Molecular Profiles of Neurofibromatosis Type 1-Associated Plexiform Neurofibromas: Identification of a Gene Expression Signature of Poor Prognosis

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ABSTRACT

Purpose: Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder with a complex variety of clinical symptoms. The hallmark of NF1 is the development of heterogeneous benign neurofibromas, which may appear as dermal neurofibromas or plexiform neurofibromas. NF1 patients with plexiform neurofibromas are at risk of developing malignant peripheral nerve sheath tumors.

Experimental Design: To obtain additional insight into the molecular pathogenesis of plexiform neurofibromas, we used real-time quantitative reverse transcription-PCR assays to quantify the mRNA expression of 349 selected genes in plexiform neurofibromas in comparison with dermal neurofibromas and patient-matched malignant peripheral nerve sheath tumors.

Results: Thirty genes were significantly up-regulated in plexiform neurofibromas compared with dermal neurofibromas. None were down-regulated. The up-regulated genes mainly encoded transcription factors and growth factors, and secreted proteins, cytokines, and their receptors, pointing to a role of paracrine and autocrine signaling defects in the genesis of plexiform neurofibromas. We also identified a gene expression profile, based on MMP9, FLT4/VEGFR3, TNFRSF10B/TRAILR2, SHH, and GLI1, which discriminated those plexiform neurofibromas most likely to undergo malignant transformation.

Conclusion: Our study has identified a limited number of signaling pathways that could be involved, when altered, in plexiform neurofibromatosis development. Some of the up-regulated genes could be useful diagnostic or prognostic markers or form the basis of novel therapeutic strategies.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurocutaneous disorder affecting 1 in 3000 individuals worldwide (1). The main clinical features of NF1 are café au lait macules, skinfold freckling, and iris Lisch nodules. NF1 patients also develop benign peripheral nerve sheath tumors, termed neurofibromas. In a small proportion of NF1 patients, neurofibromas (mainly plexiform neurofibromas) may progress to malignant peripheral nerve sheath tumors (MPNST).

NF1 is caused by germ-line mutations of the NF1 gene, which contains 60 exons and is located on chromosome arm 17q11.2 (2, 3). The encoded protein, neurofibromin, is expressed in all of the tissues but is most abundant in neurons, astrocytes, oligodendrocytes, Schwann cells, adrenal medullary cells, WBCs, and gonadal tissue (4). Neurofibromin contains a central domain homologous to a family of proteins known as Ras-GTPase-activating proteins, which function as negative regulators of Ras proteins (5). Many NF1-deficient tumors contain elevated levels of Ras-GTP, supporting the notion that unregulated Ras signaling may contribute to their development (5, 6). In keeping with its role as a classical tumor suppressor gene, NF1 loss of heterozygosity has been found both in benign and malignant tumors in NF1 patients (7–10).

The hallmark of NF1 is benign neurofibromas, which vary greatly in both number and size, and which may take the form of dermal or plexiform neurofibromas (11, 12). In contrast to dermal neurofibromas, which are typically small and grow as discrete lesions in the dermis, plexiform neurofibromas can develop internally along the plexus of major peripheral nerves and become quite large (13, 14).

Neurofibromas are heterogeneous tumors mainly composed of Schwann cells (60–80%), together with neurons, fibroblasts, mast cells, and other cells. Plexiform neurofibromas are composed of the same cell types but have an expanded extracellular matrix and, often, a rich vascular supply. Schwann cells are considered to be the progenitors of neurofibromas. Recent data also support the possibility that whereas defects in one cell type (Schwann cells) might initiate tumorigenesis, other cell types may contribute to their development (15).

NF1-associated MPNSTs arise mainly within preexisting plexiform neurofibromas. These differences between dermal and plexiform neurofibromas suggest that the timing of somatic
NF1 mutations may determine the clinical course of these tumors, early mutations being associated with more aggressive tumor development and with malignant transformation. Little is known of the additional cooperating genetic events potentially required for full neurofibroma formation, and tumorigenic mechanisms of benign dermal neurofibromas and plexiform neurofibromas have rarely been compared (16). Finally, no clinical, pathological, or biological criteria predictive of the clinical course of plexiform neurofibromas have been identified to date.

