricle IV	F/P
17	aE; III	f	42	14	Ventricle IV	P/N
18	aE; III	m	4	75	Ventricle IV	P/N
19	aE; III	f	4	31	Ventricle IV	F/P
19R	aE; III	f	5	ND	Ventricle IV	F
20	aE; III	f	2	64	Ventricle II	F/P
21	aE; III	f	6	65	Ventricle II	P
22	aE; III	m	59	33.5	Ventricle IV	F

Table 1. *Summary of selected Clinical Data, Histopathology of Ependymomas.*
Abbreviations: R, recurrence; M, metastasis of second recurrence; m, male; f, female; F, DNA extracted from fresh frozen tumor; P, DNA extracted from paraffin-embedded tumor; N, as internal control DNA extracted from non-tumorous areas (normal karyotype by CGH); ND, no data.

present study, the genomic alterations in ependymal tumors of three different malignancy grades were investigated in a series of 26 tumors by means of comparative genomic hybridization (CGH). The CGH data were supplemented by fluorescence *in situ* hybridization (FISH).

Materials and Methods

Tumor Samples. Twenty-six ependymomas from 22 patients were investigated (10 females, 12 males; age at diagnosis 0.5-70 years; median 29 years) (Table 1). Nine recurrences were included (nos. 5, 13, 13R, 13M, 15, 18, 19, 19R, 20). The samples were obtained from the archives of the following institutions: Institute of Pathology, University of Ulm; Institute of Neuropathology, University of Göttingen; Department of Neurosurgery, University of Heidelberg. Two patients (nos. 11 and 18) had chemotherapy and one (no. 13) had radiotherapy before or between operations. In one patient (no. 16), there was evidence of an association with neurofibromatosis type 2 (NF2) because of previous operations of several schwannomas. The tumors were classified according to the WHO classification of tumors of the central nervous system (22). The series group consisted of 7 myxopapillary ependymomas (myE, grade I), 6 ependymomas (E, grade II), and 13 anaplastic ependymomas (aE, grade III) (Table 1). Histologic evaluation of these samples revealed an estimated tumor cell content of >90%. Tumor samples were available in 21 cases as formalin-fixed and paraffin embedded and in 13 cases as fresh frozen tissue. Up to 20 serial sections, each of 10µm in thickness, were used for DNA isolation. In cases no. 2, 17 and 18 non-tumorous tissue was microdissected separately from tumor areas under microscopic view. In these cases non-tumorous and tumorous tissue was subsequently used for DNA extraction. In 22/26 material for FISH investigations was available. The data are summarized in Table 1.

Immunohistochemistry. All tumors were evaluated on formalin-fixed paraffin sections and in a few cases on fresh frozen sections for the expression of the proliferation associated nuclear antigen Ki-67 (MIB1; Dianova, Hamburg, Germany). The Ki-67 proliferation index was evaluated by counting labeled nuclei in three different high power fields in the tumor area with the highest density of labeled cells. The counted labeled nuclei were expressed as a percentage of tumor nuclei.

Chromosome Preparations. Metaphase chromosome spreads were prepared from primary blood cell cultures of healthy donors (46, XX or 46, XY) using standard protocols (2). Slides were stored at -80°C until use.

DNA Preparations. Paraffin-embedded tissues were treated in xylene to remove wax, washed in methanol, dried, and incubated overnight with sodium thiocyanate (1M) at 37°C. Samples were incubated for the following 3 days at 50°C with isolation buffer (75 mM NaCl, 25 mM EDTA, 0.5% Tween) and proteinase K (500µg/ml) was added every 24 hours. DNA extraction from paraffin-embedded material using a commercially available DNA extraction Clean-Mix kit (Talent, Trieste, Italy) was performed for small amounts of tissue or, if large amounts of tissue were available, we used phenol-chloroform-isoamylalcohol (24:24:1) methods. Isolation of reference genomic DNA from fresh frozen material (normal human tonsil) and fresh tumor was extracted by a phenol-chloroform standard protocol.

Labeling of DNA Probes and CGH. Nick translation of DNA probes was performed following standard protocols (2). Tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim, Germany) and reference

Table 2. *Characteristics and Results of CGH and FISH Analysis of Ependymomas.*
Abbreviations: R, recurrence; M, metastasis of second recurrence; ND, no data; FISH data are given as the mean of interphase signals of at least 100 cells counted, expressed as the mean of 100 cells. CGH data expressed in bold letters of gains and losses are confirmed by FISH. In cases 1, 3, 4 and 6 a copy number of three was considered normal, instead of the usual copy number of two.

