Integrative genomics reveals frequent somatic NF1 mutations in sporadic pheochromocytomas

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Pheochromocytomas are neuroendocrine tumors of the adrenal medulla which can occur either sporadically or in the context of hereditary tumor syndromes. Whereas the genetic background of hereditary pheochromocytomas is becoming rather well-defined, very little is known about the more common sporadic form of the disease which constitutes ∼70% of all cases. In this study, we elucidate some of the molecular mechanisms behind sporadic pheochromocytoma by performing a comprehensive analysis of copy number alterations, gene expression, promoter methylation and somatic mutations in the genes RET, VHL, NF1, SDHA, SDHB, SDHC, SDHD, SDHAF2, KIF1Bβ, TMEM127 and MAX, which have been associated with hereditary pheochromocytoma or paraganglioma. Our genomic and genetic analyses of 42 sporadic pheochromocytomas reveal that a large proportion (83%) has an altered copy number in at least one of the known susceptibility genes, often in association with an altered messenger RNA (mRNA) expression. Specifically, 11 sporadic tumors (26%) displayed a loss of one allele of the NF1 gene, which significantly correlated with a reduced NF1 mRNA expression. Subsequent sequencing of NF1 mRNA, followed by confirmation in the corresponding genomic DNA (gDNA), revealed somatic truncating mutations in 10 of the 11 tumors with NF1 loss. Our results thus suggest that the NF1 gene constitutes the most frequent (24%) target of somatic mutations so far known in sporadic pheochromocytomas.

INTRODUCTION

Pheochromocytomas are neuroendocrine tumors arising from neural crest-derived cells in the adrenal medulla. They usually cause hypertension due to excessive production of catecholamines, and ∼10% of the tumors are considered to be malignant and give rise to distant metastases (reviewed in 1–3). About a third of pheochromocytomas are thought to be caused by hereditary mutations in any of the genes RET, VHL, NF1, SDHA, SDHB, SDHC, SDHD, KIF1Bβ, TMEM127 and MAX. However, the cause of the sporadic tumors, which constitute ∼70% of pheochromocytomas, is still largely unknown. As opposed to many other tumor forms (4,5), somatic mutations in any of the known familial disease genes appear to be rare (0–10% for the RET, VHL, SDHx and MAX genes) in sporadic pheochromocytomas (6–12), although some of the familial disease genes have not yet been investigated.

Previous microarray studies of genome-wide transcription patterns have revealed that hereditary pheochromocytomas and paragangliomas cluster into two distinct groups (8,13–15): VHL- and SDHx-related tumors share a similar gene expression profile linked to hypoxia and angiogenesis through a stabilization of hypoxia-inducible factor alpha, whereas RET-and NF1-related tumors express genes associated with an activation of kinase signaling pathways including the RAS/RAF/MAPK and the PI3K/AKT/mTOR cascades. Subsequently, hereditary tumors with KIF1Bβ (16), TMEM127 (17) and MAX (18) mutations have been demonstrated to cluster with the RET/NF1 group. Interestingly, several studies have

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shown that sporadic pheochromocytomas can cluster into either of the two distinct gene expression profile groups, but since somatic mutations appear to be rare, the molecular mechanisms behind this behavior are not yet understood (8,13−15). In addition, the same pattern has been observed when clustering pheochromocytomas based on the patients’ plasma or urine catecholamine levels, where the subgroups of sporadic tumors cluster with the VHL/SDHx or the RET/NF1 group, respectively (19).

In this study, we have investigated the roles of the known familial pheochromocytoma genes in sporadic pheochromocytomas by performing an extensive analysis of copy number alterations, gene expression, promoter methylation and somatic mutations in these genes. We present a comprehensive characterization of the familial disease genes in sporadic pheochromocytomas, and our results suggest that they have a more important role than has previously been thought.

