Comparative genomic hybridization indicating two distinct subgroups of pilocytic astrocytomas

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Object. The authors investigated the spectrum of chromosomal imbalances of pilocytic astrocytoma by using comparative genomic hybridization (CGH).

Methods. Tumor DNA was extracted from surgically obtained samples of 18 pilocytic astrocytomas that were examined for the presence of neoplastic tissue on frozen sections. Comparative genomic hybridization was performed using standard procedures, and digital image analysis was conducted using out by custom-made software. The chromosomal alterations were determined by a statistical procedure in which Student's t-test (99% confidence interval) was used. Details on CGH analysis and individual ratio profiles are available at <http://amba.charite.de/cgh/>.

Conclusions. The results suggests the presence of two distinct genetic subgroups of pilocytic astrocytoma, with imbalances of chromosome 19 being the major change for differentiation. In the first group (10 samples), deletions on chromosome 19 were shown as well as multiple gains mainly on chromosomes 5 and 6q but also on chromosomes 4, 7, 8, 10, and 11. The second group (eight samples) was characterized by overrepresentation on chromosomes 19p and 22q, which were associated with deletions on 4q, 5q, 6q, 9p, 13q, and 18q. To understand the diverse biological and clinical behavior exhibited by this tumor type, it is important that pilocytic astrocytomas be classified into distinct subgroups according to their genetic makeup.

KEY WORDS • pilocytic astrocytoma • DNA • comparative genomic hybridization • chromosome 19

Pilocytic astrocytomas occur most frequently in children and young adults. Although most pilocytic astrocytomas affect the cerebellum, they also show a predilection for the optic nerves and chiasm, as well as for the hypothalamus. Less often affected are the cerebral hemispheres or the spinal cord. The occurrence of supratentorial pilocytic astrocytomas remains uncommon in children and rare in adults.^{1,5,12,15} Although, macroscopically, pilocytic astrocytomas are well circumscribed, in some tumors parenchymal infiltration and invasion of leptomeninges^{8,18,25,32,34,35,39,42} or even the nuchal musculature has been shown.²² A very small proportion of pilocytic astrocytomas (<1% of cases) do show histological features of anaplastic progression. After the patient undergoes radiotherapy, this incidence has been observed to rise to 1.8%; however, the percentage still remains relatively low.⁵² This correlates with the observation that 70 to 90% of patients experience long-term survival after surgery.¹⁰ Although

many tumors are indolent and potentially curable, there is a distinct probability that the tumor may recur even after a very long latency period of up to five decades.^{24,61} Based on histological features, some authors have differentiated between two distinct group of patients with “excellent” and “moderately good” prognoses.^{14,16,17,27,50,60}

There are no consistent genetic aberrations known to characterize pilocytic astrocytomas.^{9,40} Although cytogenetic analysis of this most benign variant of astrocytoma has revealed the presence of normal karyotypes in the majority of cases it is notable that Thiel, et al.,⁴⁹ have observed that in two of eight tumors they studied there was loss of the sex chromosome and tandem associations between different chromosomes. In one case there was a 46, XY, inv(9) karyotype. Moreover, White, et al.,⁵⁹ and Zattara-Cannoni, et al.,⁶³ have observed that one third of pilocytic astrocytomas were characterized by chromosomal gains, most commonly chromosomes 7, 8, and 11, suggesting that trisomy 7 and 8 are relatively common events in pilocytic astrocytomas.

In the present study, we investigated the spectrum of chromosomal imbalances found in pilocytic astrocytomas

Abbreviations used in this paper: CI = confidence interval; CGH = comparative genomic hybridization; duTP = deoxyuridine triphosphate; GFAP = glial fibrillary acidic protein.

by performing CGH, which suggested the presence of two distinct genetic subgroups.

