Glmus Tumors in Neurofibromatosis Type 1: Genetic, Functional, and Clinical Evidence of a Novel Association

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Abstract

Neurofibromatosis type 1 (NF1) is a common disorder that arises secondary to mutations in the tumor suppressor gene NF1. Glomus tumors are small, benign but painful tumors that originate from the glomus body, a thermoregulatory shunt concentrated in the fingers and toes. We report 11 individuals with NF1 who harbored 20 glomus tumors of the fingers and 1 in the toe; 5 individuals had multiple glomus tumors. We hypothesized that biallelic inactivation of NF1 underlies the pathogenesis of these tumors. In 12 NF1-associated glomus tumors, we used cell culture and laser capture microdissection to isolate DNA. We also analyzed two sporadic (not NF1-) tumors, we used cell culture and laser capture microdissection to isolate DNA. We also analyzed two sporadic (not NF1-) tumors. For the first time, we show that loss of neurofibromin function is crucial in the pathogenesis of glomus tumors in NF1. Glomus tumors of the fingers or toes should be considered as part of the tumor spectrum of NF1. [Cancer Res 2009;69(18):7393–401]

Introduction

Neurofibromatosis type 1 (NF1) is a common (1/3,000), autosomal dominant disorder that arises secondary to mutations in the tumor suppressor gene NF1 (1). The protein product of NF1, neurofibromin, regulates RAS through its GTPase activating protein–related domain (2). Individuals with NF1 are at an increased risk for a variety of benign and malignant tumors. Biallelic inactivation (a “second hit”; ref. 3) of NF1 due to loss of heterozygosity (LOH) or somatic mutation is pathogenic in a variety of NF1-associated tumors (4).

Glomus tumors are benign neoplasms that arise from the glomus body, a specialized thermoregulatory shunt concentrated in the fingers and toes (5). Glomus tumors in the fingers or toes are distinct from adrenal and extra-adrenal paragangliomas, also called “glomus tumors” (6). The glomus body is a highly innervated structure containing an afferent arteriole, an anastomotic Suquet-Hoyer canal, and an efferent venule. The canal is surrounded by concentric layers of contractile α-smooth muscle actin (αSMA)–positive glomus cells. Heat-induced contraction of the glomus body causes closure of the arteriovenous anastomosis and forces blood flow through the capillary network of the distal phalanx, causing heat loss (7). Cold temperatures prompt relaxation of the glomus body, opening the anastomosis and conserving body heat.

Sporadic glomus tumors of the fingers are solitary and predominantly affect middle-aged women (5, 8, 9). Affected individuals present with a triad of severe paroxysmal pain, cold intolerance, and localized tenderness. The first association of NF1 and glomus tumors (at any location) was published in 1938 (10). To date, there are eight published cases of an NF1 association and glomus tumors of the fingers or toes in the English-language literature (11–15). There were no examples of multifocal glomus tumors in two large retrospective reviews of 86 sporadic cases (8, 9). However, of the eight individuals with NF1 and glomus tumors of the fingers or toes, seven harbored multiple tumors (11, 12, 14, 15), suggesting an association.

We hypothesized that biallelic inactivation of NF1 is pathogenic in NF1-associated glomus tumors (14). In this report, we searched for somatic NF1 mutations, loss of neurofibromin function, and dysregulation of the RAS mitogen-activated protein kinase (MAPK) pathway in glomus cells in NF1-associated and sporadic glomus tumors. We also investigated genome copy number changes using comparative genomic hybridization (CGH).

Materials and Methods

Patient Material

Studies were performed on 21 glomus tumors from 11 individuals with NF1. Fresh tissue was available from nine tumors and was used for primary...
cell cultures. The three glomus tumors from NF1-G10 were fixed in HistoChoice (an alcohol-based fixative; Sigma) prior to laser capture microdissection (LCM). Primary glomus cell cultures were also established from two tumors from two individuals without NF1. Skin fibroblast culture from a normal control was also available.

**Cell Culture**

Surgically excised glomus tumors were treated overnight with collagenase (160 units/mL) and dispase (0.8 units/mL) at 37° C. Glomus cells were grown to confluency in DMEM/F12 + 10% fetal bovine serum + penicillin + streptomycin and harvested.