RESULTS

Patients and tumors

This study included 42 pheochromocytomas that were diagnosed as sporadic in the absence of family history and syndromic features (Supplementary Material, Table S1), including careful examination to rule out neurofibromatosis type 1 (NF1) disease according to current diagnosis criteria (20). The samples were investigated for germline and somatic mutations in any of the genes RET, VHL, SDHA, SDHB, SDHD and MAX by Sanger sequencing of coding tumor DNA. No germline mutations were present, but one tumor (2.4%) had a somatic RET mutation (c.1893_1898delC-GAGCT) not present in the blood DNA of the patient. The in-frame deletion probably has an activating effect by changing the spacing between cysteine residues, and has been reported once previously (21). One patient had a nonsynonymous single nucleotide polymorphism (SNP) in SDHB (rs11203289, p.Ala3Gly) which was predicted to be non-pathogenic by bioinformatic analysis in Polyphen (22). Another patient had a nonsynonymous SNP in SDHD (rs11214077, p.His50Arg) which was predicted to be potentially damaging, but since the SNP has been found in ~3% of healthy individuals and no clear association with pheochromocytoma/paraganglioma has been identified (23), we did not exclude the sample from further analysis. It cannot be excluded that a minority of patients could carry mutations in genes not investigated. Of 21 samples which were analyzed with immunohistochemistry (including the one with the SDHB polymorphism), all showed positive staining of SDHB (Supplementary Material, Fig. S1), which has previously been demonstrated to characterize tumors without mutations in any of the SDHx genes (24−26).

Genome-wide copy number analysis reveals alterations in familial disease genes

We used high-density SNP microarrays to analyze 250,000 markers throughout the genome for copy number alterations in tumor DNA. We then investigated the copy number state of the genes that have so far been associated with susceptibility to pheochromocytoma and/or paraganglioma: RET, VHL, NF1, SDHA, SDHB, SDHC, SDHD, SDHAF2, KIF1Bβ, TMEM127and MAX, and could conclude that a number of these genes had frequently altered copy number, i.e. were affected by deletions or gains (Fig. 1 and Supplementary Material, data file). In total, as many as 35 of 42 samples (83%) had an altered copy number in at least one of the genes associated with familial pheochromocytoma. Several alterations were focal deletions covering only ~100–500 kbp (Supplementary Material, data file). In agreement with earlier studies using array comparative hybridization, we also detected a number of larger chromosomal alterations such as deletion of 1p (64% of the tumors), 3q (36%), 11p (31%) and 22q (31%) with similar frequencies as has previously been reported (27,28).

Heterozygous deletion of NF1 and other familial disease genes is associated with a reduced gene expression

For each of the familial disease genes, we used reverse transcription quantitative real-time PCR to compare the messenger RNA (mRNA) expression between the tumor samples with and without an altered copy number (SDHA, SDHC and TMEM127 were excluded due to no or few relevant copy number alterations). For NF1 (P < 0.00001), SDHAF2 (P = 0.0062), SDHD (P = 0.0066), KIF1Bβ (P = 0.019) and VHL (P = 0.045), we observed significantly lower mRNA levels in tumors with a loss of one gene copy compared with those with a normal copy number, which was particularly apparent for NF1 (Fig. 2). No significant difference was observed between tumors with and without loss of MAX (P = 0.14) and SDHB (P = 0.19), although the mean expression levels were somewhat reduced in tumors with gene loss (Supplementary Material, Fig. S2). With regard to RET, one tumor displayed gain and one displayed loss, but no differences could be seen at the mRNA level (Supplementary Material, Fig. S2).

