The pilocytic astrocytoma
Dirven, Clemens Maria Franciscus

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1998

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
CHAPTER 7

UP-REGULATION OF SPECIFIC NF1 GENE TRANSCRIPTS IN SPORADIC PILOCYTIC ASTROCYTOMAS

Platten M, Giordano MJ, Dirven CMF, Guttman DH, Louis DN
INTRODUCTION

Neurofibromatosis 1 (NF1) is a common, autosomal dominant disorder with an incidence of approximately 1 in 3000 individuals (1). The disease is characterized by neurofibromas, café-au-lait spots, hamartomas of the iris (Lisch nodules), axillary freckling, and distinct osseous lesions. Patients with NF1 are also predisposed to a host of tumors, including malignant peripheral nerve sheath tumors (neurofibrosarcomas), pheochromocytomas, rhabdomyosarcomas and gliomas. Of the gliomas in NF1 patients, the most characteristic and common is pilocytic astrocytoma of the optic nerve (optic nerve glioma).

The \textit{NF1} gene comprises 59 exons and spans more than 350 kb of genomic DNA on the long arm of chromosome 17 (17q11.2) (2-7). The approximately 13-kb \textit{NF1} mRNA transcript encodes neurofibromin, a protein with a central 400-amino-acid region that is homologous with human GTPase-activating proteins (GAPs). The GAP-related domain (GRD) of neurofibromin stimulates the intrinsic GTPase activity of p21-ras, implicating neurofibromin in the transduction of proliferating and differentiation signals (2-7). The \textit{NF1} gene is alternatively spliced, leading to multiple transcript isoforms, some of which are highly expressed in brain. For instance, the GRD region contains an alternatively spliced 63-nucleotide exon, designated 23a. \textit{NF1} transcripts that include exon 23a (type 2) and those that do not include exon 23a (type 1) are both expressed in normal brain (8-10). Another \textit{NF1} splice variant involves a 30-nucleotide exon between exon 9 and exon 10a, designated 9br (also termed 9a). Exon-9br-containing transcripts are specifically expressed in brain (11). Additional alternative splicing of the \textit{NF1} gene has been described, but these additional transcripts are not found in the central nervous system.

Loss of neurofibromin expression occurs in malignant peripheral nerve sheath tumors with \textit{NF1} gene inactivation (12) and is associated with elevated levels of p21-ras (13-15). Neurofibromin is also reduced or absent in other sporadic tumors, including NF1-related tumors such as pheochromocytoma and non-NF1-related tumors such as melanoma and neuroblastoma (16-18). Interestingly, exon-23a-containing isoforms may have reduced GAP activity, compared with those isoforms lacking this exon (19), and variable expression of these isoforms may correlate with malignant transformation and differentiation (8-10, 19-22).

Furthermore, transgenic mice heterozygous for \textit{NF1} gene inactivation have a higher incidence of malignant tumors than wild type mice (23). These observations all support the prediction that the \textit{NF1} gene functions as a tumor suppressor gene.

The association of optic nerve glioma with NF1 raises the hypothesis that the \textit{NF1} gene is involved in optic nerve glioma tumorigenesis. Optic nerve gliomas and histologically identical pilocytic astrocytomas of the cerebellum and third ventricular region occur in non-NF1 patients (24). These observations further raise the possibility that the \textit{NF1} gene is altered in sporadic (non-NF1-associated) optic nerve gliomas and other pilocytic astrocytomas. This hypothesis is supported by the finding of allelic loss of the \textit{NF1} region on chromosome 17q in NF1-associated and sporadic pilocytic astrocytomas (25). Direct analysis of the \textit{NF1} gene, however, has not been undertaken to confirm its role in pilocytic astrocytoma formation. To evaluate the role of the \textit{NF1} gene in these tumors, therefore, we examined quantitative and qualitative aspects of \textit{NF1} gene expression in a series of sporadic pilocytic astrocytomas.
MATERIALS AND METHODS

Fresh portions of 6 pilocytic astrocytomas and 3 glioblastoma multiforme (GBM) were obtained from the University of Groningen Hospital and the Massachusetts General Hospital. Of the 6 pilocytic astrocytomas, three were optic nerve gliomas (patient ages 5 months, 14 months and 4 years) and 3 were cerebellar pilocytic astrocytomas (patient ages 5, 11 and 13 years). None of the patients with pilocytic astrocytomas had a family history of NF1 or NF1-related skin lesions. Normal frozen postmortem brain specimens of the superior parietal lobe (5 samples) were obtained from the Brain Tissue Resource Center at McLean Hospital (Belmont, MA) and of the superior frontal lobe (2 samples) from MGH.