**Immunocytochemistry**

For immunofluorescent staining, cells cultured from glomus tumors were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 10% fetal bovine serum. The cells were washed with PBS with 1% fetal bovine serum and incubated with mouse anti-α-SMA antibody (1:400; Sigma). Wash with PBS with 1% fetal bovine serum was followed by incubation with a fluorescent goat anti-mouse antibody (AlexaFluor 488, Invitrogen). Slides were mounted with mounting medium (Vectashield, Vector Laboratories) and cell nuclei were visualized with 4',6-diamidino-2-phenylindole.

**DNA Isolation from Paraffin-Embedded Tissue**

Eight-micron sections from the three glomus tumors from patient NF1-G10 were mounted onto PENT membrane slides (Zeiss) and stained with H&E. Lesional tumor cells were microdissected from surrounding stroma and vasculature with either the PALM II (Zeiss) or the PixCel II (Molecular Devices) LCM systems. The tissue fragments were digested in protease K and DNA extracted according to the instructions of the manufacturer (Picopure DNA extraction kit, Molecular Devices). For single nucleotide polymorphism-CGH (SNP-CGH), LCM material was resuspended in buffer containing 0.5 mol/L of Tris-HCl (pH 9.0), 0.5% SDS, and 5 mmol/L of EDTA. Protease K (Invitrogen) at 400 μg/mL was added, and samples were incubated at 55°C for 16 h. Samples were extracted with phenol/chloroform and DNA was precipitated. The DNA concentration was quantified using Picogreen (Invitrogen) and/or by spectrophotometer (Nanodrop ND-1000, Thermo Scientific) analysis at 260 nm.

**Whole Genome Amplification**

The DNA extracted from tumor nos. 1, 2, and 3 from patient NF1-G10 were subjected to whole genome amplification by either multiple displacement amplification with the Repli-G kit (Qiagen) or a PCR-based method with the GenomePlex kit (Sigma). The instructions of the manufacturer were followed with appropriate controls.

**Germ Line and Somatic NF1 Mutation Detection and LOH Analysis**

Germ line mutation screening of NF1 was performed on cDNA from puromycin-treated lymphocytes (16). Detected mutations were confirmed on genomic DNA. NF1 somatic mutation analysis was performed using the same technique on cell cultures derived from glomus tumors. Somatic NF1 mutation analysis was performed on whole genome amplification DNA from tumor nos. 1, 2, and 3 subjected to LCM from participant NF1-G10 as per published methods (17, 18). Somatic mutations of NF1 were confirmed on nonamplified tumor genomic DNA. Multiplex ligand-dependent probe amplification was used to assay exonic deletions (19). LOH analysis on DNA from cell cultures from glomus tumors (tumor no. 1, NF1-G4; tumor no. 1, NF1-G6; tumor no. 1, NF1-G7; tumor no. 1, NF1-G9) was performed by genotyping microsatellite markers telomeric to (SNP1-3, SNP1-1; ref. 20) and within NF1 (Alu, IVS27AC33.1, IVS38GT53.0, IVS27TG24.8; refs. 21–24).

**Biallelic Assignment of Somatic and Germ Line NF1 Mutations**

Germ line and somatic NF1 mutations may occur on the same or different alleles. In non–whole genome amplification tumor DNA from NF1-G10, SNPs rs2269855 and rs7350946 were coamplified by PCR with NF1 somatic mutation nos. 2 and 3; in germ line DNA from NF1-G10, SNP rs2265565 was coamplified with the NF1 germ line mutation (Table 1). The PCR products were subcloned into the TOPO-TA vector (Invitrogen), and transformed into DH5α cells, harvested, and sequenced. To create NF1 haplotypes, nine informative NF1 SNPs were genotyped in participant NF1-G10 and family members (Fig. 1). Assignment of status (wild-type or NF1 mutation) to the haplotypes was then determined by segregation analysis within the family.

**Bisulfite Modification and Human Androgen Receptor Assay Methylation-Specific PCR to Assess the Clonality of Glomus Tumors**

We used the human androgen receptor assay methylation-specific PCR (25), with minor modifications (Supplemental Table S1) to determine the clonality of the three tumors from NF1-G10 (26). Bisulfite modification was performed using the Epitect bisulfite modification kit (Qiagen) according to the instructions of the manufacturer. Bisulfite-modified DNA was used in the human androgen receptor assay methylation-specific PCR assay using AmpliTaq Gold 2× PCR mastermix (Applied Biosystems) in a thermal cycler (MJ Research). PCR products were analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems) using GeneMapper software (version 3.1, Applied Biosystems). Germ line DNA from NF1-G10 was used as a polyclonal control. As a monomorphic control, germ line DNA from three females with oculofaciocardiodental syndrome was used (27).