To obtain additional insight into the molecular pathogenesis of plexiform neurofibromas, we used real-time quantitative reverse transcription-PCR (RT-PCR) assays to quantify the mRNA expression of 349 selected genes in pooled plexiform neurofibroma samples, in comparison with pooled dermal neurofibroma samples. Fifty-seven genes of interest were additionally investigated in 14 plexiform neurofibromas, in comparison with 22 dermal neurofibromas and 4 patient-matched MPNSTs.

**MATERIALS AND METHODS**

**Patients and Samples**

Tissue specimens were obtained from 27 patients with NF1 at Henri Mondor Hospital (Creteil, France). They included 22 dermal neurofibromas and 14 plexiform neurofibromas.

Dermal neurofibromas were obtained after laser excision from patients who did not develop plexiform neurofibromas. They affected the dermis and subcutis and were soft, slightly elevated, painless, and smaller than 20 mm.

Plexiform neurofibromas and MPNSTs were obtained after surgical excision. Plexiform neurofibromas, deep lesions involving a plexus of nerves, were large, and had a nodular aspect and severely deformed affected tissues. They immunostained positively for S100 and neurofilament protein.

The median follow-up of the 14 patients with plexiform neurofibromas was 35 months (range, 10–81). Four patients had developed MPNST, 2 patients at the plexiform neurofibroma surgery time and 2 patients 16 and 23 months, respectively, after the plexiform neurofibroma surgery. The clinical and histological characteristics (including S100 and neurofilament protein immunostaining) were identical between the 4 plexiform neurofibromas of the patients who did not develop MPNST and the 10 plexiform neurofibromas of the patients who did not develop MPNST.

Four patient-matched MPNSTs were used as “malignant” controls. The main clinical and histological characteristics of these 4 MPNSTs are shown in Table 1. The MPNST samples were also characterized by a very weak S100 immunostaining.

Immediately after surgery the tumor samples were flash-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

**Real-Time RT-PCR**

**Theoretical Basis.** Reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than the amount of PCR product accumulated after a fixed number of cycles. The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. The parameter threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence generated by cleavage of a TaqMan probe (or by SYBR green dye-amplicon complex formation) passes a fixed threshold above baseline. The increase in fluorescent signal associated with exponential growth of PCR products is detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA), using PE Biosystems analysis software according to the manufacturer’s manuals.

The precise amount of total RNA added to each reaction mix (based on absorbance) and its quality (i.e., lack of extensive degradation) are both difficult to assess. Therefore, we also quantified transcripts of two endogenous RNA control genes involved in two cellular metabolic pathways, namely TBP (GenBank accession no. NM.003394), which encodes the TATA box-binding protein (a component of the DNA-binding protein complex TFIIID), and RPLP0 (also known as 36B4; NM.001002), which encodes human acidic ribosomal phosphoprotein P0. Each sample was normalized on the basis of its TBP (or RPLP0) content.

Results, expressed as N-fold differences in target gene expression relative to the TBP (or RPLP0) gene, and termed “Ntarget,” were determined as $N_{target} = 2^{\Delta C_t \text{sample}}$, where the $\Delta C_t$ value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the TBP (or RPLP0) gene (17, 18).

The Ntarget values of the samples were subsequently normalized such that the mean of the dermal neurofibroma Ntarget values was 1.

**Primers and Controls.** Primers for TBP, RPLP0, and the 349 target genes (Supplementary Data) were chosen with the assistance of the Oligo 5.0 computer program (National Biosciences, Plymouth, MN).

We conducted searches in dbEST (database of Expressed Sequence Tags), htgs (High Throughput Genomic Sequences) and nr (the nonredundant set of GenBank, EMBL, DDBJ and
PDB database sequences) databases to confirm the total gene specificity of the nucleotide sequences chosen as primers and the absence of single nucleotide polymorphisms. In particular, the primer pairs were selected to be unique relative to the sequences of closely related family member genes or of the corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons, if possible. In general, amplicons were between 70 and 120 nucleotides long. Gel electrophoresis was used to verify the specificity of PCR amplicons.