Case	Diagnosis; Grade	Signals of FISH probes Mean of 100 cells	Chromosomal imbalances by CGH	
			Gains	Losses
1	myE; I	9q34: 3.4; 22q11: 3.0	**9q32-qter**; Xq27-qter	4; 8q11.2-q24.1; 13q; 14q12-q21
2	myE; I	9q34: 2.1; 22q11: 2.0	4; 5; 6; 7p13-p21; 7q22-qter; 9p-9q33; 18q22-qter	-
3	myE; I	3 cen: 2.1; 6 cen: 2.1; 9 cen: 2.7; 9q34: 2.6; 22q11: 2.1 Xcen: 2.0	4p16; 5q31-qter	**3**; **6**; 8q; 10; 13q; **X**
4	myE; I	6 cen: 2.9; 9 cen: 3.2; 9q34: 4.3; 10 cen: 2.1; 22q11: 2.2	3p21; **9q34**; 15q22-qter; 17pter-q22; 20	**10**; 13q
5	myE; I	9q34: 2.1; 22q11: 3.3	17p; 17q25; 20q; **22q**; X	1p31; 2q22; 4q21-q28; 5q14-q23; 6q12-q23; 13q14-q31
6	myE; I	6 cen: 2.9; 9q34: 3.9; 22q11: 2.9	**9q34**; 12q24; 17p; 17q25; 20	2q21-q33; 4p15; 4q; 6q12-q25; 8q11.2-q23; 13q14-q32; 14q11.2-q21
7	myE; I	9q34: 3.3; 22q11: 2.9	7; 8q24.2-q24.3; **9**; 11; 17; 18; 20; 21q; **22q**; X	1p31; 1q31-q32; 2q22-q33; 3p12-p13; 3q24-q26; 6q21; 12q15-q21, 13q12-q32
8	E; II	9q34: 2.4; 22q11: 2.3	12q24; 17	-
9	E; II	9q34: 2.0; 22q11: 2.1	1q41-qter; 12q24; 14q32; 17; 20q; Xq28	-
10	E; II	ND	1q; 6p21.3-p25; 12q23-qter; 20; 22q	3; 4; 9p21-p23; 13q12-q32; X
11	E; II	6 cen: 1.2; 9q34: 1.7; 22q11: 2.1; X cen: 1.3	-	**6**; **X**
12	E; II	ND	4p; 7; 9q; 12; 15q; X	4q28-qter
13	E; II	3 cen: 2.0; 9q34: 2.0; 10 cen: 1.7; 22q11: 2.4	**amp(2p23-p24)**; 12q24; 20; **22q**	13q14-q31
13R	aE; III	9q34: 2.0; 22q11: 2.1	amp(2p23-p24)	10
13M	aE; III	6 cen: 1.8; 9 cen: 2.0; 9q34: 1.9; 10 cen: 0.9; 22q11: 2.0	amp(2p23-p24)	10
14	aE; III	9q34: 2.2; 22q11: 3.0	-	-
15	aE; III	9q34: 1.9; 22q11: 2.0	1q	-
15R	aE; III	ND	1q; 9q; 15q	13q21-q22
16	aE; III	6 cen: 1.9; 9q34: 2.0; 22q11: 2.1	**amp(2p23-p24)**	10
17	aE; III	ND	5p15.1-p15.2; 5q31-qter	2q24-q34
18	aE; III	9q34: 2.1; 22q11: 2.2	17p11.2-p12	-
19	aE; III	9q34: 2.0; 22q11: 2.0	10q26	-
19R	aE; III	9q34: 2.3; 22q11: 3.8	17; **22q**	-
20	aE; III	9q34: 2.3; 22q11: 2.3	1q32-q42	7q31-qter
21	aE; III	3 cen: 1.2; 6 cen: 2.1; 9q34: 1.9; 10 cen: 2.1; 22q11: 2.1; X cen: 2.1	11q23-qter	**3**; 9p-q33
22	aE; III	9q34: 1.9; 10cen: 2.1; 22q11: 2.0; Xcen: 1.1	-	-

genomic DNA was labeled with digoxigenin-11-dUTP (Boehringer Mannheim).

CGH was performed as described elsewhere (2). Biotinylated DNA sequences were visualized by fluorescence using fluorescein isothiocyanate (FITC) conjugated avidin (Vector, Burlingame, CA) and digoxigenin-labeled reference DNA was visualized by anti-digoxigenin-rhodamine (Boehringer Mannheim). Chromosome preparations were counterstained with 4′-6′-diamindino-2-phenylinodole dihydrochloride hydrate (DAPI) (Sigma, St. Louis, MO). For each case, at least 15 metaphases were evaluated. Chromosome regions known to give false-positive signals in CGH analyses, such as chromosome region 1p32-pter, chromosomes 16 and 19 were excluded from the study for reasons specified elsewhere (21).

Interphase Cytogenetics. Interphase FISH was performed for fresh-frozen tissue on 4 to 6µm thick sections. Nuclei from paraffin-embedded tissue were separated according to the protocol of Liehr *et al* (27). Paraffin-sections were 40µm thick. The slides were stored at -20°C.