**RAS-MAPK Pathway Analysis of Glomus Cells**

Cell culture of glomus tumors [NF1-associated glomus tumor–derived glomus cells, NF1-associated glomus tumor–derived fibroblasts, sporadic (non-NF1) glomus tumor–derived glomus cells, and control fibroblasts (skin fibroblasts from an individual not affected with NF1)] were grown to confluency, starved overnight in serum-free medium and stimulated with acidic fibroblast growth factor (aFGF, 10 ng/mL; Sigma) for 5, 15, 30, 45, and 60 min. Cell lysates were analyzed by Western blot. DNA was also extracted from the NF1-associated glomus tumor–derived glomus cells to confirm the somatic and germ line NF1 mutations. Antibodies used for immunoblotting included anti–phosphorylated MAPK kinase 1 and 2 (MEK1/2; Cell Signaling Technology), anti-MEK1/2 (Santa Cruz Biotechnology), anti–phosphorylated extracellular signal-regulated kinase 1 and 2 (ERK1/2; Cell Signaling), and anti–β-actin (Sigma). Quantitative analysis of Western blot images was performed using Scion software (Scion Corp.). Experiments were performed in triplicate. Statistical significance was determined by multivariate repeated measures ANOVA.

**CGH Illumina HumanHap550 SNP-CGH**

Unmapped DNA (400 ng) microdissected from both glomus tumor nos. 1 and 3 and germ line DNA from NF1-G10 was hybridized to Illumina HumanHap550 Genotyping BeadChips (Illumina). Data was analyzed using Illumina BeadStudio software version 3.1 with genotyping module version 3.2.23. To assess LOH and copy number changes, we used the “LOH score” and “CNV partition” algorithms. The recommended thresholds for a significant LOH score is >5 and a confidence score of 100 (“DNA Copy Number Analysis Algorithms,” Illumina publication no. 970-2007-008, March 12, 2008). Genomic coordinates for all SNPs were derived from dbSNP build 129.

We also identified discordant loci by comparing SNP genotypes of tumor and corresponding germ line DNA at each SNP locus. For those SNPs with apparent LOH, we then identified the nearest centromeric and telomeric heterozygous (informative) SNPs to establish an interval with putative LOH.

**Agilent oligo Array-CGH**

Array-CGH using the Agilent Human Genome Microarray kit 244A (Agilent Technologies) was performed on tumor DNA from primary glomus tumor cell cultures from participants NF1-G1 (tumor no. 1), NF1-G3 (tumor no. 3), NF1-G8 (tumor no. 1), non–NF1-G1, and non–NF1-G2. The presence of a somatic NF1 mutation was confirmed in each culture of NF1-associated glomus tumor–derived glomus cells tested by array-CGH. Matching genomic DNA was available for the three tumors.

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from individuals with NF1. For the tumors from individuals not affected with NF1, we used gender-matched genomic DNA from a healthy control. Digestion, labeling, and hybridization were performed according to the instructions of the manufacturer ("Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis" protocol, v4.0, June 2006). Microarrays were scanned by the GenePix 4000B scanner (Axon Instruments, Molecular Devices) and analyzed by the Agilent Feature Extraction software (v9.5.1). Results were visualized by Agilent CGH Analytics software (v3.5.14).

