Neurofibromatosis 1 (NF1) is an autosomal dominant disorder that predisposes sufferers to various forms of neoplasia. Among affected individuals, 15%–20% develop astrocytomas, especially pilocytic astrocytomas (PA), which are benign and classified as grade I by the World Health Organization. They are generally well circumscribed, and their progression is slow. NF1-associated PAs (NF1-PAs) occasionally behave as aggressive tumors. To elucidate underlying genetic events in clinically progressive NF1-PAs, we performed molecular genetic analysis on 12 PAs, including 3 NF1-PAs, for \( p53 \), \( p16 \), and epidermal growth factor receptor genes, as well as loss of heterozygosity (LOH) on chromosome 1p, 10, 17, and 19q. None of the obvious genetic alterations typically seen in higher grade astrocytomas were found in 9 sporadic PAs. However, in 2 of 3 NF1-PAs, microsatellite analysis showed LOH on chromosome 10, including the \( PTEN \) (phosphatase and tensin homolog deleted on chromosome 10) gene locus, despite the diagnosis of pilocytic astrocytoma; one of these also manifested homozygous deletion of the \( p16 \) gene. The other NF1-PA harbored only LOH of the \( NF1 \) gene locus (17q). Our preliminary results support the hypothesis that some NF1-PAs differ genetically from sporadic PAs.

Neurofibromatosis 1 (NF1; von Recklinghausen’s disease), a common autosomal dominant disease that occurs at a frequency of about 1 in 3000 to 4000 people (Friedman et al., 1999; Huson and Hughes, 1994), is characterized by café au lait spots, neurofibromas, hamartomas of the iris (Lisch nodules), axillary freckling, and distinct bone lesions (Gutmann et al., 1997). Individuals with NF1 are at increased risk for astrocytomas, especially pilocytic astrocytomas (PAs; WHO grade I) involving the optic nerve, chiasm, hypothalamus, and brain stem (Listernick et al., 1997). The majority of these tumors behave in a benign fashion with relatively little evidence of growth and no associated symptoms (Molloy et al., 1995). However, the biological behavior of PA in patients with NF1 is occasionally more aggressive histopathologically as well as clinically, and in some NF1 patients, PAs undergo malignant transformation to high-grade tumors (Cohen et al., 1990–1991;
Ilgren and Stiller, 1987; Listernick et al., 1999; Ruggieri and Packer, 2001).

Patients with NF1 have a defect in the NF1 gene, which is located on the long arm of chromosome 17 (17q11.2) and was identified by positional cloning in 1990 (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990). Neurofibromin is the 220–250-kDa cytoplasmic protein product of the NF1 gene (Daston et al., 1992; DeClue et al., 1991; Gutmann et al., 1991), and a small portion of the molecule has striking sequence similarity to the catalytic domain of a family of proteins termed GTPase-activating protein (GAP), such as the mammalian p120-GAP. GAP proteins function as negative regulators of small GTPase proteins (e.g., p21-ras) by accelerating their conversion from active GTP-bound forms to inactive GDP-bound forms (Zhu and Parada, 2001). Lau et al. (2000) reported that loss of neurofibromin expression was observed in NF1-PA and was associated with elevated levels of activated Ras, MAPK, and PI3-K.

Genetic alterations of the p53, p16, and epidermal growth factor receptor (EGFR) genes are frequently observed in diffuse astrocytomas, and loss of heterozygosity (LOH) on chromosome 10 is one of the most frequent chromosomal abnormalities in human high-grade astrocytomas, especially glioblastomas (WHO grade IV) (Kleihues and Ohgaki, 1999; Tada et al., 2001). We analyzed mutation of the p53 gene, homozygous deletion of the p16 gene, amplification of the EGFR gene, and LOH on both arms of chromosome 10 in 12 PAs using 12 fluorescent microsatellite markers on both arms of chromosome 10. We also estimated LOH on both arms of chromosome 17 where the p53 gene (17p) and the NF1 gene (17q) are located, on the short arm of chromosome 1 (1p), and on the long arm of chromosome 19 (19q), alterations that are specific predictors of a chemotherapeutic response and better prognosis in anaplastic oligodendrogloma patients (Cairncross et al., 1998).