FISH analysis of interphase nuclei extracted from paraffin-embedded tissue was performed as described elsewhere (27). We modified digestion times (pepsin 10 to 20 min; proteinase K 40 to 60 min) using the same enzyme concentrations. Digoxigenin-labeled probes were visualized by a Cy3-conjugated monoclonal mouse anti-digoxi-

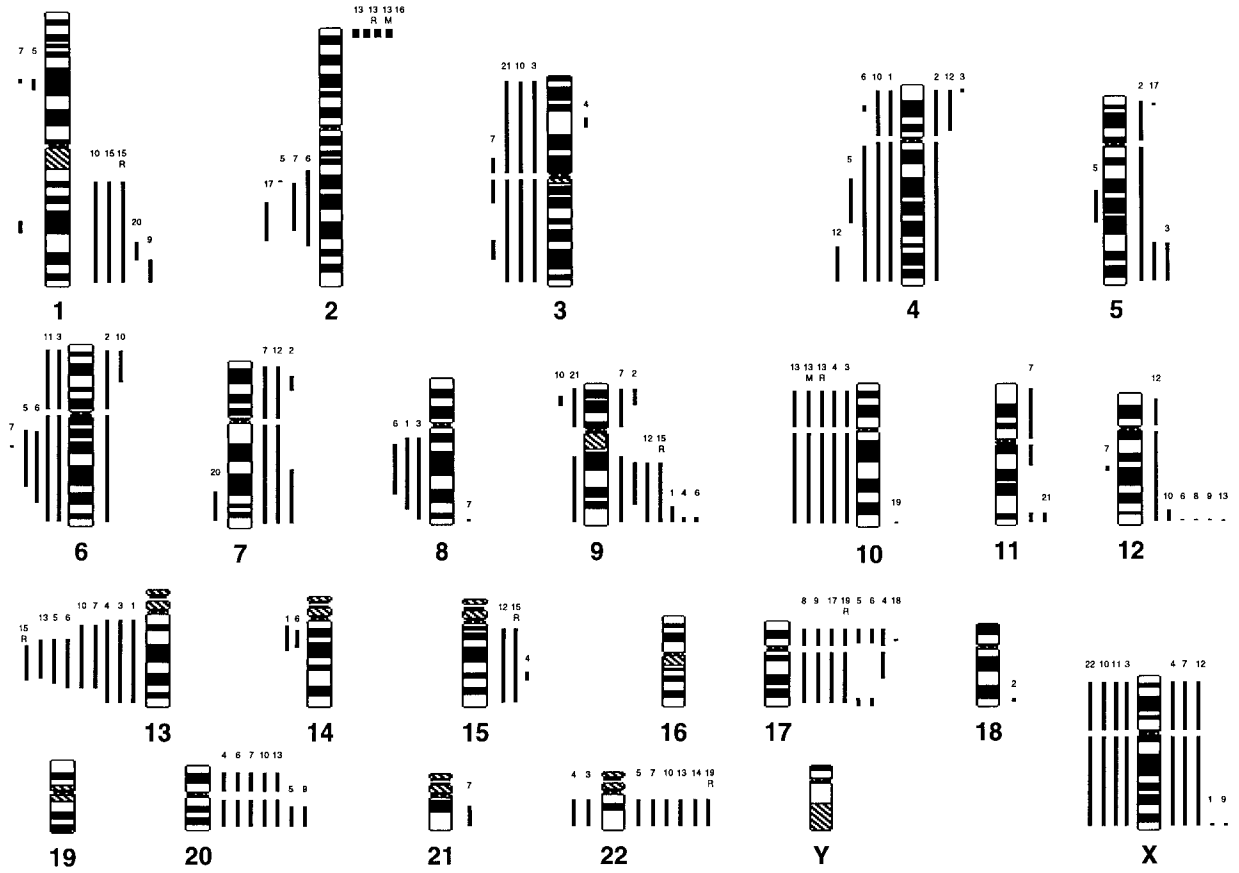

Figure 1. *Summary of genome wide screening of gains and losses detected by CGH in 26 cases (22 patients) of ependymomas.* Vertical lines on the left side of the chromosome indicate losses, whereas vertical lines on the right correspond to gains of chromosomal material. The chromosomal region 1p34-p36, chromosomes 16 and 19 were excluded from evaluation for reasons specified elsewhere (23). All lines at 2p23-p24 represent DNA amplifications of MYCN. The numbers above each line refer to the case analyzed.

genin antibody (IgG; Jackson Immuno-Research Laboratories Inc., West Grove, PA) and then by a Cy3-conjugated affinity purified goat anti-mouse IgG antibody (Jackson). Biotin-labeled probes were visualized by FITC-conjugated avidin (Vector), then biotinylated anti-avidin D "affinity purified" (Vector) and finally amplified by FITC-conjugated avidin. A minimum of 100 nuclei per case and probe were evaluated.