Table 1. Study participants with glomus tumors of the fingers and toes

<table>
<thead>
<tr>
<th>Participant</th>
<th>Gender</th>
<th>Age</th>
<th>Finger/toe</th>
<th>Tumor no.</th>
<th>NF1 germ line mutation</th>
<th>NF1 somatic mutation</th>
<th>Mutation effect</th>
<th>Presenting clinical symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF1-G1</td>
<td>F</td>
<td>42</td>
<td>R F4</td>
<td>1</td>
<td>Partial skip exon 29</td>
<td>c.403delC</td>
<td>PTC</td>
<td>Progressive and localized pain for more than 1 y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R F5</td>
<td>2</td>
<td>—</td>
<td>NA</td>
<td>LOH at introns 27-38</td>
<td>Loss of wild-type NF1 allele</td>
</tr>
<tr>
<td>NF1-G2</td>
<td>M</td>
<td>35</td>
<td>R F3</td>
<td>1</td>
<td>c.7395_7404del10</td>
<td>NA</td>
<td>Loss of wild-type NF1 allele</td>
<td>Progressive and localized pain in distal phalanges for &gt;2 y, exacerbated by cold temperatures; also reddish discoloration at the nail bed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R F4</td>
<td>2</td>
<td>—</td>
<td>c.7395-2A&gt;G</td>
<td>NA</td>
<td>Progressive and localized pain in two phalanges</td>
</tr>
<tr>
<td>NF1-G3</td>
<td>F</td>
<td>53</td>
<td>R F4</td>
<td>1</td>
<td>c.2546delG</td>
<td>NA</td>
<td>LOH at introns 27-38</td>
<td>Loss of wild-type NF1 allele</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L F3</td>
<td>2</td>
<td>—</td>
<td>NA</td>
<td>LOH at introns 27-38</td>
<td>Loss of wild-type NF1 allele</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L F4</td>
<td>3</td>
<td>Fresh</td>
<td>c.5539_5546del8</td>
<td>Splice-site</td>
<td>Split nail and progressive and localized pain for 1 y</td>
</tr>
<tr>
<td>NF1-G4</td>
<td>M</td>
<td>57</td>
<td>R F4</td>
<td>1</td>
<td>c.2252-11T&gt;G</td>
<td>ND</td>
<td>Splice-site</td>
<td>Progressive and localized pain for &gt;5 y, exacerbated by cold temperature; mild distortion of the nail bed with increased curvature of the nails</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L F3</td>
<td>1</td>
<td>c.2256A&gt;G</td>
<td>NA</td>
<td>Splice-site</td>
<td>Progressive and localized pain in distal phalanx for 4 y, worse in cold temperature</td>
</tr>
<tr>
<td>NF1-G5</td>
<td>F</td>
<td>41</td>
<td>L F3</td>
<td>1</td>
<td>c.2451-2A&gt;T</td>
<td>c.3113+1G+C</td>
<td>ND</td>
<td>Progressive and localized pain in distal phalanx for 1–2 y in distal phalanx, worse in winter, with few cutaneous neurofibromas; segmental NF1</td>
</tr>
<tr>
<td>NF1-G6</td>
<td>F</td>
<td>36</td>
<td>R F3</td>
<td>1</td>
<td>c.2041C&gt;T(50%)*</td>
<td>ND</td>
<td>ND</td>
<td>Severe pain in distal phalanx for &gt;2 y and swelling of distal phalanx</td>
</tr>
<tr>
<td>NF1-G7</td>
<td>F</td>
<td>11</td>
<td>L F5</td>
<td>1</td>
<td>c.2304delT</td>
<td>ND</td>
<td>ND</td>
<td>Unexplained pain for many years; patient developed depression</td>
</tr>
<tr>
<td>NF1-G8</td>
<td>F</td>
<td>26</td>
<td>L F4</td>
<td>1</td>
<td>c.311T&gt;G</td>
<td>c.7727C&gt;A</td>
<td>PTC</td>
<td>Progressive and localized pain for 1–2 y in distant phalanx, worse in winter, with few cutaneous neurofibromas; segmental NF1</td>
</tr>
<tr>
<td>NF1-G9</td>
<td>F</td>
<td>29</td>
<td>hallux</td>
<td>1</td>
<td>c.1541_1542delAG</td>
<td>ND</td>
<td>ND</td>
<td>Pain for several years</td>
</tr>
<tr>
<td>NF1-G10</td>
<td>F</td>
<td>35</td>
<td>R F3</td>
<td>1</td>
<td>c.6789_6792delTTAC</td>
<td>ND</td>
<td>ND</td>
<td>Severe, debilitating pain in both hands for 5 y with chronic regional pain syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L F5</td>
<td>2</td>
<td>PE</td>
<td>c.204+1G&gt;A</td>
<td>Splice site</td>
<td>Severe, progressive pain in left hand for 20 y and right thumb for 5 y with complex regional pain syndrome in left hand and arm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L F4</td>
<td>3</td>
<td>PE</td>
<td>c.7600_7621del22</td>
<td>PTC</td>
<td>Severe, progressive pain in left hand for 20 y and right thumb for 5 y with complex regional pain syndrome in left hand and arm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F F4</td>
<td>4</td>
<td>—</td>
<td>NA</td>
<td>ND</td>
<td>Severe, progressive pain in left hand for 20 y and right thumb for 5 y with complex regional pain syndrome in left hand and arm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R F1</td>
<td>3</td>
<td>—</td>
<td>NA</td>
<td>ND</td>
<td>Severe, progressive pain in left hand for 20 y and right thumb for 5 y with complex regional pain syndrome in left hand and arm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R F4</td>
<td>4</td>
<td>—</td>
<td>NA</td>
<td>ND</td>
<td>Severe, progressive pain in left hand for 20 y and right thumb for 5 y with complex regional pain syndrome in left hand and arm</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; R, right; L, left; PE, paraffin-embedded; ND, not detected; NA, not analyzed; LOH, loss of heterozygosity; PTC, premature termination codon.