Materials and Methods

Patients and Tissue Samples

Tumor tissue samples and matched blood samples were obtained from 12 unrelated patients with PAs who were treated at Kumamoto University Hospital between March 1998 and January 2002. In 9 patients the tumors were sporadic; in 3 they were associated with NF1 as diagnosed according to NIH criteria (NIH, 1988). After resection, tumor tissues were frozen at −80°C until use for isolation of genomic DNA or mRNA. Blood was drawn from each patient, and peripheral blood lymphocytes were separated by using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). Written informed consent was obtained from all patients and/or family members before the patients were entered into this study.

Histopathologic Evaluation

Histological sections from all tumors were classified by a histopathologist from our research group according to WHO criteria (Kleihues and Cavenee, 2000). We recorded findings regarding the presence or absence of cellular elongation as fine hair-like bipolar processes, of eosinophilic granular bodies, and of Rosenthal fibers. One of these samples (case 11) was also evaluated by a histopathologist outside our group.

DNA and mRNA Preparation

Genomic DNA was extracted as previously described (Tada et al., 2001). Briefly, frozen samples were reduced to powder and treated with sodium dodecyl sulfate and proteinase K; this was followed by phenol and chloroform extraction. mRNA was also extracted from frozen samples by using the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, N.J.), according to the manufacturer’s instructions (Shiraishi et al., 2002).

Analysis of p53 Gene Mutation

To investigate the p53 gene status, we employed yeast functional assay as previously described (Shiraishi et al., 2002). Briefly, amplified p53 cDNA and linearized p53-expression vector pSS16 were cotransfected into the reporter yeast strain yIG397 by following the lithium acetate procedure. The transformed yeast cells were plated, incubated at 30°C for 2 days to generate colonies, and stored at 4°C overnight to develop color. At least 200 colonies were examined for each plate. When more than 15% of the colonies were red, we judged the sample positive for p53 functional loss and proceeded to sequencing analysis.

Analysis of p16 Gene Homozygous Deletion

We used our previously described multiplex polymerase chain reaction (PCR) for the analysis of homozygous deletion of the p16 gene (Kamiryo et al., 2002). Briefly, PCR amplification was performed by using primer pairs for p16 exon 2 and for a sequence-tagged site (STS) on the long arm of chromosome 9 (9qSTS). After PCR, 5 ml of the reaction product was separated by electrophoresis on a 2% agarose gel and stained with 0.5 µg/ml ethidium bromide under UV illumination. The resulting images were next density-scanned. Band intensities for the areas corresponding to the amplimers of p16 and 9qSTS were then determined with the NIH Image 1.62 program (Wayne Rasband Analytics, National Institutes of Health, Bethesda, Md.), and the intensity ratio of p16/9qSTS was calculated.