FISH was performed on 22/26 samples (Tables 2, 3). The following commercially available digoxigenin- or biotin-labeled probes were used with assignment to human chromosomes: 3 cen, 6 cen, 9 cen-q12, 10 cen, X cen, 2p23-24 (MYCN locus); the combined probe m-bcr/abl with assignment to 9q34 (ABL1 locus) and 22q11 (BCR locus) (all probes by Oncor Appligene, Illkirch Cedex, France). FISH experiments were performed as dual-color hybridization. Hybridization experiments were evaluated only when the second FISH probe exhibited two or three signals in more than 75% of single interphase nuclei. Cut-off levels were analyzed using five normal tonsillar glands, prepared in the same way as tumor samples.

Fluorescence Microscopy and Digital Image Analysis. A Zeiss Axioskop microscope equipped with a 100 W mercury lamp was used for epifluorescence microscopy. The microscope was attached to the commercially available image analysis system ISIS (Meta-Systems, Altlussheim, Germany).

Ratio values of 1.25 and 0.8 were used as upper and lower thresholds for the identification of chromosomal imbalances (2).

Statistics. The median, mean, standard error of the mean (SEM) and the range for the number of chromo-

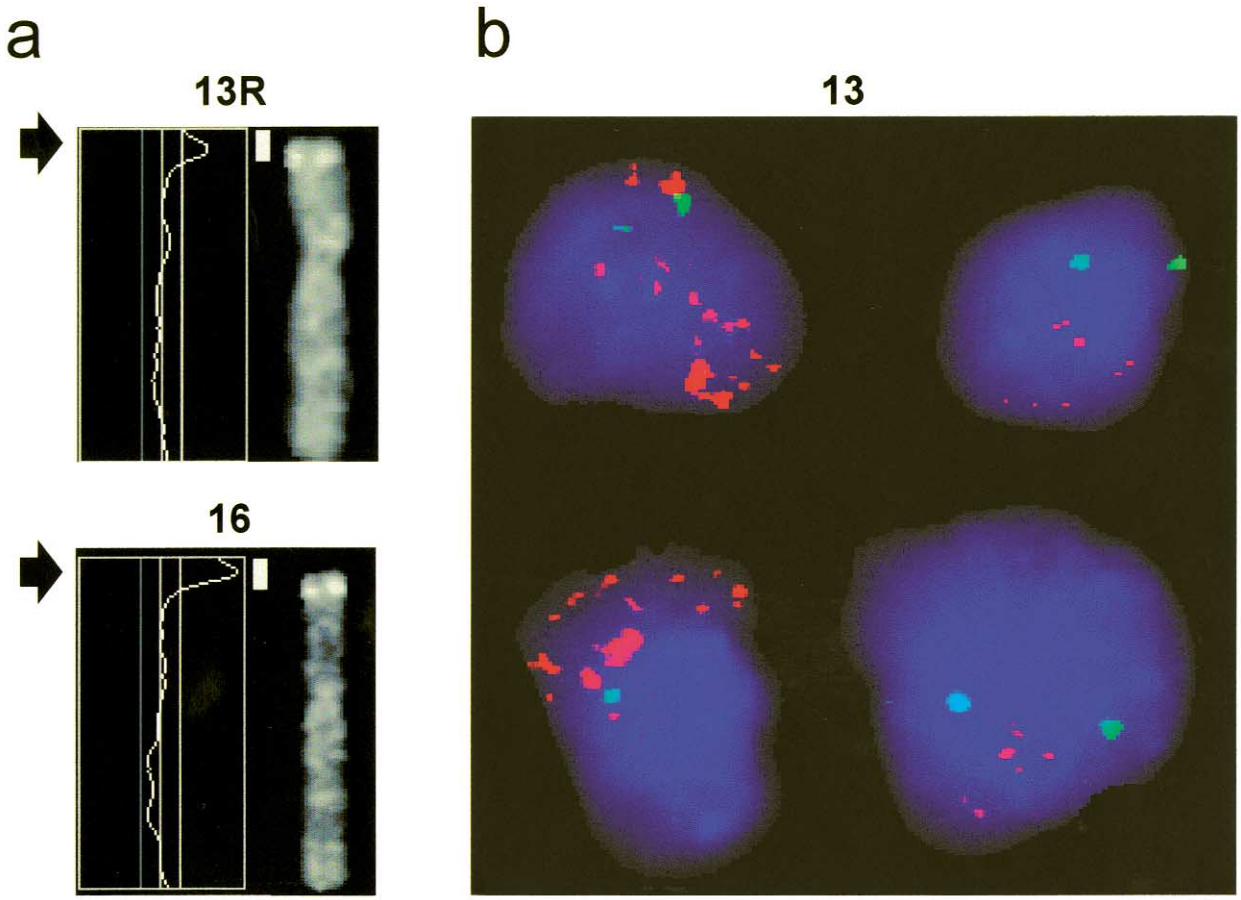

Figure 2. *Over-representations mapping to 2p23-p24.* **a**, on the left: partial CGH profile of chromosome 2 of case no. 13R (see above) a recurrent aE, and case no. 16 (see below) showed a DNA amplification on 2p23-p24. The band-like hybridization signals (arrow) indicate highly amplified chromosomal sequences. On the right side of the ideogram, the average ratios of FITC/rhodamine fluorescence of 17 chromosomes are plotted ("ratio profiles"). The central line indicates a ratio of 1.0; line to the right indicate ratio of 1.25; and line to the left indicate a ratio of 0.8. **b**, on the right: Image of a dual-color hybridization of case no. 13 obtained with MYCN proto-oncogene (2p23-p24) detected via Cy3 (red) and the centromer-specific probe for chromosome 10 (10 cen; green) visualized by FITC. In four nuclei a tight cluster of red signals is detected indicating the presence of an amplification of the MYCN proto-oncogene. The second probe (10 cen; green) resulted in 75% of nuclei in two distinct hybridization signals.