Somatic inactivating NF1 mutations are frequent in sporadic pheochromocytomas

The loss of one NF1 copy in 11 of 42 sporadic pheochromocytomas, described in the ‘Patients and tumors’ section above (Fig. 1), in combination with a notably low mRNA expression of NF1 in the tumors with loss (Fig. 2), prompted us to screen for somatic NF1 mutations in the tumors. Due to the large size of the NF1 gene, we decided to sequence NF1 mRNA after conversion to complementary DNA (cDNA). The results revealed genetic alterations in 10 of the 11 tumors with NF1 loss: two nonsense mutations, three frame-shift mutations and five cases of abnormal splicing (Table 1; Supplementary Material, Fig. S3). The mutations, which were all predicted to result in a truncated protein, could be confirmed by targeted sequencing of the corresponding genomic tumor DNA (Supplementary Material, Fig. S4). Sequencing of blood or normal tissue DNA (in one case normal cells were obtained through microdissection) revealed that all mutations were somatic (Supplementary Material, Fig. S4). Most tumors displayed loss of the normal allele, but in two cases there was no sign of loss of heterozygosity.
in the sequence (Supplementary Material, Fig. S4), although mRNA expression was clearly reduced (Fig. 2; Supplementary Material, Table S1). Array copy number analysis showed that all the mutated tumors had a copy number of one, and the normal sequence may be due to the presence of normal cells and/or heterogeneity of the tumors.

Among the cases of abnormal splicing, one sample had a point mutation in the 5′ splice site of intron 15, resulting in skipping of exon 15, whereas another sample was missing both exons 15 and 16, due to a point mutation in the 3′ splice site of intron 15. A mechanism for a similar dual-exon skipping has previously been suggested (29). In two other samples, genomic DNA (gDNA) analysis would suggest point mutations to be missense (c.1466A>G, Tyr489Cys and c.1885G>A, p.Gly629Arg, respectively), but cDNA evidence shows that the mutations in fact cause the introduction of de novo splice sites resulting in partial skipping of exons 13 and 17, respectively, and bioinformatic analysis suggests that both novel splice sites are stronger than the corresponding natural splice sites. In another sample, a part of intron 13 was present between exons 13 and 14 in the cDNA. Analysis of gDNA revealed a point mutation deep within intron 13 (c.1527+1159C>T), giving rise to a strong de novo 5′ splice site which, together with an existing upstream cryptic 3′ splice site, results in the inclusion of a 54 bp long cryptic exon containing a stop codon (Supplementary Material, Fig. S5). cDNA sequencing yielded a mix between the wild-type and the mutated sequence, perhaps due to tumor heterogeneity, presence of normal tissue and/or nonsense-mediated decay (30) of the mutated mRNA. By means of TA cloning, the mutated and wild-type cDNA could be sequenced separately to show the presence of the cryptic exon (Supplementary Material, Fig. S5).

![Figure 1](link)

**Figure 1.** Copy number state of familial disease genes in 42 sporadic pheochromocytomas analyzed by SNP microarrays. Chromosomal losses (copy number = 1) are denoted by red color and chromosomal gains (copy number = 3) are shown in blue. An asterisk indicates a local alteration covering <500 kbp around the gene. For comparison, the copy number profile of a pheochromocytoma from an NF1 patient (number 12) is shown in the bottom row.
As a control, we carried out copy number analysis on a pheochromocytoma from a patient clinically diagnosed with NF1, which also displayed loss of NF1 (Fig. 1) and a decreased NF1 mRNA expression (Fig. 2). Upon sequencing, a splice-site mutation was detected in both tumor and blood DNA of the patient (Table 1; Supplementary Material, Fig. S6). No NF1 mutations were detected in 20 investigated sporadic tumors without loss of NF1. Three synonymous polymorphisms, rs1801052, rs2285892 and rs17881168, were detected, but their frequencies did not differ significantly from those of CEU HapMap (31) individuals.

Figure 2. Gene expression of NF1, VHL, SDHD, SDHAF2 and KIF1Bβ in pheochromocytomas. Expression is shown for samples with a loss of one gene copy (circles) and normal copy number (squares), respectively. One sample also had gain (three copies) of the SDHD locus (triangle). Relative mRNA expression was calculated based on the expression of two reference genes, GUSB and HPRT1. The difference in mean was tested using a two-tailed Student’s t-test. For NF1, the control pheochromocytoma from an NF1 patient with a germline mutation is denoted with an open circle for comparison (this sample was not included in P-value calculation for NF1 expression).