RNA POLYMERASE CHAIN REACTION (PCR) ANALYSIS.

RNA was extracted from histologically verified tumor or normal tissue using TRIzol Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. NF1 cDNA was generated from 3 to 5 µg of RNA using 200 U of SuperScript II reverse transcriptase (Life Technologies, Gaithersburg, MD), 0.1 µg of oligo-dT (Pharmacia, Piscataway, NJ), and 50 U of RNAase inhibitor (Perkin Elmer, Norwalk, CT) in a total volume of 50 µl containing 2.5 mmol/L of each dNTP, 0.1 mol/L dithithreitol, 5 µg bovine serum albumin, 250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, and 15 mmol/L MgCl at 37°C for 1 hour. For all reverse transcriptase reactions, a control was performed omitting the reverse transcriptase to exclude DNA contamination. NF1-GRD cDNA was amplified with primers 5’-CA GAATTCCCCCCTCAACTTCAAGT-3’ and 5’-TGCGTGCTGCATCAAAGTTTGCTTTTCAC-3’ for 30 cycles at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. NF1-GRD isoforms containing exon 23a (type 2) are 366 bp whereas those without exon 23a (type 1) are 303 bp in length. NF1-9br region cDNA was amplified for 30 cycles of 95°C for 45 seconds, 69°C for 3 minutes, and 72°C for 3 minutes using primers 5’-CTGAGCAATCTCTGGCATGTGTGACTGA-3’ (located in exon 10c) and 5’-TGGA CAGTCTACGAAAAGCCTCTTGCTGG-3’ (located in exon 7). The isoforms with and without exon 9br are 761-bp and 731-bp amplicons, respectively. As an internal control for the amount of RNA in each sample, cDNA from the cyclophilin gene was amplified using primers 5’-ATGGTCAACCCCACCGTGTT-3’ and 5’-CGGTGTTAAGTCACCACCT-3’, which yield a 206-bp amplicon after 22 cycles of 95°C for 30 seconds, 55°C for 40 seconds, and 72°C for 30 seconds. Previous studies revealed linear template amplification under these circumstances for 25 cycles (26). PCR was performed in a programmable thermal cycler (MJ Research, Watertown, MA). The PCR mixes contained 0.05 mmol/L of each dNTP, 1 pmol of each primer, 20 mmol/L Tris, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl, and 1 U of Taq polymerase. The PCR products were radiolabeled with 1 µCi of [³²P]-dCTP, which was added before the DNA-denaturing step of the final 2 PCR cycles to provide linear incorporation of the radioisotope. The radiolabeled PCR products were electrophoresed on 6% polyacrylamide denaturing sequencing gels, visualized by autoradiography, and quantitated by densitometry using an LKB 2222-020 UltraScan XL laser densitometer (Pharmacia). Each experiment was repeated at least twice. NF1 gene expression level was defined as the ratio between the NF1-GRD and cyclophilin transcripts. In addition, as another control, NF1 gene expression was also evaluated by analyzing the ratio of the NF1-9br region transcript with cyclophilin.
**SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS.**

SSCP was performed on the 6 cases as detailed elsewhere (27). PCR of the NF1-GRD and NF1-9br region was performed as above, except for the addition of 1µCi of α-32PdCTP at the start of the reaction. The NF1-GRD product was digested with *Hin*f1 (New England BioLabs, Beverly, MA) to yield 238- (including exon 23a), 175-, and 128-bp fragments to improve sensitivity of the SSCP. Similarly, the NF1-9br region SSCP products were digested with *Hin*f1 and *Pst*1 and yielded 238-, 211-, 175- (including 9br), and 137-bp fragments. The digested products were run on 6% and 8% nondenaturing polyacrylamide gels containing 10% glycerol at 4 and 6 W overnight. The gels were dried and visualized autoradiographically.

**IMMUNOHISTOCHEMISTRY.**

Immunohistochemistry was performed on 5 of the 6 specimens, as detailed elsewhere (28), using an antibody (B3A.1) directed against the carboxyl-terminal region of neurofibromin. Twelve-micron-thick frozen sections were placed on SuperFrost-Plus slides (Fisher Scientific, Pittsburgh, PA) and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.2, for 10 minutes. After incubation with 10% goat serum in 0.1 mol/L PBS containing 0.02% Triton X-100 for 60 minutes, the sections were incubated overnight at 4°C in a 1:500 dilution of rabbit polyclonal B3A.1. The slides were then washed in PBS and incubated in a 1:200 dilution of anti-rabbit biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 60 minutes. After PBS washes, the reaction was visualized using the Vectastain Elite kit (Vector) and diaminobenzidine. The sections were then dehydrated and mounted in 50% DPX:xylene without counter staining. Positive controls include immunohistochemistry for glial fibrillary acidic protein (Sigma Chemical Co, St. Louis, MO), and negative controls involved omission of the primary antibody.