somes with imbalanced regions per tumor detected by CGH for each group (grade I to III) were calculated. For groups grade II and grade III, a two-dimensional Wilcoxon test was performed.

Results

In pilot studies we hybridized the extracted tumor DNA from fresh tumor tissue in five cases (nos. 2, 3, 13M, 16 and 19). We compared these data with hybridization experiments of DNA extracted from formalin-fixed tissue in the same tumor. The ideograms of these hybridization experiments were identical. CGH experiments were carried out, together with control experiments, in which differently labeled normal DNAs of non-tumorous tissue from case nos. 2, 17 and 18 as well as normal DNA (human tonsil) were hybridized to normal metaphase chromosomes. Twenty-two samples in 69 localizations were selectively analyzed using FISH. Compared to CGH data, the FISH experiments demonstrated 3 further diagnostic alterations (losses of 22q in case nos. 3 and 4 and a gain of 22q in case 14). In the remaining analyses, FISH experiments confirmed CGH data. The cut-off level (median plus 3 $\times$ SEM) for isolated nuclei from paraffin-embedded normal tissue (human tonsil) concerning loss of 22q11 (BCR locus) was estimated as 5.1% (4.7% for frozen sections of human tonsil; in total, cut-off ranged from 4.7% to 13.7% for different FISH probes; highest level for 3cen and 6cen probe). Cut-off level for gains ranged from

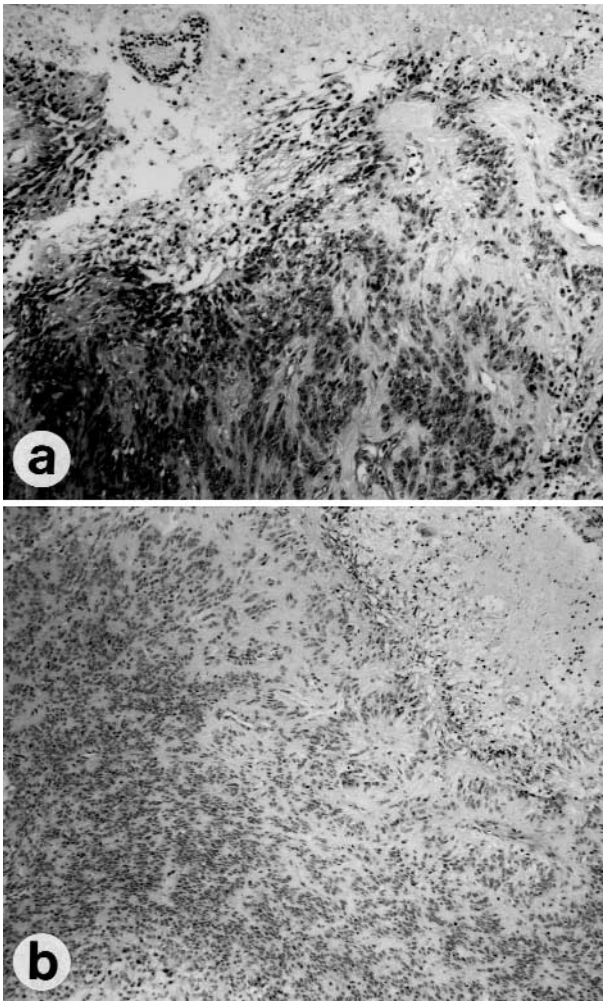

Figure 3. *Histologic appearance of the ependymomas with MYCN gene amplification.*
a, case no. 13M; supratentorial metastasis of a spinal aE. The tumor demonstrated large areas of necrosis (upper margin), perivascular pseudorosettes, moderate cellularity, oval nuclei with moderately dense chromatin and prominent nucleoli (H&E × 94). Ki-67 proliferation rate was 15% (Table 1); **b**, case no. 16; spinal aE. Large areas of necrosis (see upper right corner), areas of increased cellularity, perivascular pseudorosettes and pale fibrillarity of the perithelial zones (H&E × 73). Ki-67 proliferation index was 19% (Table 1).

10.7% to 24.6% (highest level for MYCN probe). The frequency and distribution of chromosomal imbalances of ependymomas are presented in Table 2 and Figure 1.