Analysis of EGFR Gene Amplification

For Southern blot analysis, 10 µg of each genomic DNA sample was restriction-digested with EcoRI, electrophoretically separated on 0.8% agarose gels, and transferred to nylon membranes (Roche, Mannheim, Germany). To prepare digoxigenin (DIG)-11-dUTP-labeled random-primed DNA probes, we labeled full-length human EGFR cDNA (Okutani et al., 1994) and full-length human α-tubulin cDNA (pEGFP-Tub Vector, BD Biosciences Clontech, Palo Alto, CA) with DIG-dUTP by using the DIG DNA Labeling Kit (Roche). After hybridization at 37°C overnight, the membranes were washed twice in 5× SSC and 0.5% SDS and once in 0.1× SSC and 0.5% SDS, then hybridized with anti-DIG-alkaline phosphatase (Roche), and exposed to X-ray film (Kodak). The resulting images were next density-scanned. Band intensities for the areas corresponding to the amplimers of p53 and 9qSTS were then determined with the NIH Image 1.62 program (Wayne Rasband Analytics, National Institutes of Health, Bethesda, Md.), and the intensity ratio of p53/9qSTS was calculated.
Alto, Calif.) using a DIG-DNA labeling kit (Roche) according to the manufacturer’s instructions. Cross-linked blots were hybridized at 65°C with the DIG-labeled \( EGFR \) cDNA probes. After washing, the membranes were probed with Anti-Digoxigenin-AP (Roche), and luminescence reaction of the blots was then performed with 0.5% CSPD (a chemiluminescent substrate, Roche). We took autoradiographs using X-ray film with intensifying screens at room temperature. Next, the blots were stripped and rehybridized with \( \alpha \)-tubulin reference probes.

We used the Scion Imaging Software Program (Scion Corp., Frederick, Md.) to compare the signal intensities of the 6.1-kb fragment of \( EGFR \) from a tumor tissue sample and a normal human brain sample, and the relative ratio of the signal intensity of the \( EGFR \) fragments was determined. Similarly, the signal intensities of a 3.0-kb \( \alpha \)-tubulin fragment from a tumor sample and a normal brain sample were compared, and the relative ratio was determined. The relative ratio of the signal intensity of the \( EGFR \) fragments was normalized by the relative ratio of the signal intensity of the \( \alpha \)-tubulin fragments. After the \( EGFR \) gene status of normal human brain tissue samples was determined, a cut-off value was assigned. As this value ranged from more than 0.5 to less than 2.0 (data not shown), 2.0 was chosen as the threshold. Relative ratios equal to or exceeding 2.0 were taken as indicative of amplification.

Analysis of LOH on Chromosomes 1p, 10, 17, and 19q

LOH in tumors was investigated on chromosomes 1p, 10, and 19q by using 22 microsatellite markers (D1S214, D1S244, D1S2667, D10S189, D10S547, D10S538, D10S557, D10S215, D10S541, D10S200, D10S566, D10S544, D10S545, D10S209, D10S587, D17S804, D17S1176, D17S1849, D17S1863, D17S1880, D19S219, and D19S596 [Genome Database, http://www.gdb.org]). Markers D10S215 and D10S541 flank the \( PTEN \) gene. DNA fragments were PCR-amplified by using primer pairs. Forward primers were labeled with the fluorescent dye FAM (blue) or HEX (green) at the 5’-end. PCR reactions were performed in a 25-\( \mu \)l volume with \( rTth \) DNA polymerase according to the manufacturer’s instructions (ABI, Foster City, Calif.) for 35 cycles under optimized PCR conditions. After amplification was confirmed by electrophoresis on 2% agarose gels, the PCR products were mixed with 400HD-ROX size standard (ABI, Applied Biosystems, Foster City, Calif.) and formamide-loading buffer, loaded into denaturing (6M urea) 4% acrylamide gels, and analyzed with an ABI-377 fluorescent DNA sequencer. The results were analyzed with GeneScan software (ABI). In informative cases, allelic loss was determined by comparing the peak areas of both alleles in blood and corresponding tumors.

Results

Patient Characteristics

Between 1998 and 2002, we diagnosed 12 patients seen at our department with PA; 3 patients had PAs associated with NF1 (NF1-PAs) (Table 1). The median age of patients with sporadic PAs and NF1-PAs at the time of diagnosis was 18.8 years (range 6–70 years) and 30.3 years (range 8–47 years), respectively. There were 10 males and 2 females; the site of tumor origin varied among the patients. All 9 sporadic PAs manifested the usual histological pattern. Two of the 12 patients (cases 3 and 11) had recurrent tumors; case 3, a 6-year-old boy, had previously undergone subtotal removal and had been diagnosed at another hospital as having PA. His residual tumor showed progression 3 years later and was totally removed at our institution. Case 11, a 36-year-old male, had pre-