MATERIAL AND METHODS

Clinical and Pathological Data

The 18 pilocytic astrocytomas included in the present study were surgical specimens obtained in the Neurosurgical Department, University of Medical Sciences of Poznan. At the time of surgery six patients were in the pediatric-age group, and the other 12 patients, except for one, were young adults. The mean age of the patients at the time of surgery was 23 years (range 6–48 years). The sex ratio was almost equal: 10 female and eight male patients. Ten tumors were of cerebellar and six were of supratentorial location, whereas two were optic gliomas. Except in one case, all samples were obtained from primary tumors. The specimen obtained in Case 15 (lab no. 15168) originated from a tumor that recurred after the patient underwent radiotherapy following the first surgery. The clinicopathological data are summarized in Table 1. All tumors were classified according to the World Health Organization grading system.²³

Histological Examination

Surgically removed tumor tissue samples were immediately fixed in 4% formalin in 0.1 M phosphate buffer, pH 7.4, dehydrated in absolute ethanol, and embedded in paraffin. For light microscopy, 4-µm sections were stained with hematoxylin and eosin, Masson’s trichrome, and the periodic acid Schiff method.

Immunohistochemical studies were conducted using paraffin-embedded sections and a peroxidase–antiperoxidase method in which we used commercially available

monoclonal and polyclonal antibodies. We investigated the following antigens: GFAP, HNK-1, A2B5, S-100, and vimentin. Tissue sections were pretreated in a wet autoclave for antigen retrieval.

Comparative Genomic Hybridization Analysis

Tumor DNA was extracted from samples obtained during surgery. The samples were verified for the presence of a high amount of tumor tissue by the intraoperative frozen section diagnosis and, if necessary, were trimmed to reduce normal contamination prior to freezing and storing in liquid nitrogen. Comparative genomic hybridization preparation and digital image analysis were performed as previously described.^{36,43} Detailed information on the CGH procedure is available at our web site: <http://amba.charite.de/cgh/>. Briefly, 5 µg each of tumor DNA and normal reference DNA obtained from the peripheral blood lymphocytes of a healthy female donor were labeled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively, by standard nick translation. Then 1 µg of tumor and normal DNA together with human *Cot1* DNA were hybridized to slides with normal chromosome metaphase spreads, which were either prepared from peripheral blood lymphocytes or commercially purchased. After 3 days of hybridization at 37°C, the tumor and normal DNA samples were visualized using fluorescein and rhodamine, respectively.

The fluorescence images were captured as 12-bit gray level images by using a fluorescence microscope and a cooled charged-coupled device camera connected to a Macintosh Quadra 950. Image processing was conducted using custom-made CGH software that is based on other karyotyping software written in a Windows 95 version of the image software AMBA. The software was implemented on a 400-MHz Pentium II PC running Microsoft Windows 95. The mean ratio profiles were determined from the analysis of at least 10 metaphases. Analysis was accomplished by a procedure that automatically detects chromosomal aberrations based on the average ratio profiles with the use of Student’s distribution function, 1.0: 1.0 thresholds and the 99% and/or 95% CI.^{37,38}

Sources of Supplies and Equipment

The monoclonal antibodies were obtained from DAKO (Glostrup, Denmark) and the polyclonal antibodies from Becton-Dickinson (Mountain View, CA). The biotin-16-dUTP was purchased from Böhrringer Mannheim (Mannheim, Germany), as was the digoxigenin-11-dUTP. The peripheral blood lymphocytes were bought from Vysis (Downers Grove, IL). The fluorescence microscope (Axiphot) was aquired from Carl Zeiss (Thornwood, NY) and the charged-coupled software device camera from Photometrics (Tucson, AZ). Our karyotyping software is based on that (KARYOTYP) produced by IBSB GmbH (Berlin, Germany).

RESULTS

Light Microscopy

The characteristic biphasic pattern was observed in almost every tumor. In the more solid portions of the tumor, piloid cells were arranged in bundles, whereas stellate