**RNA Extraction.** Total RNA was extracted from frozen tumor samples by using the acid-phenol guanidinium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, the 18S and 28S RNA bands being visualized under UV light.

**cDNA Synthesis.** Total RNA was reverse transcribed in a final volume of 20 μl containing 1× RT buffer [50 μM each deoxynucleoside triphosphate, 3 mM MgCl2, 75 mM KCl, and 50 mM Tris-HCl (pH 8.3)], 20 units of RNasin RNase inhibitor (Promega, Madison, WI), 10 mM DTT, 100 units of Superscript II RNase H-reverse transcriptase (Invitrogen, Cergy Pontoise, France), 3 μM random hexamers (Pharmacia, Uppsala, Sweden), and 100 ng of total RNA. The samples were incubated at 20°C.
for 10 min and 42°C for 30 min, and reverse transcriptase was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

**PCR Amplification.** All of the PCR reactions were performed using a ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems) and the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). Ten μl of diluted sample cDNA (produced from 2 ng of total RNA) was added to 15 μl of the PCR master-mix. The thermal cycling conditions were composed of an initial denaturation step at 95°C for 10 min, and 50 cycles at 95°C for 15 s and 65°C for 1 min.

**Statistical Analysis**

As the mRNA levels did not fit a Gaussian distribution: (a) the mRNA levels in each subgroup of samples were characterized by their median values and ranges, rather than their mean values and coefficients of variation; and (b) relationships between the molecular markers and clinical and histological parameters were tested using the nonparametric Kruskal-Wallis (19) or Mann-Whitney U test (20). Differences between two populations were judged significant at confidence levels >95% (P < 0.05).

To visualize the capacity of a given molecular marker to discriminate between two populations (in the absence of an arbitrary cutoff value), we summarized the data in a receiver operating characteristic (ROC) curve (21). The area under the curve (AUC) was calculated as a single measure for the discriminatory capacity of each molecular marker. When a molecular marker had no discriminatory value, the ROC curve lies close to the diagonal, and the AUC is close to 0.5. In contrast, when a molecular marker has strong discriminatory value, the ROC curve moves to the upper left-hand corner (or to the lower right-hand corner) and the AUC is close to 1.0 (or 0).

Hierarchical clustering was performed using GenANOVA software (22). The data sets were rank-transformed before analysis by the Unweighted Pair Group Method of Analysis method (23).

**RESULTS**

The mRNA levels of the 349 candidate genes in the plexiform neurofibroma pool were first compared with the mean mRNA levels in the two pools of dermal neurofibromas, and genes displaying markedly different expression (> 4-fold) were selected for additional study. The mRNA expression of the genes thus identified was then determined in 14 plexiform

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**Table 2** List of the significantly altered expressed genes in the plexiform neurofibromas relative to the dermal neurofibromas