Table 1. Summary of clinical data and gene analysis of 12 pilocytic astrocytomas

<table>
<thead>
<tr>
<th>Genetics</th>
<th>Case No.</th>
<th>Age (years) /Sex</th>
<th>Localization</th>
<th>Surgery</th>
<th>p53 Mut.</th>
<th>p16 Del.</th>
<th>EGFR Amp.</th>
<th>LOH*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic</td>
<td>1</td>
<td>6/M</td>
<td>brain stem</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(11)–(2)–(1)–(1)–(2)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6/M</td>
<td>lt. temporal lobe</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(6)–(1)–(1)–(1)–(1)</td>
</tr>
<tr>
<td></td>
<td>3#</td>
<td>6/M</td>
<td>3rd ventricle</td>
<td>T</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(7)–(2)–(2)+(2)–(2)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7/F</td>
<td>lt. temporal lobe</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(7)–(2)–(2)–(2)–(2)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9/F</td>
<td>4th ventricle</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(6)–(2)–(2)–(2)–(2)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10/M</td>
<td>cerebellum</td>
<td>T</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(5)–(3)–(2)–(2)–(3)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>15/M</td>
<td>cerebellum</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>–(6)–(1)–(1)–(1)–(1)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>40/M</td>
<td>suprasellar</td>
<td>B</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td>–(9)–(2)–(2)+(2)+(2)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>70/M</td>
<td>rt. thalamus</td>
<td>B</td>
<td>—</td>
<td>ND</td>
<td>—</td>
<td>–(5)–(2)–(2)+(2)+(2)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8/M</td>
<td>cerebellum</td>
<td>T</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td>–(5)–(2)–(2)+(2)+(2)</td>
</tr>
<tr>
<td></td>
<td>11#</td>
<td>36/M</td>
<td>rt. parieto-occipital lobe</td>
<td>T</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td>–(5)–(2)–(2)+(2)+(2)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>47/M</td>
<td>spinal cord</td>
<td>B</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td>–(7)+(2)+(1)+(1)+(1)</td>
</tr>
</tbody>
</table>

Abbreviations and symbols: B, biopsy; Amp., amplification; Del., homozygous deletion; LOH, loss of heterozygosity; Mut., mutation; ND, not determined because of insufficient DNA or RNA sample; NF1, neurofibromatosis 1; S, subtotal removal; T, total removal; — = normal; + = abnormal.

*Numbers in parentheses are the number of informative markers.

#Recurrent case.
Previously undergone total removal; regrowth was noted 5 years later. In 2 of 3 NF1-PA patients (cases 11 and 12), there was a family history of NF1-PA.

Genetic Alterations

In patients with sporadic PA, none of the obvious genetic alterations typically seen in higher grade astrocytomas were noted (Table 1). One patient with tumor recurrence (case 3) manifested LOH17p; another patient with sporadic PA, a 70-year-old male (case 9), had LOH on both arms of chromosome 17.

Allelic loss of chromosome 10 was observed in 2 of the 3 NF1-PAs (cases 11 and 12); both of these patients also had LOH on 1p, 17p, 17q, and 19q. Case 11 also manifested homozygous deletion of the \( p16 \) gene. The remaining NF1-PA patient (case 10) manifested only LOH of the \( NF1 \) gene locus.

Tumors with LOH showed loss for almost all informative markers except for D10S566 in case 11 and D10S189 in case 12 in this study (data not shown). Typical electropherogram images of marker D10S566 in cases 10, 11, and 12 are shown in Fig. 1.