Somatic NF1 mutations correlate with biochemical phenotype

Tumors with somatic NF1 mutations were associated with higher plasma levels of normetanephrine ($P = 0.0050$) and metanephrine ($P = 0.0025$) than tumors without mutations (Fig. 3). No corresponding relationship was seen for urine measurements of catecholamines and their metabolites; however, these measurements were only available for a few tumors (Supplementary Material, Table S1) and have been suggested to be less sensitive (32). No relationship could be
seen between NF1 mutations and age, gender or malignancy, but there was a (nonsignificant, \( P = 0.098 \)) tendency of NF1-mutated tumors to be slightly larger (Fig. 3). One of 10 patients with somatic NF1 mutations had a malignant pheochromocytoma with metastases; compared with one of 32 patients without NF1 mutations; however, follow-up may be too short for most of the patients.

**Promoter methylation of familial disease genes is rare in sporadic pheochromocytomas**

All the genes associated with hereditary pheochromocytoma except RET are thought to function as tumor suppressors. Inactivation of both copies of a tumor suppressor gene is usually required for tumor formation (33), but the deletions discovered by microarray analysis were generally heterozygous. Since we ruled out somatic mutations in most of the familial disease genes, and only 10 of 11 samples with NF1 loss had somatic mutations, we analyzed the immediate promoter region for hypermethylation as an alternative mechanism of gene inactivation. The genes VHL, NF1, SDHB, SDHD, SDHAF2, KIF1B and MAX, which were subjected to heterozygous deletions, all have 5′-CpG islands covering their promoter regions. Using methylation-specific PCR of bisulfite-treated tumor DNA, we did not detect methylation in any of the familial disease genes. We did detect promoter methylation of HIC1 (hypermethylated in cancer 1) in 67% of the tumors, which is in agreement with a previous report (34), and can thus conclude that the absence of methylation in the other investigated genes is not due to a technical error during sample preparation (Supplementary Material, Fig. S7). However, methylation of CpG sites that we did not analyze cannot be excluded.

**DISCUSSION**

In this study, we used high-density SNP microarrays to investigate copy number alterations in sporadic pheochromocytomas, and show that a large proportion (>80%) of the sporadic tumors have copy number alterations in at least one of the known susceptibility genes for hereditary pheochromocytoma and/or paraganglioma. With regard to NF1, 26% of the sporadic tumors displayed loss of one gene copy, which correlated significantly with a reduced NF1 mRNA expression. Further, we could disclose somatic, truncating NF1 mutations in 91% of these tumors, of which a majority displayed loss of the wild-type allele in the tumor DNA sequence.

The NF1 gene encodes the protein neurofibromin, which is mainly expressed in the nervous system and suppresses cell proliferation by promoting the conversion of RAS into its inactive form, thereby inhibiting the RAS/RAF/MAPK signaling pathway (20,35). Germline mutations in NF1 are the genetic cause of NF1, a dominantly inherited tumor syndrome in which pheochromocytoma is a rare (up to 6%) manifestation (20). Despite this well-established role of NF1 in a subset of hereditary pheochromocytomas, the gene has not, until now, been examined for somatic mutations in the sporadic tumors, probably due to its very large size. Among a few other tumor types, large genomic characterizations have recently revealed somatic NF1 mutations in glioblastomas (36,37), lung adenocarcinomas (38) and ovarian carcinomas (39). A multiple sequence alignment of the neurofibromin amino acid sequence from different species shows several highly conserved regions throughout the protein, including the C-terminal part (Supplementary Material, Fig. S8). The mutations identified in this study are all predicted to truncate the protein, and are thus likely to abolish its structure and function. The mutation types and their distribution throughout almost the entire NF1 gene (Supplementary Material, Fig. S8) reflect the mutational spectra observed in patients with the NF1 syndrome (40–43). Four of the mutations have previously been reported in germline form (42–45), whereas the remaining six were not found in the literature or in the Ensembl, HGMD or COSMIC databases. Interestingly, 11 tumors displayed loss of one NF1 copy coupled with a notably low NF1 mRNA expression, but somatic mutations were only detected in 10 (91%) of these. This observation could be in agreement with an earlier study showing that
only 86% of NF1 patients carry intraexonic or splice-site germline NF1 mutations, whereas the remaining patients have exon deletions or duplications which could potentially be missed by our analysis approaches (40), or alternatively, it might be the result of a random deletion of the region. Our observation of increased plasma metanephrine levels associated with somatic NF1 mutations is in agreement with the high levels previously observed in NF1 patients with pheochromocytomas (46). Our study also suggests that normetanephrine levels are higher in patients with somatic NF1 mutations compared with sporadic pheochromocytomas in general, indicating both catecholamine metabolites as potential biochemical markers for NF1 mutations in pheochromocytomas.