Chromosomal Imbalances of Myxopapillary Ependymomas (myE), Grade I. The clinicopathologic data of the 7 patients are summarized in Table 1. In total, 5 to 16 (median 9) chromosomes with imbalanced regions per tumor were detected by CGH (Table 1). The most frequently detected genomic losses were found on chromosome 13q14-31 (6/7). Gains on chromosome region 9q were found in 5/7 samples. The gains of 9q34 were confirmed in all cases (ABL1 probe; Table 2). In 4/7 cases we detected mainly losses of chromosomal material on chromosomes 6q. In 4/7 cases we found gains of chromosome 17. FISH analysis of cases nos. 1, 3, 4 and 6 suggesting aneuploid karyotype in these samples (Table 2).

Chromosomal Imbalances of Ependymomas (E), Grade II. In E we found 2 to 10 chromosomes with imbalances per tumor (median 5.5). 5/6 cases demonstrated a gain on the terminal region on the long arm of chromosome 12. One E (no. 13) showed an amplification of 2p23-p24 (Figure 2b). This tumor was a local recurrence of an E within a year after radiotherapy and transformed into an anaplastic ependymoma the following year, with subarachnoidal dissemination (see results grade III). Gains on chromosome 22q were detected by CGH and confirmed by FISH in 2 cases nos.10 and 13 (Table 2). Only two cases (nos. 8 and 11) demonstrated less than 5 chromosomes with imbalances per tumor in this group. Using FISH analysis a state of near-diploid of these samples was found (Table 2).

Chromosomal Imbalances of Anaplastic Ependymomas (aE), Grade III. Using CGH, in 13 aE of 10 patients, we found up to 4 chromosomes with imbalances per tumor (median 2; range 0 to 4; Table 1). One case (no. 22) did not demonstrate any aberrations. In 3 spinal aE of two patients (nos. 13R/M; no. 16) we found a loss of chromosome 10 together with a DNA amplification mapping to chromosomal region 2p23-p24. Using FISH analysis we identified MYCN as part of this amplicon (Figure 2a). In case no. 13M the loss of chromosome 10 was confirmed by FISH analysis (one signal of centromeric region of chromosome 10 in 72% of nuclei), whereas 9 cen, 9p34 and 22q11 showed two signals (86 to 96%). 3 samples of pediatric aE in two different patients (nos. 15/15R and 20) showed an overrepresentation of chromosome 1q. The gain of 1q in sample no. 15 was detected in both the primary aE and its recurrence within the same year (no. 15R).

Identification of DNA Amplification Mapped by CGH. Using CGH, four specimens from two patients revealed band-like hybridization signals which are the hallmarks of DNA amplifications. The first patient (no. 13) had a recurrence of an E transformed into an aE (no. 13R) with subarachnoidal dissemination (no. 13M; Fig-

ure 3a). CGH profiles of chromosome 2 were identical for cases no. 13, 13R and 13M. The second patient (no. 16; Figure 3b) had a primary aE. For all four samples these band-like hybridization signals mapped to chromosomal bands 2p23-p24. FISH analysis was performed (nos. 13R and 16) using a probe assigned to MYCN locus. This probe detected a tight cluster of at least 20 signals in 84% of nuclei (more than 10 in 93%) for case no. 13R. At least 20 signals in 42% of nuclei (more than 10 in 81%) were found for case no. 16. This indicated the presence of an MYCN amplification of this gene. The centromeric specific probes (6 cen in patient no. 16; 10 cen in patient no. 13R) resulted in two distinct hybridization signals (87% for no. 16; 75%; for no. 13R; Figure 2).

FISH Analysis of 22q11. CGH data demonstrated gains of 22q in 5/26 cases (nos. 5, 7, 10, 13, 19R) of our cohort. FISH was performed with the 22q11 (BCR locus) probe. Experiments confirmed these CGH results and we found 3 further diagnostic imbalances. In a near-diploid aE (case no. 14) we found a gain of 22q11. Losses of 22q11 were found in 2 otherwise aneuploid myE (case nos. 3 and 4; Tables 2, 3). In total, 8 cases had minor subpopulations of tumor nuclei (7 to 25%), without or with only one 22q11 signal. The data are listed in Table 3.

Comparison of CGH and FISH Data with the Grades of Ependymal Tumors. Overall, 120 chromosomal imbalances (gains/losses ratio, 1.5:1) were detected in 26 tumors by CGH. In 22 cases FISH analysis was performed (Table 2). The number of genomic imbalances detected per tumor was higher in the groups of myE (median 9; mean 9.14, SEM 4.4) and E (median 5.5; mean 5.33, SEM 3.0) than for the aE (median 2; mean 1.77, SEM 1.01). These results relating to the biologically comparable tumors, ependymomas grade II and III, were significantly different ($p<0.01$) calculated by the two-dimensional Wilcoxon test.