*Mosaic.
Quantitative PCR at the CRTAC1, GUCY1A2, and WASF1 Loci

Three loci (CRTAC1, GUCY1A2, and WASF1) with SNP-CGH evidence of copy number changes were evaluated with custom TaqMan PCR assays (Applied Biosystems) according to the instructions of the manufacturer. Unamplified genomic DNA (1 ng) isolated by LCM from glomus tumor nos. 1 and 3 and germline DNA (1 ng) were used in the quantitative PCR reactions. All reactions were performed in triplicate in a 7900HT Fast Real-time PCR instrument (Applied Biosystems). Relative amounts of DNA in each sample/locus were calculated using the standard “double delta” Ct method and expressed as a percentage of the DNA in the germline sample. Primers are listed in Supplemental Table S1. RNase P RNA component H (RPPH1) was used as a normalizing control. For each gene, a standard t test was used to test for the significance of differences in relative DNA amount between germ line and tumor samples.

Results

Clinical Characterization of NF1-Associated Glomus Tumors

We evaluated 11 individuals (three males and eight females; ages, 11-59 years; mean age excluding the child, 40 years) with signs and symptoms of NF1 with 21 pathologically confirmed glomus tumors of the fingers and toes (Table 1). One individual (NF1-G6) was diagnosed with mosaic NF1 (previously reported patient SNF1-1; ref. 28). The remaining 10 individuals fulfilled the consensus criteria for the diagnosis of NF1. The clinical data of two of these 10 individuals have been previously reported (NF1-G2 as “case 2” and NF1-G3 as “case 1”; ref. 14). A pathogenic germ line mutation in

Figure 1. Pedigree of NF1-G10 with phased 11 SNP NF1 haplotypes spanning ~203 kb (5’ end at top, 3’ end at bottom). SNP alleles subcloned with germ line and somatic mutations are in boldface. The maternal haplotype harboring the NF1 germ line mutation (c.6789_6792 del TTAC, subcloned with SNP rs2525565; in black) cosegregates with the NF1 affection status in the two affected sons (Son 1 and Son 2). The maternal haplotype harboring the two somatic NF1 mutations (light gray) is transmitted to the unaffected son (Son 3).
NF1 was identified in all individuals, with the exception of N1-G6 (mosaic NF1), for whom the mosaic NF1 mutation was found in neurofibroma-derived Schwann cells (28).

Five of the 11 individuals harbored multiple glomus tumors. Twenty of the 21 glomus tumors were located in the fingertips, with the exception of NF1-G9, whose tumor was located in the right hallux. Ten tumors were located in the right hand, and 10 in the left hand. The aggregate distribution of tumors was similar for both hands: F1/thumb (5%), F2 (5%), F3 (25%), F4 (50%), and F5 (15%).

NF1-Associated Glomus Tumors Show Typical Glomus Tumor Morphology

Histologic examination of all 21 tumors revealed small vessels surrounded by uniform cuboidal cells without cytologic atypia, necrosis, or increased mitotic activity (Fig. 2A). Immunostaining with αSMA showed uniformly positive cytoplasmic staining in the tumor cells (Fig. 2B).