TABLE 1			
Clinicopathological data obtained in patients with pilocytic astrocytoma classified into two subgroups			
Case No.	Age (yrs), Sex	Diagnosis	Localization
group w/ pronounced gains			
1	14, F	pilocytic astrocytoma	suprasellar
2	22, M	pilocytic astrocytoma	cerebellum
3	32, F	pilocytic astrocytoma	frontocentral
4	16, F	pilocytic astrocytoma	cerebellum
5	38, M	pilocytic astrocytoma	thalamus & 3rd ventricle
6	27, M	pilocytic astrocytoma	central
7	16, F	pilocytic astrocytoma	cerebellum
8	21, M	pilocytic astrocytoma	cerebellum
9	19, F	pilocytic astrocytoma	cerebellum
10	19, F	pilocytic astrocytoma	cerebellum
group w/ multiple losses			
11	29, F	pilocytic astrocytoma (optic glioma)	opticus
12	48, M	pilocytic astrocytoma	central
13	37, M	pilocytic astrocytoma	frontocentral
14	6, M	pilocytic astrocytoma	cerebellum
15	15, F	pilocytic astrocytoma	cerebellum
16	11, M	pilocytic astrocytoma	cerebellum
17	23, F	pilocytic astrocytoma (optic glioma)	opticus
18	21, F	pilocytic astrocytoma	cerebellum

CGH patterns in pilocytic astrocytomas

Fig. 1. A: Sum karyograms of the pilocytic astrocytoma (Case 2) seen in Fig 1C. Deletions are depicted in *red*, amplifications in *green*, and equilibrium between the tumor and normal DNA in *blue*. Changes include losses on chromosomes 9q13-q21, 13q12, 14q11.2-q12, and 19p13.1-p13.2 and gains on chromosomes 1q24-q32, 2q22-q24, 3p14, 4q26-q27, 4q31.1-q31.2, 5, 7p11.2-p21, 7q, 8q12-q21.3, 9p21, 10p14-pter, 10q21-q22, 10q24, 11q22-q23, 13q21-q22, 15q15-q21, and 15q25. The *red curves* depict the average ratio profiles. For each chromosome, the *middle green line* corresponds to the normal state (fluorescence ratio 1.0). The *blue line* on the left represents the theoretical values of a monosomy in 50% of the tumor cells of an otherwise diploid tumor (fluorescence ratio 0.75). The *blue line* on the right represents a trisomy in 50% of the tumor cells of an otherwise diploid tumor (fluorescence ratio 1.25). B: Sum karyograms of the pilocytic astrocytoma (Case 14) seen in Fig. 1D. Deletions are depicted in *red*, amplifications in *green* and equilibrium between the tumor and normal DNA in *blue*. Changes include losses on chromosomes 4q24-qter, 5p15.2-q34, 6q22-qter, 10, 13q12-q33, 14q, 15q15-q22, 16q13-q24, and 18 and amplifications on chromosomes 1p31-pter, 1p13-q25, 2p16-p21, 2p11.2-q21, 3p13-p21, 3p12-q23, 4p16, 6p21.3-q12, 7q11.2, 8p22-pter, 8p12-q11.2, 9p12-q34, 11q12-q14, 15q24-q25, 16p, 17, 19p13.3-q13.3, 20p11.2-q13.3, and 22q11.2-q13. C: Photomicrograph showing the somewhat loose pattern of solid area of pilocytic astrocytoma consisting of elongated, fibrillated cells (Case 2). H & E, original magnification $\times 100$. D: Photomicrograph showing the compact, paucicellular region of pilocytic astrocytoma consisting of elongated and highly fibrillated cells (Case 14). H & E, original magnification $\times 100$.

cells formed loose-textured tissue, often with microcystic changes. These textures was associated with Rosenthal fibers and granular bodies, respectively.

Immunohistochemical Reaction

Neoplastic astrocytes with long hairlike extensions were stained strongly with GFAP, HNK-1, S-100, and vimentin and did not stain for A2B5. The reaction of cells in the looser, microcystic or spongy areas were weaker with antibodies to GFAP.

Frozen Section Analysis

For all genetic molecular studies, neoplastic material was confirmed by light microscopic analysis of the frozen sections. If present, tissue from adjacent brain was removed in all cases. Thus, dilution effects by normal tissue contamination can be largely excluded in this study.

Comparative Genomic Hybridization Analysis

The CGH sum karyogram and the ratio profile with its 99% CI were mainly used to define the chromosomal imbalances found in each tumor (Fig. 1).