Representative Cases of NF1-PA

Case 10. This 8-year-old boy with NF1 had no familial history of the disease. At the age of one month he manifested many café au lait spots and freckling and was diagnosed as having NF1. He presented to us with headache, nausea, and gait disturbance. After MRI revealed a cystic tumor in the left cerebellar hemisphere (Fig. 2a), the patient underwent gross total removal. Histological findings on the surgical specimen are shown in Fig. 2b.

Case 11. This 36-year-old male with NF1 has a familial history of the disease; both his father and brother have NF1. From infancy, this patient manifested many café au lait spots and neurofibromas. At age 31 he presented at another hospital with left homonymous hemianopsia and underwent gross total removal. The pathological diagnosis at the time was PA, and he received no adjuvant therapy. He subsequently presented to us with a history of headache and episodes of loss of consciousness. Admission examination revealed mild left homonymous hemianopsia, MRI disclosed a right parieto-occipital tumor (Fig. 2c), and the patient underwent gross total removal of the recurrent tumor at our hospital. His diagnosis was recurrent PA associated with NF1. Histological findings on the surgical specimen are shown in Figs. 2d–2g.

Case 12. This 47-year-old male with NF1 had a familial history of the disease; both his father and his son have NF1. He too manifested many café au lait spots from infancy; neurofibromas were recognized when he was 20 years old. He reported to us with a history of abnormal sensation in the lower back that was followed by dysesthesia of the left hand, and he presented with mild left hemiparesis. After MRI revealed an enhanced lesion in the cervico-medullary cord (Fig. 2h), open biopsy was carried out. Histological findings are shown in Fig. 2i. The tumor was highly resistant to therapy and progressed, even after multidisciplinary treatment that included radiotherapy and chemotherapy. The patient died one year after undergoing biopsy.