NF1-Associated Glomus Tumors Arise Secondary to Somatic NF1 Mutations in αSMA-Positive Glomus Cells

We used two strategies to collect DNA from small glomus tumors, (a) fresh tissue culture to derive glomus cells and fibroblasts (nine tumors) and (b) LCM of paraffin-embedded tissue (three tumors).

In the cultured tumors, the glomus cells were αSMA-positive (Fig. 2B). In addition to the germ line mutations, five somatic mutations were identified in five of the nine tumors (Table 1). No NF1 mutations were detected in glomus cells or fibroblasts from two sporadic glomus tumors. In the three tumors subject to LCM, two different somatic mutations were identified in two tumors (Table 1) in addition to the germ line mutation. All seven somatic mutations are predicted to inactivate NF1. Using segregation analysis of NF1 SNP haplotypes harboring the NF1 germ line and somatic mutations in the pedigree of NF1-G10, we proved that the germ line and somatic mutations arose on separate copies of chromosome 17 (Fig. 1).

NF1-Associated Glomus Tumors are Monoclonal by Human Androgen Receptor Assay Methylation-Specific PCR

Because NF1-associated glomus tumors arise from biallelic inactivation of NF1, we reasoned that they should harbor evidence of a monoclonal expansion from a single cell. In three different glomus tumors from three fingers of one female (NF1-G10), a single allele was detected using the methylated- and unmethylated-specific primer pairs (Supplemental Fig. S1), consistent with monoclonal tumor expansion.

Biallelic Inactivation of NF1 in NF1-Associated Tumor-Derived Glomus Cells Increased Activation of the RAS-MAPK Pathway Compared with Other NF1-Associated Cells and Control Skin Fibroblasts

Because biallelic inactivation of NF1 was observed in NF1-associated tumor-derived glomus (αSMA positive) cells, we reasoned that MAPK pathway activity should be elevated when compared with NF1-associated glomus tumor–derived (αSMA negative) fibroblasts, sporadic glomus tumor–derived glomus (αSMA positive) cells, and control skin fibroblasts.

Consistent with the predicted effects of NF1 biallelic inactivation, we observed significantly higher MEK1/2 phosphorylation ratios in NF1-associated glomus tumor–derived glomus cells when compared with the three cell types described above at 5, 15, and 30 minutes after stimulation with aFGF (Fig. 3). The NF1-associated glomus tumor–derived fibroblasts showed less MEK1/2 activation in comparison to the NF1-associated glomus tumor–derived glomus cells but a higher activation at 5 and 15 minutes when compared with sporadic glomus tumor–derived glomus cells and control skin fibroblasts.

Similarly, we observed increased activation of ERK1/2 phosphorylation after stimulation with aFGF (Fig. 3). The maximum pERK/ERK ratio was detected in the NF1-associated glomus tumor–derived glomus cells 15 minutes after stimulation; the pERK/ERK ratios did not return to prestimulation levels even 60 minutes after stimulation. The NF1-associated glomus tumor–derived fibroblasts, sporadic glomus tumor–derived glomus cells,

Figure 2. A, photomicrograph of glomus tumor no. 1 from NF1-G7 showing a uniform population of tumor cells with rounded nuclei and eosinophilic cytoplasm. Note the perivascular arrangement of the tumor cells (inset). H&E staining, original magnification, ×250 (inset, ×400). Leica DMLB microscope. Bar, 15 μm. B, immunocytochemistry of glomus tumor–derived αSMA-positive cells from tumor no. 3 of NF1-G3. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue); αSMA-positive structures are green. Zeiss Axiophot fluorescent microscope. Bar, 15 μm.
and skin fibroblasts showed a similar but significantly lower increase in pERK/ERK ratios after stimulation with aFGF at all time points; 30 minutes after stimulation, the pERK/ERK ratio in these three cell types returned to prestimulation levels. Taken together, these data are consistent with the effects of \textit{NF1} biallelic inactivation on the MAPK pathway in NF1-associated glomus tumor–derived glomus cells (29, 30).