The spectrum of imbalances is exemplified by the two CGH results depicted in Fig. 1 in one tumor (Case 2). Whereas in one tumor (Fig. 1 A) there were only a few imbalances, in the second one (Case 14; Fig. 1 B) the multiple and pronounced changes resembled a highly malignant astrocytoma. Histological and immunohistochemical analyses in both cases, however, revealed typical features of pilocytic astrocytoma as shown in Fig. 1 C and D. These two cases represent the spectrum of changes found in pilocytic astrocytoma. The pattern of the first tumor subtype was observed in the majority of cases. Individual profiles as well as sum profiles, line representations, and histograms obtained in the tumor group can be viewed at our CGH online tumor database (<http://amba.charite.de/cgh/>).

When we used histograms to describe the overall genetic changes, we were able to distinguish two subgroups of pilocytic astrocytoma. The first, which we called the "group with multiple gains," represented 10 of the 18 cases. The typical changes observed in this group were multiple gains mainly on chromosomes 5 and 6 but also on chromosomes 4, 7, 8, 10, and 11. In addition we observed deletions on chromosome 19 as well as DNA losses on chromosome 22. The histogram indicated (99% CI) overrepresentation with peak incidences at 5p12, 5q23, and 5q31 in nine of 10 cases and 6q23 in eight of 10 cases and losses at 19p13.1 (Fig. 2 *upper and lower*).

The second (eight cases) was called the "group with multiple losses." These tumors were associated with various multiple losses predominantly on chromosomes 3, 4, 5, 6q, 7p, 9p, 10q, 13q, 14q, 17, and 18. In contrast to the group characterized by multiple gains, in these cases overrepresentations were most frequently found on chromosomes 19 and gains on chromosomes 16, 17, and 22q (Fig. 2 *center and lower*). The histogram indicated the highest frequency of changes for the losses on 4q24, 5q14-21, 6q12, 9p21-2, and 13q21 in six of eight cases, as well as the overrepresentation at 19p13.3-centr and 19q13.1 in six

Fig. 2. *Upper*: Super karyograms of the chromosomal imbalances detected in the group of 10 pilocytic astrocytomas with prominent gains. Typical changes are visible include the DNA gains on chromosomes 5 and 6q and losses on chromosome 19. *Center*: Super karyograms of the chromosomal imbalances detected in the group of eight pilocytic astrocytomas with prominent losses. Typical changes visible in this group and include the DNA losses on chromosomes 4q, 5, 6q, 13q, and 18q and gains on chromosome 19. *Lower*: Difference histograms of two subgroups of pilocytic astrocytoma represented in the *upper* and *center* super karyograms. The left side of each histogram shows the genetic losses, and the right side shows the genetic gains. The three *black lines* on both sides of a histogram represent the 0% (closest to the ideogram), the 50%, and the 100% (farthest from the ideogram) relative frequency levels. The percentage of changes only occurring in the tumor subgroup with prominent gains is shown in *green*. The excess of changes in the second group with multiple losses is shown in *red*. The blank *areas* beneath the colored regions indicate the percentage of changes in both groups. *Light gray* and *dark gray peaks* refer to chromosomal locations where the difference between tumor groups are statistically significant at the 5% and 1% significance level according to the results of Chi-square analysis. The most prominent changes are seen on chromosomes 5, 6, and 19.

of eight, and 22q11.1-2 in seven of eight cases. The dichotomy between both tumor subgroups is most clearly visible in the difference histogram presented in Fig. 2 *lower*.

When we considered subgroups according to the topographic localization of the tumor (cerebellar compared with supratentorial), patients age (in childhood as compared with adult blood), or sex, no preferential gains or losses were observed.

DISCUSSION

Investigations of genetic changes in neuroectodermal tumors have already led to a fairly good characterization of several tumor types of the central nervous system such as diffuse astrocytomas,^{13,21,31,57} oligodendrogliomas,² oligoastrocytomas,^{26,29} ependymomas,¹¹ medulloblastomas,³⁰ and meningiomas.⁵⁸ In contrast, the genetic abnormalities of the many benign tumors such as pilocytic astrocytoma have remained largely enigmatic. However, understanding of these tumors may be especially important to understand the early carcinogenesis of astrocytic tumors.