<table>
<thead>
<tr>
<th>Genes</th>
<th>Dermal neurofibromas (n = 22)</th>
<th>Plexiform neurofibromas (n = 14)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ROC-AUC&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOS</td>
<td>0.61 (0.15–6.30&lt;sup&gt;f&lt;/sup&gt;)</td>
<td>8.79 (1.90–344)</td>
<td>0.000553</td>
<td>0.904</td>
</tr>
<tr>
<td>JUN</td>
<td>0.83 (0.51–2.35&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.56 (1.28–11.7)</td>
<td>0.000075</td>
<td>0.896</td>
</tr>
<tr>
<td>CDKN1A/P21</td>
<td>0.70 (0.22–3.05&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.80 (0.83–59.9)</td>
<td>0.000111</td>
<td>0.886</td>
</tr>
<tr>
<td>TNFRSF10B/TRAILR2</td>
<td>0.97 (0.41–1.67)</td>
<td>1.62 (1.28–8.42)</td>
<td>0.000131</td>
<td>0.883</td>
</tr>
<tr>
<td>IL8</td>
<td>0.86 (0–7.80&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>62.9 (2.96–2493)</td>
<td>0.000281</td>
<td>0.864</td>
</tr>
<tr>
<td>NOS3</td>
<td>0.98 (0.25–3.00&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.83 (1.15–11.4)</td>
<td>0.000531</td>
<td>0.862</td>
</tr>
<tr>
<td>GRO1/CCNL1</td>
<td>0.54 (0–4.80&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.05 (0.04–99.0)</td>
<td>0.00101</td>
<td>0.828</td>
</tr>
<tr>
<td>PTGS2/COX2</td>
<td>0.74 (0.25–3.17&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.47 (0.41–83.6)</td>
<td>0.00151</td>
<td>0.818</td>
</tr>
<tr>
<td>EGR1/KROX24</td>
<td>0.53 (0.24–3.89&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.57 (0.17–23.4)</td>
<td>0.00171</td>
<td>0.813</td>
</tr>
<tr>
<td>SERPINE1/PAI1</td>
<td>0.68 (0.11–4.41&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.98 (0.36–35.6)</td>
<td>0.00211</td>
<td>0.808</td>
</tr>
<tr>
<td>JUNB</td>
<td>0.77 (0.19–5.57&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>3.76 (0.42–151)</td>
<td>0.00411</td>
<td>0.787</td>
</tr>
<tr>
<td>LIF</td>
<td>0.88 (0.35–7.87&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.83 (0.63–48.0)</td>
<td>0.00411</td>
<td>0.787</td>
</tr>
<tr>
<td>MYC</td>
<td>0.89 (0.40–2.18&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.73 (0.41–28.5)</td>
<td>0.00481</td>
<td>0.782</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>0.77 (0.26–3.22&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.60 (0.64–13.8)</td>
<td>0.00631</td>
<td>0.771</td>
</tr>
<tr>
<td>H19</td>
<td>0.50 (0.06–3.28&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.66 (0.13–93.2)</td>
<td>0.00991</td>
<td>0.759</td>
</tr>
<tr>
<td>SHC1</td>
<td>0.93 (0.55–2.46&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.17 (0.85–6.03)</td>
<td>0.0121</td>
<td>0.750</td>
</tr>
<tr>
<td>THBD/Thrombomodulin</td>
<td>1.04 (0.30–4.43&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.72 (0.65–22.3)</td>
<td>0.0134</td>
<td>0.748</td>
</tr>
<tr>
<td>THBS1/Thrombospondin 1</td>
<td>0.67 (0.31–2.76&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.49 (0.20–31.5)</td>
<td>0.0141</td>
<td>0.745</td>
</tr>
<tr>
<td>IGF2</td>
<td>0.66 (0.22–10.7&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.70 (0.58–68.5)</td>
<td>0.0164</td>
<td>0.742</td>
</tr>
<tr>
<td>NOS1</td>
<td>0.56 (0–7.23&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>4.82 (0.03–89.5)</td>
<td>0.0164</td>
<td>0.742</td>
</tr>
<tr>
<td>RASSF3</td>
<td>0.99 (0.14–11.0)</td>
<td>3.37 (0.37–12.1)</td>
<td>0.0164</td>
<td>0.740</td>
</tr>
<tr>
<td>IL1B</td>
<td>0.67 (0.08–2.76&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>8.91 (0.518)</td>
<td>0.0174</td>
<td>0.739</td>
</tr>
<tr>
<td>SHH</td>
<td>0.54 (0–14.3&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.31 (0–1464)</td>
<td>0.0184</td>
<td>0.737</td>
</tr>
<tr>
<td>GJA1/Connexin 43</td>
<td>0.80 (0.48–4.71&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.21 (0.49–76.7)</td>
<td>0.0184</td>
<td>0.737</td>
</tr>
<tr>
<td>TNF</td>
<td>0.69 (0.19–3.17&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.20 (0.41–13.9)</td>
<td>0.0204</td>
<td>0.732</td>
</tr>
<tr>
<td>FOSb</td>
<td>0.28 (0.03–3.80&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>6.84 (0.01–1007)</td>
<td>0.0224</td>
<td>0.729</td>
</tr>
<tr>
<td>TGFA</td>
<td>0.62 (0.08–15.1&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.63 (0.36–83.1)</td>
<td>0.0234</td>
<td>0.727</td>
</tr>
<tr>
<td>TNFRSF10A/TRAILR1</td>
<td>0.87 (0.13–2.51&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>1.37 (0.64–12.8)</td>
<td>0.0304</td>
<td>0.718</td>
</tr>
<tr>
<td>CEBPB/NF-IL6</td>
<td>0.85 (0–2.82&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>2.32 (0.19–18.1)</td>
<td>0.0434</td>
<td>0.703</td>
</tr>
<tr>
<td>IL6</td>
<td>0.64 (0.01–10.2&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>6.48 (0.03–8901)</td>
<td>0.0464</td>
<td>0.700</td>
</tr>
<tr>
<td>S100B</td>
<td>0.98 (0.44–1.68&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>0.77 (0.14–3.03)</td>
<td>NS</td>
<td>0.424</td>
</tr>
<tr>
<td>TPSB/Tryptase β</td>
<td>0.70 (0.32–4.38&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>0.96 (0.03–2.57)</td>
<td>NS</td>
<td>0.549</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kruskal-Wallis test.