Interestingly, in aE we found little variability in the numbers of imbalances per tumor detected by CGH (0 to 4, median 2). One aE (no. 15R) had 4 chromosomal imbalances. This tumor was a local recurrence of an aE which showed, as the sole genomic alteration, a gain on chromosome 1q (no. 15). On the other hand, we found only two E with 2 imbalanced chromosomes detected by CGH. The first exception was E (no. 11) with 2 chromosomal imbalances. In this case the patient had chemotherapy before surgery. The second exception was case no. 8. This grade II tumor had the highest proliferation index (13%) in that group.

Case	22q11: Mean of 100 cells	22q11: Numbers of Signals Per Cell (%)											
		0	1	2	3	4	5	6	7	8	9	10	12
1	3.04	0	4	34	28	23	10	1	0	0	0	0	0
2	1.97	0	4	95	1	0	0	0	0	0	0	0	0
3	2.05	0	13	72	12	3	0	0	0	0	0	0	0
4	2.24	2	2	78	10	5	2	1	0	0	0	0	0
5	3.26	4	3	32	18	23	13	4	0	3	0	0	0
6	2.94	1	3	14	69	10	2	0	0	0	0	0	0
7	2.94	17	8	19	19	17	8	7	1	3	0	1	0
8	2.29	0	3	77	10	8	0	0	0	0	0	0	0
9	2.05	0	3	94	0	1	2	0	0	0	0	0	0
11	2.12	3	3	77	13	4	0	0	0	0	0	0	0
13	2.40	0	5	52	41	2	0	0	0	0	0	0	0
13R	2.11	1	2	83	13	1	0	0	0	0	0	0	0
13M	1.98	1	6	87	6	0	0	0	0	0	0	0	0
14	2.95	6	9	34	19	18	4	5	2	2	1	0	0
15	2.02	1	0	95	4	0	0	0	0	0	0	0	0
16	2.07	1	3	88	4	4	0	0	0	0	0	0	0
18	2.20	2	6	71	12	9	0	0	0	0	0	0	0
19	2.02	0	0	99	0	1	0	0	0	0	0	0	0
19R	3.80	4	6	30	12	22	6	10	1	2	1	2	4
20	2.26	0	2	77	14	7	0	0	0	0	0	0	0
21	2.13	1	10	70	15	2	2	0	0	0	0	0	0
22	1.99	1	4	92	1	2	0	0	0	0	0	0	0

Table 3. *Results of FISH analysis of chromosomal region 22q11 (BCR locus).* Cut-off level for losses or monosomy of 22q were evaluated as 5%. FISH experiments were performed with commercially available dual m-bcr/abl FISH probe. Most samples were near-diploid. In cases 1, 3, 4 and 6 a copy number of three was considered normal, instead of the usual copy number of two using FISH and CGH analysis (Table 2).

Discussion

The histologic grading of myE (grade I) is based on well-defined histologic criteria. This tumor has a good outcome. In contrast, in ependymomas of grades II and III, the histologic grading continues to be a difficult issue and little consensus has been reached on the prognostic value of specific histologic features (7, 22). Interphase cytogenetics might be helpful tools in this issue. We performed a genome wide screening for genomic alterations on a series of 26 ependymomas grades I to III in 22 different patients using CGH and, in part, supplemented by FISH analysis.

In our study, the most frequently detected gains of chromosomal material were found on chromosomes 9 and 17. In 7/26 (27%) cases we showed gains on chromosome 9 (especially 9q). This is consistent with previously published CGH analysis of pediatric ependymomas which demonstrated gains on chromosome 9 (33,

34). Several conventional cytogenetic studies (3, 4, 8, 10, 11, 16, 19, 24, 30, 34, 37-40, 43, 45, 47, 53, 54) described gains of chromosome or translocations involving chromosome 9 in approximately 15% of ependymomas. In our study, the gains on 9q were almost exclusively detected in the two groups of low-grade tumors. The terminal region of chromosome 9 includes the cellular oncogene ABL1 (9q34.1) involved in the translocation t(9;22) typically found in Philadelphia-positive chronic myeloid leukemia. Investigations focusing on ABL1 transcript expression in other gliomas failed to detect increased expression of this gene in about 20 tumors (31, 32), whereas a glioblastoma cell line expressed high level DNA and RNA of ABL1 (6). Using FISH analysis we did not detect any ABL1 high-level amplification or split signal suggesting t(9;22)(q34;q11).

Gains on 1q were found selectively in childhood ependymomas grade II and III. These data support the findings of Reardon *et al* (35), thus demonstrating that genomic imbalances in pediatric ependymomas most often involve gains of either 1q or 9. In our study, only one recurrence demonstrated both, the gain of 1q and 9q (case no. 15R).