In the present study we investigated, for the first time, pilocytic astrocytomas by using CGH, and the results indicated the presence of two distinct genetic subtypes. Interestingly, the single major change that distinguishes these two subsets is DNA imbalances on chromosome 19. This chromosome has been identified as playing an important role in astrocytoma tumorigenesis.^{44,56} An extensive allelotyping study of gliomas revealed loss of heterozygosity in almost every astrocytoma subtype: three of 19 World Health Organization Grade II tumors, 12 of 27 anaplastic astrocytomas, 16 of 76 glioblastomas multiforme, four of nine oligodendrogliomas, three of five anaplastic oligodendrogliomas, five of nine mixed oligoastrocytomas, and eight of 10 anaplastic oligoastrocytomas.⁵⁷ The fact that chromosome 19-related data obtained from studying pilocytic astrocytoma are still inconclusive is most probably related to our observation that distinct genetic subtypes exist.

One group of tumors was characterized by deletions on chromosomes 19 and 22 and frequent overrepresentations of chromosomes 5 and 6 as well as chromosomes 4, 7, 8, 10, and 11. In this regard, it is interesting to note that numerical gains on chromosomes 7, 8, and 11 have already been detected in cytogenetic studies,^{59,63} whereas deletions on chromosome 22q, as detected using allelotyping, have recently been described in astrocytoma.²¹ The analysis of candidate genes found on chromosome 19 such as *BAX*, *MIA*, and *ANOVA* has not shown conclusive evidence of tumor suppressor properties.^{4,7,54}

The second subgroup of pilocytic astrocytomas was characterized by gains on chromosome 19, deletions at 4q, 5q, 6q, 9p, 13q, and 18q, and gains of 22q. High-copy amplifications on chromosome 19 in human malignant gliomas were observed by Schrock, et al.⁴⁷ This change has been mainly associated with glioma tumor progression as well as lung carcinoma.^{8,41,46,51} In this regard, it is important to note that amplifications are not typically found in pilocytic astrocytoma. Instead more often we observed numerical chromosome gains or low-level overrepresenta-

CGH patterns in pilocytic astrocytomas

tions except for single cases (see Fig. 2), which may represent the tumor subset that will be characterized by recurrence and/or tumor progression. Follow-up information about our tumor collective will be available in the future. Again, still lacking is conclusive evidence for a role of the many protooncogenes that reside on chromosome 19 such as *AKT2*, *BCL-3*,⁶ *RRAS*,^{28,53} *HKR1*, *HKR2*,³³ *FOSB*,^{20,48} and *JUNB*⁶² in the development of astrocytic tumors.

Consistent with previous findings, a certain percentage of cases carried deletions on chromosome 9p, which has also been found by allelotyping, although the role of the *p16* gene is still unknown.⁵⁵ Chromosome 9p deletions have also been described in previous CGH studies as being found in low and high malignant diffuse astrocytoma and glioblastoma.^{3,31,45} However, for other chromosomes, their CGH patterns differ from pilocytic astrocytomas. The fact that we observed, infrequently, deletions on chromosome 17p might corroborate the finding that the *p53* gene is rarely mutated in pilocytic astrocytomas, although this view has recently been challenged.¹⁹ Regardless, *p53* mutation does not seem to be associated with gross chromosomal deletions.

The existence of genetic subtypes of pilocytic astrocytoma is corroborated by clinicopathological studies that succeeded in differentiating subgroups of patients with excellent and moderate prognosis. However, we were not able to recognize specific morphological features that may characterize the two genetic subtypes, and this may be related to the limited number of cases analyzed to date.

In summary, our study revealed that pilocytic astrocytoma is characterized by two distinct genetic subgroups. We believe that this is an important finding, although it raises many questions. For example, which genes are affected by the chromosomal imbalances with importance for the tumor biology? How are the genetic alterations related to the clinical outcome? Are there any morphological or immunohistochemical markers that might be used to differentiate these tumor subgroups? We hope to answer these questions in future studies.

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