<sup>b</sup> ROC, receiver operating characteristics; AUC, area under curve; NS, not significant.

<sup>f</sup> Median (range) of gene mRNA levels.
neurofibromas, in comparison with 22 dermal neurofibromas and 4 patient-matched MPNSTs.

Validation of the Methodology. Two different dermal neurofibroma pools and one plexiform neurofibroma pool were each prepared by mixing identical amounts of tumoral RNA from 4 patients. The means of TBP gene Ct values for the 4 tumor samples from each pool sample were 26.49 ± 0.29 (dermal neurofibroma pool 1), 26.30 ± 0.47 (dermal neurofibroma pool 2), and 26.23 ± 1.45 (plexiform neurofibroma pool).

Very low levels of target gene mRNA that were only detectable but not reliably quantifiable by real-time quantitative RT-PCR assays based on fluorescence SYBR Green methodology (Ct > 30), were observed for 40 (11.5%) of the 349 genes in both the plexiform and dermal neurofibroma pools.

We chose to express the results concerning the 309 gene expression levels in the plexiform neurofibromas relative to the dermal neurofibromas. Indeed, this latter type of neurofibroma is not at risk of developing malignant MPNSTs. Neurofibromas are heterogeneous benign tumors composed of Schwann cells, together with neurons, fibroblasts, mast cells, and other cells, and no "normal" tissue equivalent exists.

To test the reliability of our large-scale quantitative gene expression methodology using pooled RNA samples, we comparatively analyzed a second pool of dermal neurofibromas. Only 4 of the 309 (1.3%) quantifiable genes showed at least a 4-fold difference in expression between the two pools of dermal neurofibromas.

mRNA Expression of 309 Genes in the Pool of Plexiform Neurofibromas Relative to the Two Pools of Dermal Neurofibromas. The results for the 309 genes expressed in neurofibromas are summarized in Fig. 1, according to the relevant biological or cellular processes. Fifty-seven of the 309 (18.4%) genes were up-regulated in the plexiform neurofibroma pool as compared with the dermal neurofibroma pools (>4-fold increase); 29 (9.4%) genes showed 4–10-fold up-regulation, 23 (7.4%) 10–100-fold up-regulation, and 5 (1.6%) >100-fold up-regulation. None of the 309 target genes was down-regulated in the plexiform neurofibroma pool compared with the dermal neurofibroma pools. It is noteworthy, however, that several of these 309 genes were down-regulated in the MPNST samples (data not shown).