Discussion

Pilocytic astrocytomas associated with NF1 are sometimes biologically different from sporadic PAs (Cohen et al., 1990–1991; Ilgren and Stiller, 1987; Listernick et al., 1999; Molloy et al., 1995), yet there is scant published evidence for their genetic difference (Gutmann et al., 2002; Ruggieri and Packer, 2001). Li et al. (2001) demonstrated that PAs from individuals affected with NF1 lack the alteration of \( p53, p16, RB \) (retinoblastoma), \( EGFR \),
Fig. 2. Magnetic resonance images and photomicrographs illustrating tumors and tumor specimens from 3 patients. Case 10: (a) Admission gadolinium-enhanced axial T1-weighted MR image obtained from an 8-year-old boy reveals a large cystic tumor with ringlike enhancement in the left cerebellar hemisphere. (b) Photomicrograph of the surgical specimen shows a biphasic combination of microcystic and compact areas with nuclear atypia (H & E stain × 100). An eosinophilic granular body can also be seen. Neither mitosis nor necrosis is present. Case 11: (c) Admission gadolinium-enhanced axial T1-weighted MR image obtained from a 36-year-old male reveals a large multicystic and irregularly enhanced tumor in the right parieto-occipital lobe. (d) Photomicrograph of the first surgical specimen (H & E stain × 100). The cellular elongation, “piloid cells,” and eosinophilic granular bodies identify this as a pilocytic astrocytoma. The cellularity is not high, and neither mitosis nor necrosis is present. (e) Photomicrograph of the specimen obtained at surgery for recurrence (H & E stain × 200). The tumor cells show bizarre nuclear and cytoplasmic pleomorphism. (f) High magnification of the same specimen shows mitotic activity (H & E stain × 400). (g) Another portion of the same specimen shows a remnant of the former pilocytic astrocytoma with lobular tissue pattern (H & E stain × 200). Case 12: (h) Admission gadolinium-enhanced sagittal T1-weighted MR image obtained from a 47-year-old male reveals an intramedullary mass lesion extending from the medulla to the upper cervical cord with surface dissemination. (i) Photomicrograph of the surgical specimen shows a loose array of polar cells and many Rosenthal fibers (H & E stain × 100). However, histopathological malignant features cannot be seen.
CDK4 (cyclin-dependent kinase 4), PDGF-A (platelet-derived growth factor A), and PDGF-βR (PDGF receptor β), each of which has been associated with clinically more aggressive fibrillary astrocytomas (Cheng et al., 2000). Therefore, we examined LOH10, the most common chromosomal abnormality in high-grade gliomas, in patients with PA, including NF1-PA. We detected allelic loss of chromosome 10 in 2 of 3 NF1-PAs that appeared to involve the PTEN gene locus (Li et al., 1997; Steck et al., 1997), despite the diagnosis of pilocytic astrocytoma. At present, although we are unable to explain the reason for the occurrence of these alterations in NF1 patients, one possibility is that they are the result of aging, as Ly et al. (2000) proposed, especially in these familial diseases. Gutmann et al. (2000) demonstrated the loss of neurofibromin expression in NF1-PAs. On the other hand, studies on sporadic PAs detected a slight up-regulation of either NF1 or NF1 expression levels that was comparable to that for other astrocytomas (Gutmann et al., 1996, 2000; Lau et al., 2000). The loss of neurofibromin may lead to functional up-regulation of the Ras pathway in patients with NF1, although Bajenaru et al. (2002) suggested that this does not explain the formation of astrocytomas in mice, and that other genetic or environmental factors play a role in NF1-associated glioma tumorigenesis. Their interpretation is consistent with results of Holland et al. (2000), who showed that the introduction of an activated p21-ras molecule alone failed to produce astrocytic tumors in mice unless there were other coexisting genetic events. However, activated p21-ras combined with activation of the phosphatidylinositol-3-kinase (PI3-K) pathway, which mimics loss of the PTEN tumor suppressor (Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000), results in the development of aggressive astrocytomas. Although Duerr et al. (1998) demonstrated the PTEN gene mutation in 1/30 (3%) PAs, to the best of our knowledge, ours is the first demonstration of LOH10 including the PTEN gene locus in patients with NF1-PA. Our data support the hypothesis of additional biological differences between sporadic PAs and NF1-PAs. Interestingly, both of our NF1-PA patients with LOH10 (cases 11 and 12) also manifested LOH on 1p and 19q. Allelic loss of chromosome 1p and 19q is a distinctive feature of oligodendrogliomas and is associated with heightened chemosensitivity and a better prognosis (Cairncross et al., 1998). At present we cannot interpret the clinical significance of these chromosomal abnormalities because candidate genes on these chromosomes remain to be identified. However, the identification of an antibody epitope (PEN5; Figarella-Branger et al., 1999) that specifically distinguishes between juvenile PAs and fibrillary astrocytomas suggests that PAs may arise from the same O2A lineage that gives rise to oligodendrocytes. Nielsen et al. (1999) pointed out the participation of the p16 gene in the malignant transformation of neurofibromas in NF1. Although our case 11 had a recurrent NF1-PA, our finding of homozygous deletion of the p16 gene in this patient is consistent with their interpretation. Although allelic loss of chromosome 17p was observed in 4 of the 12 PAs studied here, the incidence of 17qLOH was higher in NF1-PA than sporadic PA (2 of 3 NF1-PA cases vs. 2 of 9 sporadic PA cases). With respect to NF1 alleles (17q), although all 3 NF1-PA patients manifested 17qLOH, only one of 9 patients with sporadic PA, a 70-year-old man, had 17qLOH. Our findings coincide with those reported by Kluwe et al. (2001) and suggest that aging may play a role. While our study population was small and the documented genetic alterations were restricted, our study provides preliminary evidence of an additional genetic difference between NF1-PAs and sporadic PAs.

Acknowledgements

We thank Dr. Yoichi Nakazato (Gunma, Japan) for histopathological evaluation and Dr. Keisuke Ueki (Tokyo, Japan) for technical advice.

References


Tada et al.: Gene analysis for pilocytic astrocytomas