**RESULTS**

Of the 7 specimens of normal brain, 5 showed predominant expression of the type 1 GRD transcript and 2 (both from the parietal lobe) had type 2 isoform predominance. On the other hand, all 6 pilocytic astrocytomas showed marked type 2 predominance (table 1). Densitometric analysis on the pilocytic tumors revealed two- to fivefold excess of type 2 transcripts whereas the 3 GBMs showed four- to fivefold excess of type 2 transcripts (table 1). Six of the 7 normal brain specimens showed the 9br-containing transcript in addition to the 9br-negative transcript, whereas only 1 pilocytic astrocytoma and 1 GBM expressed the 9br-containing transcript (table 1). All controls with omission of reverse transcriptase were negative.

The mean ratio of NF1-GRD to cyclophilin transcripts was 0.72 for the normal brain samples (range, 0.26 to 1.17). All tumor specimens had increased *NFI* gene expression; relative expression levels ranged from 1.4 to 4.2 times that of the normal brain mean ratio (table 1). In general, *NFI*
gene expression was somewhat higher in the pilocytic astrocytomas than in the GBMs and higher in the cerebellar pilocytic astrocytomas than in the optic nerve tumors (table 1). The mean ratio of NF1-9br region to cyclophilin transcripts paralleled the NF1- GRD/ cyclophilin ratios. No SSCP migration shifts were noted in any of the fragments from tumors or from normal cDNA controls. The controls with no DNA, done to exclude PCR contamination, were negative. Immunohistochemistry revealed strong cytoplasmic neurofibromin positiveness in all 5 tumors studied, implying the presence of full-length neurofibromin. Control reactions for glial fibrillary acidic protein showed a similar strong cytoplasmic reaction, and negative controls showed no staining.

**TABLE 1. Clinical and NF1 gene expression data.**

<table>
<thead>
<tr>
<th>Case</th>
<th>age</th>
<th>site</th>
<th>relative NF1 expression</th>
<th>type 1/2 ratio</th>
<th>9br expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>5 years</td>
<td>cerebellum</td>
<td>1.9</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>5 months</td>
<td>optic nerve</td>
<td>1.9</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td>14 months</td>
<td>optic nerve</td>
<td>1.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>13 years</td>
<td>cerebellum</td>
<td>2.4</td>
<td>0.3</td>
<td>+</td>
</tr>
<tr>
<td>27</td>
<td>11 years</td>
<td>cerebellum</td>
<td>4.2</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>4 years</td>
<td>optic nerve</td>
<td>1.4</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>GBM 1514</td>
<td>74 years</td>
<td>temporal lobe</td>
<td>1.7</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>GBM 1518</td>
<td>30 years</td>
<td>temporal lobe</td>
<td>1.5</td>
<td>0.2</td>
<td>+</td>
</tr>
<tr>
<td>GBM 1520</td>
<td>59 years</td>
<td>temporal lobe</td>
<td>1.4</td>
<td>0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The NF1 gene functions as a tumor suppressor gene in a variety of tumors in both NF1 and non-NF1 patients (12-14,16,17,26). Because of the close association between pilocytic astrocytomas and NF1 (1) and because allelic losses at the NF1 region occur in these tumors (25), the NF1 gene was also hypothesized to act as a tumor suppressor in pilocytic astrocytomas. We therefore predicted that NF1 gene and neurofibromin expression would be reduced or absent in pilocytic astrocytomas. Surprisingly, however, NF1 gene transcripts were elevated up to fourfold in the tumors when compared with NF1 transcript levels in normal brain. Increased expression or accumulation of other tumor suppressors, such as p53 (29), occurs in human tumors. Such accumulation, however, may have different causes and sequelae, depending on whether the protein is mutant or wildtype. For example, most tumors that accumulate p53 protein have missense mutations that inactivate the DNA-binding ability of the molecule (30,31). To evaluate whether the NF1 gene was mutant in our cases, we screened for approximately 1 kb of
coding sequence, including the critical GRD region. Although we did not detect sequence alterations, our negative screening of less than 8% of the coding sequence does not exclude mutations in other regions of the gene. We therefore evaluated the same tumors immunohistochemically using an antibody directed against the carboxy terminus of the protein, as most NF1 gene mutations lead to a truncated protein (2) that would not be detected by a carboxyl-terminal antibody. Consequently, the presence of full-length neurofibromin on immunohistochemical analysis in all 5 of the studied pilocytic tumors argues that the accumulating protein is most likely wildtype.