The 57 up-regulated genes mainly coded for transcription factors and growth factors or cytokines and their receptors (Fig. 1). Interestingly, no difference in expression between the dermal and plexiform neurofibroma pools was observed for NF1 or NF2, genes involved in cell proliferation (CCND1, CCNE1, CDKN2A/p16, ARF/p14, RB1, TOP2A, SFN, MKI67, and so forth) or genes specifically expressed in Schwann cells (PMP22, MPZ, L1CAM, S100B, and so forth) or mast cells (TPSB and CMA1).

mRNA Expression of the 57 Up-Regulated Genes in 14 Plexiform Neurofibromas and 22 Dermal Neurofibromas. The expression level of the 57 genes identified by the pooled sample analysis was then determined individually in 14 plexiform and 22 dermal neurofibromas. Thirty (52.6%) of the 57 genes were up-regulated in the 14 plexiform neurofibromas relative to the 22 dermal neurofibromas.

The capacity of each of these 30 genes to discriminate between plexiform neurofibromas and dermal neurofibromas was then tested by using ROC curve analysis. Comparison of overall diagnostic values of the 30 candidate molecular markers was assessed by the AUC value (Table 2). Fig. 2 shows the mRNA levels of the 5 most discriminatory genes, namely FOS (AUC-ROC, 0.904), JUN (AUC-ROC, 0.896), CDKN1A/p21 (AUC-ROC, 0.886), TNFRSF10B/TRAILR2 (AUC-ROC, 0.883), and IL8 (AUC-ROC, 0.864), in each neurofibroma sample.
The gene mRNA levels indicated in Table 2 (calculated as described in “Materials and Methods”) were based on the amount of the target message relative to the endogenous control TBP message, to normalize the starting amount and quality of total RNA. Similar results were obtained with a second endogenous control, the RPLP0 gene (also known as 36B4). Indeed, the same 30 genes (except SHC1 and IL6) were significantly up-regulated in the plexiform neurofibromas relative to the dermal neurofibromas.

Interestingly, we observed no difference in the expression of the cell type-specific markers S100B (a Schwann cell-specific gene) and TPSB (a mast cell-specific gene) between dermal and plexiform neurofibromas (Table 2). These results suggest that the observed up-regulation of the 30 target genes in plexiform neurofibromas was not due to an increase in one of the cellular components relative to the dermal neurofibromas.

### mRNA Expression of the 57 Up-Regulated Genes in 4 Plexiform Neurofibromas Associated with MPNST and in 10 Plexiform Neurofibromas Not Associated with MPNST.

Four of the 14 patients with plexiform neurofibromas had also developed MPNST. The mRNA levels of 12 genes differed significantly between the 4 plexiform neurofibromas associated with MPNST and the 10 plexiform neurofibromas not associated with MPNST (Table 3). It is noteworthy that all of these genes were up-regulated in the plexiform neurofibromas of the patients who developed MPNST relative to those who did not develop it.

Fig. 3 shows mRNA levels of the 5 most discriminatory genes, namely MMP9 (AUC-ROC, 1.000), FLT4/VEGFR3 (AUC-ROC, 1.000), TNFRSF10B/TRAILR2 (AUC-ROC, 0.975), SHH (AUC-ROC, 0.950), and GLI1 (AUC-ROC, 0.950) in each plexiform neurofibroma sample and in the 4 patient-matched MPNSTs. It is noteworthy that only MMP9 mRNA levels were higher in the MPNSTs than in the plexiform neurofibromas associated (or not) with MPNST; TNFRSF10B/TRAILR2 mRNA levels were similar in the two tumor types, FLT4/VEGFR3, SHH, and GLI1 mRNA levels were lower in the MPNSTs. These results ruled out the possibility that the observed up-regulation of the 5 genes in the plexiform neurofibromas associated with MPNST relative to the plexiform neurofibromas not associated with MPNST were due to contamination of the benign plexiform neurofibroma samples by malignant cells.

Finally, hierarchical clustering of the samples, based on the expression of these 5 genes (displayed as a dendrogram in Fig. 4), perfectly discriminated among the 10 plexiform neurofibromas not associated with MPNST, the 4 plexiform neurofibromas associated with MPNST, and the 4 MPNSTs.