We can also confirm previous results showing that somatic mutations in RET, VHL, SDHB, SDHD and MAX are rare in sporadic tumors: one of 42 tumors (2.4%) had a somatic RET mutation, whereas no mutations were detected in the other investigated genes. Further, we show that promoter methylation in any of the familial disease genes is infrequent in sporadic pheochromocytomas.

We also report frequent copy number alterations, primarily one-copy deletions, of familial disease genes other than NF1 in sporadic pheochromocytomas, of which deletions of VHL, SDHD, SDHAF2 and KIF1Bβ were also associated with a significantly lower expression of the corresponding mRNA. Some of the copy number alterations are likely to be passenger events rather than drivers of tumorigenesis. For example, gain of putative tumor suppressor genes such as SDHA and SDHC would appear likely to be passenger events, and the same could naturally be the case for other alterations. Some of the familial disease genes are also present on chromosomes that tend to have large deletions in pheochromocytomas, e.g. SDHB and KIF1Bβ on chromosome 1p (27,28), and deletions of those can thus in many cases be linked to the larger events where the driving genes are still unknown. Apart from the NF1 gene, no second hit in terms of mutations or promoter methylation could be detected to accompany the heterozygous loss. Even so, the fact that the copy number alterations often were focal may suggest that the familial genes are the targets of the events in some cases and that loss of one gene copy, resulting in decreased gene expression, may be enough to provide some
proliferative or survival advantage to the tumor cells. Several tumors displayed heterozygous loss of more than one of the genes involved in familial pheochromocytoma which could imply that some of them lack importance, or possibly that they could have a combined effect on tumor progression. It can, however, be noted that loss of NF1 and VHL were mutually exclusive in the sporadic pheochromocytomas (though the tumor from an NF1 patient did display loss of both genes), which would be in agreement with the two distinct gene expression patterns that have been described in sporadic pheochromocytomas (8,13–15). A loss of heterozygosity at the VHL locus has also recently been observed in sporadic pheochromocytomas displaying VHL-like gene expression profiles (8), suggesting that VHL loss may constitute part of the explanation for a ‘pseudo-VHL’ behavior of some sporadic tumors. In contrast to that study, however, we also observed that VHL loss had an effect on VHL gene expression itself. Further functional studies will be required to determine whether the different copy number alterations are of pathogenic importance.

In conclusion, our results suggest that the genes involved in hereditary pheochromocytoma may be important players also in the sporadic disease. Specifically, we show that somatic NF1 mutations are frequent in sporadic pheochromocytomas, 23.8% in this study. In consistence with Knudson’s two-hit model (33), we also show that most of these mutations are coupled with a loss of the wild-type allele. The NF1 gene thus constitutes the most frequent target of somatic mutations so far identified in pheochromocytomas, suggesting that neurofibromin deficiency in neuroendocrine cells of the adrenal gland accounts for a substantial portion of these tumors. Somatic NF1 mutations are also likely to be responsible for the NF1/RET-like gene expression patterns that have been observed in a subset of sporadic pheochromocytomas (8,13–15). Our results of frequent mutations and loss of heterozygosity of NF1 are further supported by a previous observation of frequent loss of the 17q11 region in pheochromocytomas (28). The outcome of our study affirms the usefulness of high-density copy number analysis as a tool in discovering genes involved in tumor development and shows that copy number analysis of NF1 can be used to uncover the presence of NF1 mutations in pheochromocytomas, and possibly in other tumor forms. The fact that almost one-third of the mutations were point mutations activating cryptic splice sites, one of which was located deep within an intron, stresses the importance of studying the NF1 gene both at DNA and mRNA level.