These observations raise the possibility that wildtype neurofibromin is being overexpressed as part of a physiological response. Such a situation may be similar to the accumulation of wildtype p53 in malignant astrocytomas and other tumors, which presumably reflects a physiological response by p53 to genomic damage or deregulated proliferation (32,33).

In the case of the NF1 gene, overexpression in tumors may represent an intact mechanism to reduce p21-ras-mediated proliferative signals. Recent data on the NF1 gene in malignant diffuse, fibrillary astrocytomas support this assumption (34). In a study of 21 malignant astrocytomas, all showed high levels of NF1 gene expression. Moreover, increased neurofibromin correlated with elevated levels of activated p21-ras, and reduction of activated p21-ras in vitro yielded a reduction in the levels of neurofibromin. In addition, neurofibromin expression may also be increased in activated non-neoplastic astrocytes, which may indicate an epigenetic mechanism of NF1 gene regulation (35). Combined, the findings imply that non-neoplastic, benign neoplastic, and malignant neoplastic astrocytes may share a common pathway in which the NF1 gene is up-regulated, although the precise significance of this up-regulation remains unclear.

Because NF1 isoforms may have different tumor suppressor capabilities, we examined pilocytic astrocytomas for those alternative transcripts that are normally expressed in the central nervous system. Early studies suggested that type 1 isoforms (without the exon 23a insert) are the predominant variant in fetal brain and neuroectodermal tumors, whereas the type 2 isoform was more common in differentiated cells (8). Other studies, however, showed type 1 predominance in normal brain and type 2 predominance in brain tumors (9). Our examination of 7 normal brain specimens showed type 1 isoforms as the more common transcript in 5 cases and type 2 in 2 cases. Although NF1 expression may be elevated in reactive astrocytes (35) and astrocytes predominantly express type 2 isoforms (36), the 2 brains in our study with type 2 predominance did not have evidence of reactive astrocytosis. All of the pilocytic astrocytomas, however, had conspicuous type 2 transcript predominance, as had been noted in malignant astrocytomas as well (this study and ref. 9). Type 2 predominance has also been demonstrated in a series of 12 pilocytic astrocytomas studied qualitatively at the GRD region (D.H. Gutmann and R.L. Heidemann, unpublished results). Although it is tempting to postulate that the type 2 isoforms have reduced tumor suppressor activity, it is equally possible that the type 2 predominance merely reflects a solely astrocytic phenotype rather than a neuronal or mixed neuronal/astrocytic phenotype as would be seen in normal brain. In fact, the type 1 isoform is predominantly expressed in neurons in rat adult brain tissue whereas type 2 transcripts are expressed in rat glial cells (36). Thus, type 2 predominance may also reflect a physiological response that is intact in both normal and neoplastic astrocytes.

Expression of the NF1 transcript containing the 9br exon was detected in 6 of 7 normal brain tissues but was reduced or absent in the pilocytic tumors. Malignant astrocytomas also do not
express the 9br-containing transcripts (this study and ref. 11). Again, however, it is unclear whether this represents oncogenic expression or a less active molecule or physiological expression of an astrocytic isoform. Recent studies of 9br isoforms have shown that 9br-containing transcripts are expressed only by neurons (D.H. Gutmann, unpublished results), suggesting that the lack of expression of 9br-containing isoforms in astrocytomas may also simply reflect an astrocytic phenotype.

NF1 is phenotypically highly variable, even within individual families. Interestingly, monozygous twins display much closer phenotypes than distant affected relatives, which has suggested that the NF1 phenotype is modified by other genes (37). Such modification may be mediated through potential transcriptional regulatory elements in the promoter region of the NF1 gene (38). Alternatively, phenotypic variation may be mediated by mRNA editing, a phenomenon that has recently been suggested for the NF1 gene (3). Finally, genes embedded within the NF1 gene (e.g., EV12A, EV12B, and OMGP) may play a role in NF1 gene expression or in modulating the NF1 phenotype (2). The complexities of NF1 gene expression in normal and tumor cells may imply that neurofibromin has multiple functions that modify cell differentiation and proliferation. In this regard, better understanding of NF1 gene regulation will no doubt contribute to explaining the up-regulation of specific NF1 gene transcripts in pilocytic astrocytomas.

REFERENCES


23. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumor predisposition in