### DISCUSSION

We used real-time quantitative RT-PCR assays to quantify the mRNA expressions of 349 selected genes in pooled plexiform neurofibroma samples in comparison with pooled dermal neurofibroma samples. Fifty-seven genes of interest were then investigated in 14 plexiform neurofibromas, 22 dermal neurofibromas, and 4 patient-matched MPNSTs. Using the same large-scale real-time RT-PCR approach, we have identified a molecular diagnostic index for hepatocellular carcinoma (24).

Real-time quantitative RT-PCR is a promising alternative to cDNA microarrays for molecular profiling of tumors. Real-time RT-PCR is far more precise, reproducible, and quantitative than cDNA microarray technology. Real-time RT-PCR is more useful for analyzing weakly expressed genes and for distinguishing among closely related members of gene families and among alternatively spliced specific transcripts.

We included 300 major genes involved in various cellular and molecular mechanisms associated with tumorigenesis and known to be altered (mainly at the transcriptional level) in various cancers. These genes encode proteins involved in cell cycle control, cell-cell interactions, signal transduction pathways, apoptosis, angiogenesis, and so forth (about 10–20 genes/pathway type; Supplementary Data). After scrutinizing the literature, we also included the majority of genes (n = 49) reported to be involved in neurofibromas and MPNSTs and genes expressed during Schwann cell differentiation (Supplementary Data; Ref. 25).

This analysis was by no means exhaustive, and many important genes were certainly missed, but it nevertheless dem-
onstrates the usefulness of real-time RT-PCR and identified several potentially useful marker genes.

We first studied total RNA pools from dermal and plexiform neurofibromas, which were prepared by mixing equal amounts of individual neurofibroma RNAs. The comparison of the results obtained with the two dermal neurofibroma pools (only 1.3% of genes were differently expressed between the two pools) and the comparison of pool values with the mean of individual values for the samples included in each pool clearly showed that RNA pooling was an appropriate initial screening approach, significantly limiting the required number of PCR experiments.

Comparative analysis of the dermal and plexiform neurofibroma samples yielded several interesting results. First, mRNA levels of cell-specific genes (e.g., S100B for Schwann cells and TPSB for mast cells) were similar in dermal and plexiform neurofibromas (Table 2), ruling out the possibility that the observed target gene up-regulations in plexiform neurofibromas were due to a difference in the abundance of a particular cell type relative to dermal neurofibromas. Second, most of the genes involved in cell cycle control (CCND1, CCNE1, CDKN2A/p16, ARF/p14, RB1, TOP2A, SFN, MKI67, and so forth) also showed similar mRNA levels in the two types of neurofibroma, suggesting similar cell proliferation rates, in agreement with Kindblom et al. (26). Third, mRNA levels of NF1 (and NF2) were similar in the dermal and plexiform neurofibromas, probably because the NF1 gene is expressed in the different cell components of the neurofibroma and the NF1+/− Schwann cells represent only a fraction of the total Schwann cell population in neurofibromas (27).

Among the 57 genes analyzed individually in each neurofibroma, we identified 30 genes showing significant differential expression between plexiform neurofibromas and dermal neurofibromas, suggesting that several signaling pathways are specifically involved in development of plexiform neurofibromas (Table 2). Cytokine/chemokine mRNAs, including interleukin (IL)8, GRO1/CXCL1, IL1B, IL6, tumor necrosis factor, and leukemia inhibitory factor, were overexpressed in plexiform neurofibromas. The expression patterns of IL8 and GRO1 were very similar (r = +0.789, P = 0.0008; Spearman rank correlation test). Because GRO1 is physically linked to IL8 in chromosomal region 4q12-q13 (28), our results support cotranscriptional regulation and/or 4q12-q13 amplification in plexiform neurofibromas. Our results point to a role of paracrine and autocrine signaling defects involving these cytokines/chemokines in the tumorigenesis of plexiform neurofibromas.