The most frequent gain involved chromosome arm 12q in 5/6 E. The consensus region was 12q24. Gains on chromosome 12q were found in other brain tumors as well as in primary central nervous system lymphomas (51, 52). In meningiomas the gain on 12q and others is proposed to be a genomic alteration in meningioma progression from benign to atypical tumors (51). In ependymomas, a recently published PCR study by Suzuki *et al* (44) detected MDM2 gene (12q13-q14) amplification in 35% of ependymomas. These data are not yet settled. A PCR study by Tong *et al* found one tumor with an MDM2 gene amplification in their series of 26 ependymomas (46). Southern blot analysis of 8 ependymomas published by Reifenberger *et al* (36) did not reveal an MDM2 gene amplification in these tumors. In our series, we did not demonstrate high level gains at 12q involving the MDM2 gene locus. Of course, small regions of amplification may be beyond the resolution of CGH. CGH can detect and map amplified DNA sequences, if the product of amplicon size and copy number exceeds 2 Mbp (33).

Chromosome 17 was involved in gains in 8/26 cases (grades I to III). Most of them were found in myE, which do not undergo malignant transformation. In the literature, additional chromosomes 17 are documented in approximately 3.5% (3/86; two grade I and one recurrence of an ependymoma) (1, 37, 40). The relevance of the gain of 17 is not clear. In neuroblastoma the gain of 17q appears to be associated with a more aggressive subset of neuroblastoma (22) and progression of meningiomas (51).

Most frequently, regions repetitively deleted included 13q (9/26), especially in myE and E. In most cases, under-representation of 13q involved the chromosomal region 13q14-q31. One of the genes localized in this region is the tumor suppressor gene RB1 (13q14.2), known to play a critical role in the development of retinoblastomas and other tumors (12, 55). Immunohistochemically, we screened RB1 protein expression in our cohort. We did not find any correlation between RB1 expression and loss of 13q (data not shown). With conventional cytogenetics, loss of chromosome 13 was described in approximately 5% of ependymomas (4/86; 3 grade II and one recurrence) (16, 34, 40, 43).

Another under-representation found in 5/26 (19%) cases (two grades I; three grade III) was the loss of chromosome 10. Losses of chromosome 10 were reported in about 9% of ependymomas (16, 34, 40, 43, 45, 47) as well as in other gliomas, e.g., in high-grade gliomas where the incidence ranges from 25 to 90% (15, 23, 26).

A potentially relevant oncogene for aggressive growth in ependymomas is the proto-oncogene MYCN located on 2p23-p24. The region of MYCN was amplified in four samples from two different patients (nos. 13 and 16; Figure 3). Case no. 13 had three local recurrences of a spinal E with malignant transformation into aE and a supratentorial spread. The last recurrence and the metastasis had the same CGH profile, suggesting the clonality of these two tumors. Patient no. 16 suffered from different schwannomas, suggesting NF2-associated aE. In the past, only a few ependymal tumors have been examined for MYCN amplification. One study detected low accumulation of MYCN transcript in an E, whereas an elevated MYCN gene copy number was not detected (14). In seven other ependymomas (grades I to III) no amplification of MYCN was described (18, 49). Using immunohistochemistry in one cell line of an ependymoma, immunoreactivity for MYCN protein was found, whereas cell lines of malignant ependymoma and an ependymoblastoma did not express this protein (28). In neuroblastomas, indirect correlation between MYCN amplification and prognosis is well known (22, 42).

Several authors suggested that a tumor suppressor gene may be present in region 22q (See review 17). In conventional cytogenetics, monosomy 22 or structural abnormalities involving chromosome 22 are found in approximately 30% of ependymomas (29). One LOH study revealed that loss of chromosome 22q occurred

preferentially in intramedullary spinal ependymomas (13). A combined FISH and microsatellite analysis of chromosome 22 demonstrated a loss of chromosome 22 in 2/22 pediatric ependymomas (25). The incidence of monosomy 22 or breakpoint 22q11-q13 was 56% in adult tumors versus 28% in pediatric tumors (29). We performed FISH analysis using a probe assigned to 22q11. In 36% of tumors (5 pediatric/3 adults) we revealed loss of 22q11 only in small neoplastic subpopulations (7 to 25%). Altogether, we found more compelling evidence for gains of 22q (Table 3) in our cohort, rather than losses of 22q as previously characterized in several other studies.

In myE known not to undergo malignant transformation, numerous chromosomal imbalances (median 9 per tumor) were found. It is still unknown which of these imbalances are epiphenomena, e.g. part of tumor regression, rather than primary events.

Summarizing our data, we demonstrated little or no chromosomal imbalances in aE (median 2), whereas most myE and E exhibit numerous gains and/or losses by CGH (median 9 and 5.5 respectively). In 4/26 specimens we found MYCN amplification of the tumor. The prognostic value of MYCN amplification in grade II and III, especially in spinal tumors, is yet to be determined.

In conclusion, the results of the present study suggest that, unlike other brain tumors (5, 41, 50, 51), in ependymal tumors a complex genomic imbalances revealed by CGH is not an indicator of a high WHO grade of the tumor.

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