**Copy Number Changes at the CRTAC1 and WASF1 loci**

*Illumina HumanHap550 SNP-CGH and quantitative PCR with microdissected tumor DNA.* Supplemental Table S2 lists loci with evidence of copy number alterations in glomus tumor nos. 1 and 3 (from NF1-G10), but not in germ line DNA. All copy number variants (CNVs) detected were located at least 20 kb (often substantially more) from a known gene, except for four that were found to be within \textit{CRTAC1} (glomus tumor nos. 1 and 3), \textit{WDR78}, \textit{GUCY1A2}, and \textit{VPS13C} (glomus tumor no. 3 only).

Because the LOH score and CNV partition score are insensitive to the detection of copy number changes at a single SNP, we sought discrepancies in SNP genotypes between tumor and germ line samples. One SNP (rs4945851, intron 1 of \textit{WASF1}) was discrepant (Fig. 4). There was no evidence of a copy number change at the two closest flanking SNPs (rs6568634 and rs7761436), thus delimiting the size of the \textit{WASF1} putative deletion to \(\sim 10\) kb.

Due to severe limitations of the availability of tumor DNA, we performed quantitative PCR on \textit{CRTAC1}, \textit{WASF1}, and \textit{GUCY1A2}. In both \textit{CRTAC1} and \textit{WASF1}, we observed copy number changes consistent with those observed in the SNP-CGH data (Fig. 5). We were unable to confirm the homozygous deletion in tumor no. 3 of the \textit{GUCY1A2} locus.

**Agilent oligonucleotide array-CGH with DNA from the cell culture.** Oligonucleotide array-CGH analysis did not show copy number changes in cultured cells from the glomus tumors with proven \textit{NF1} inactivation (data not shown), more specifically, the loci identified by the Illumina platform on DNA extracted from paraffin-embedded tissues were normal. Similarly, the two glomus tumors from individuals not affected with \textit{NF1} and the non-\textit{NF1} control fibroblasts did not show any copy number alterations.

**Discussion**

In this report, we present the first genetic and molecular proof of an association of glomus tumors of the fingers and toes with \textit{NF1}. The mean age of adult participants in our study (40 years) is comparable to that of the sporadic glomus tumor population (9). However, one participant in our study (NF1-G7) was only 11 years old. We also observed multiple glomus tumors in 45% of our participants, a feature not observed in sporadic glomus tumors. In tumor predisposition syndromes such as \textit{NF1}, an early age of onset and the presence of multifocal tumors are evidence of an association.

Glomus tumors are small (typically \(<5\) mm). We used two techniques to obtain tumor DNA. We identified both germ line and somatic mutations in \textit{NF1} in six tumors; in a seventh tumor, we found an \textit{NF1} germ line mutation plus LOH (1/7 = 14%; 95%
confidence interval, 0.003–0.578). The rate of LOH we detected in glomus tumors is not significantly different from the expected 25% observed in neurofibromas (binomial distribution; ref. 31) due to our modest sample size. In two tumors, we showed that the wild-type chromosome harbored the somatic mutation (the “second hit” of Knudson’s two-hit hypothesis). Biallelic inactivation of NF1 is a common pathogenic mechanism of NF1-associated tumors. In four tumors from two individuals, four different somatic NF1 mutations were identified, suggesting that the multifocal NF1-associated glomus tumors arise from independent events. Presumably, NF1-nullizygosity arises in glomus cells secondary to mitotic DNA replication errors in NF1; this matters because glomus cells rely on neurofibromin-dependent RAS-MAPK–related growth factor cascades.

We also sought evidence of the functional consequences of the inactivation of NF1. Biallelic inactivation of NF1 in NF1-associated glomus tumor–derived glomus cells results in an increased activation of the MAPK pathway, as observed in other tumor cells with biallelic inactivation of NF1 (29, 30). Biochemical analysis of NF1-associated tumor-derived glomus cells (with germ line and somatic NF1 mutations) showed stronger and longer activation of the MAPK pathway after stimulation with aFGF when compared with NF1-associated tumor-derived fibroblasts (with a germ line NF1 mutation only), sporadic tumor-derived glomus cells (no NF1 mutations), and normal skin fibroblasts (no NF1 mutations). In three (of three) glomus tumors from three different fingertips from a single female, X-inactivation as detected by the human androgen

Figure 4. Comparison of log 2 R ratio of SNP rs4945851 (WASF1) and nearby SNP loci in glomus tumors no. 1 (log 2 R = −0.9) and no. 3 (log 2 R = −2.0) and germ line DNA (log 2 R = −0.3, normal) from NF1-G10. A: 0.45 Mb region (110,360,210–110,807,710 bp) surrounding rs4945851 (arrow, position 110,603,926 bp) harboring 82 SNPs on chromosome 6 (A–C; glomus tumor nos. 1, 3, and germ line sample). Locus rs4945851 and 10 adjacent SNPs (5 upstream and 5 downstream; red). The vertical axis is the log 2 R ratio of the intensity of the SNP–associated fluoros. D, the genomic position in increments of 4,475 bp, cytoband (6q21), and surrounding genes.
inactivation of a tumor suppressor gene (such as NF1) in a single cell (32).