We also found up-regulation of most components of the AP1 transcription factor family, including FOSS, FOSSB, JUN, and JUNB (Table 2). AP1 has been implicated in a variety of tumorigenic processes, including cell transformation, invasive growth, angiogenesis, and tumor spread to distant sites (29). Additional studies are necessary to identify the downstream target genes of the altered AP1 pathway in plexiform neurofibromas. Other early growth-response genes (MYC and EGR1), which code for transcription factors, were up-regulated in plexiform neurofibromas. EGR1, also known as KROX24, plays a major role in the control of Schwann cell differentiation (30). Finally, two genes involved in apoptosis, TNFRSF10A/TRAILR1 and TNFRSF10B/TRAILR2, were up-regulated. These two genes code for tumor necrosis factor receptor family members that bind to tumor necrosis factor-related apoptosis-inducing ligand (31).

We attempted to identify markers of plexiform neurofi-

![MMP9](image1.png)

![FLT4/VEGFR3](image2.png)

![TNFRSF10B/TRAILR2](image3.png)

![SHH](image4.png)

![GLI1](image5.png)

Fig. 3 mRNA levels of MMP9, FLT4/VEGFR3, TNFRSF10B/TRAILR2, SHH, and GLI1 in 10 individual plexiform neurofibromas not associated with malignant peripheral nerve sheath tumors (MPNST; light gray bars), 4 plexiform neurofibromas associated with MPNST (dark gray bars), and 4 patient-matched MPNSTs (black bars). Median values (and ranges) are indicated for each tumor subgroup.
broma aggressiveness by comparing the gene transcriptional profiles of plexiform neurofibromas associated and not associated with MPNST. We identified a 5-gene expression signature predictive of aggressiveness (MMP9, FLT4/VEGFR3, TNFRSF10B/TRAILR2, SHH, and GLI1). Larger prospective series of plexiform neurofibromas must now be studied to confirm the prognostic value of this gene expression signature. Importantly, these genes are amenable to pharmacological modulation and may provide entry points for the design of novel specific therapeutics. For example, the new synthetic matrix metalloproteinase inhibitor (Ro 28–2653), with high selectivity for MMP9, could be considered for plexiform neurofibroma therapy (32).

MMP9 and FLT4/VEGFR3 code for molecules involved in angiogenesis. Other angiogenic genes found to be up-regulated in the plexiform neurofibromas tested here were not associated with aggressiveness (PTGS2/COX2, ANGPT2, and THBD/thrombomodulin; Table 2). These findings lend additional weight to ongoing preclinical studies of antiangiogenic drugs in this setting (33).

Interestingly, two other genes (SHH and GLI1) included in the gene expression signature code for the two most important molecules of the Sonic hedgehog (Shh)-Gli signaling pathway. Our results concerning this important signaling pathway, obtained at the mRNA level, are in agreement with the recent study by Endo et al. (34), who detected Shh protein (by immunohistochemistry) in plexiform neurofibromas, but not in dermal neurofibromas. Plexiform neurofibromas also showed marked up-regulation of H19 and IGF2 (Table 2), two major downstream target genes of Gli1 transcription factor (35, 36). The Shh-Gli signaling pathway is important in regulating patterning, proliferation, survival, and growth in embryos and adults (37). Inappropriate activation of the Shh-Gli signaling pathway occurs in several tumor types, including brain and skin tumors. It is noteworthy that Berman et al. (38) investigated recently the therapeutic efficacy of cyclopamine, a Shh signaling inhibitor, in preclinical models of medulloblastoma.

In conclusion, this study points to the involvement of several altered molecular pathways in the tumorigenesis of plexiform neurofibroma. Additional studies are necessary to elucidate the genetic (or epigenetic) mechanisms responsible for the dysregulated gene expression and to determine the cellular component(s; i.e., Schwann cells, fibroblasts, mast cells, or other cells) responsible for the altered expression of each gene in neurofibromas and MPNSTs. The identified genes may provide entry points for the design of novel specific therapeutics. Finally, we identified a gene expression signature of poor-prognosis plexiform neurofibromas, which warrants testing in larger series.

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REFERENCES

Molecular Profiles of Neurofibromatosis Type 1-Associated Plexiform Neurofibromas: Identification of a Gene Expression Signature of Poor Prognosis

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