We performed a genome-wide search for copy number changes in both cultured and paraffin-embedded tumor cells. In participant NF1-G10 tumor no. 1 (no somatic NF1 mutation identified) and tumor no. 3 (biallelic NF1 inactivation), quantitative PCR was consistent with a partial deletion of a portion of the 5′-untranslated region of WASF1 (Fig. 5). Interestingly, WASF1 forms a bidirectional gene pair with the 5′ CDC40. The bidirectional promoter of WASF1 and CDC40 is located within the putative deletions of both tumors from NF1-G10. Deletion of the bidirectional promoter may plausibly affect the expression of both WASF1 and CDC40. There is significant overrepresentation of bidirectional promoters associated with cancer-related genes (33); their role in benign tumors is unknown. WASF1 is down-regulated in ovarian cancer (34). There are no reported mutations in human CDC40, an orthologue of yeast CDC40, which is a controller of cell cycle arrest (35). Both WASF1 and CDC40 are candidates for further investigation in glomus tumors.

A ~50 kb amplification within CRTAC1 was also observed in both glomus tumors from NF1-G10. CRTAC1 encodes human cartilage acidic protein 1 and is useful in distinguishing chondrocyte-like, osteoblast-like, and mesenchymal stem cells in culture (36). The PRINTS database predicts a COOH-terminal antifreeze type I domain in CRTAC1. Antifreeze proteins were identified in polar fish as an adaptation to survive in hypothermic conditions preventing cell damage (37). In prolonged subzero cryopreservation, antifreeze proteins protect the heart from freezing, improve survival, and reduce apoptosis (38). Antifreeze domains are rare in the human genome. Their role in the pathogenesis of glomus tumors, derived from cold-responsive contractile glomus cells, is unknown.

Analysis of the cultured NF1 tumor-derived glomus cells by array-CGH did not show copy number alterations. It is possible that the cell culture procedure selected for glomus cells without copy number alterations.

Lastly, many neural crest–derived cell types are involved in NF1 (39). Three observations from our data support a neural crest origin for glomus cells and their cognate tumors. First, glomus cells are αSMA-positive; progenitor cells cultured from rat sciatic nerve suggest that neural crest stem cells generate αSMA-positive myofibroblasts (40). Second, the five NF1-associated tumor-derived cell cultures with a somatic NF1 mutation showed that only αSMA-positive glomus cells, and not tumor-derived fibroblasts, harbored somatic and germ line NF1 mutations. Third, the unusual phenotype of participant NF1-G6 is consistent with somatic mosaicism mainly confined to cells of neural crest origin (28). She had a mosaic NF1 phenotype, confirmed by molecular analysis: she presented with a glomus tumor, neurofibromas on the back and an intestinal ganglioneuroma but no freckling, learning disabilities, Lisch nodules, or localized hyperpigmentation. Mosaicism in NF1 arises from a postzygotic mutation of NF1 (41). In the case of NF1-G6, that mutation event likely occurred in the neural crest or a neural crest-derived cell, because both Schwann cells (neurofibroma) and intestinal ganglion cells (ganglioneuroma) are of neural crest origin. Accordingly, we hypothesize that glomus cells (glomus tumor) arise from myofibroblasts derived from neural crest stem cells.

In summary, we show that glomus tumors in NF1 arise secondary to biallelic inactivation of the tumor suppressor gene NF1 in αSMA-positive glomus cells. We observed that NF1-inactivated glomus cells show increased MAPK signaling. Taken together, these data prove that glomus tumors of the fingers are an integral part of the tumor spectrum of NF1. We hope that an increased awareness of these tumors will improve their early diagnosis and treatment in individuals with NF1.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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