MATERIALS AND METHODS

Samples and nucleic acid isolation

This study included 42 pheochromocytomas operated between 1995 and 2011 at the Karolinska University Hospital, Stockholm, Sweden, the Linköping University Hospital, Linköping, Sweden and the Haukeland University Hospital, Bergen, Norway (Supplementary Material, Table S1). The patients were diagnosed with sporadic pheochromocytoma in the absence of syndromic features and family history. The study was started with 27 sporadic pheochromocytomas, for which all analyses were performed. Due to the findings of frequent NF1 alterations, 15 additional tumors (number 38–54 in Supplementary Material, Table S1) were included and analyzed for copy number, NF1 mRNA expression and (in case of NF1 loss) for NF1 mutations in order to confirm our initial findings. For comparison, a pheochromocytoma from a patient with the NF1 syndrome was also included in the study. The study was approved by the local ethic committees and an informed consent was obtained from all participants.

Tumors and corresponding blood samples were snap frozen in liquid nitrogen and stored at ≤ −70°C. DNA and RNA were isolated using the Maxwell 16 Tissue DNA purification kit (Promega) and the RNeasy Minikit (Qiagen), respectively. For two of the samples (7 and 20), the amount of tissue was not enough for RNA extraction, and these thus had to be excluded from expression analysis and cDNA sequencing.

Mutation analysis

Direct Sanger sequencing of RET, VHL, SDHB, SDHD and MAX was performed with previously described (6) or newly designed primers (Supplementary Material, Table S2). For each reaction, ~30 ng of tumor DNA (cDNA in the case of MAX) was PCR-amplified for 35 cycles using HotStar Taq Polymerase (Qiagen). PCR products were purified from unconsumed primers and dNTPs with ExoSAP-IT (GE Healthcare), labeled by dideoxynucleotide chain termination using BigDye Terminator 1.1 (Applied Biosystems) and separated by capillary electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems). Sequences were analyzed by alignment to the Ensembl sequence using the NCBI BLAST tool, as well as by visual inspection in Sequence Scanner v 1.0 (Applied Biosystems). The possible impact of nonsynonymous SNPs in SDHB and SDHD was tested using the Polyphen-2 software (22).

Immunohistochemistry

For 21 of the tumors (as described above) where enough tissue was available, we performed immunohistochemical staining of SDHB to exclude mutations in any of the untreated SDHx genes. Formalin-fixed, paraffin-embedded tissue sections mounted on glass slides were deparaffinized and rehydrated in xylene and a graded ethanol series, and antigen retrieval was carried out by microwave boiling in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Sections were then incubated in 0.3% H2O2 for 30 min, washed in Phosphate buffered saline and incubated with normal blocking serum for 20 min. Incubation with a primary antibody against SDHB (Abcam ab14714, diluted 1:1000) was carried out at 4°C overnight. The Vectastain Universal Elite ABC kit (Vector Laboratories) was used for subsequent staining according to the manufacturer’s recommendations, 3,3′-diaminobenzidine (Sigma) was used as a peroxidase substrate and hematoxylin (Vector Laboratories) was used for counterstaining. Sections from normal adrenal glands and a PGL4 (SDHB)-related pheochromocytoma were used as positive and negative controls, respectively. Omitting the primary antibody (negative antibody control) resulted in a complete absence of staining.
Copy number microarray analysis

Tumor DNA samples were genotyped and analyzed for copy number alterations using the Affymetrix GeneChip® Human Mapping Nsp 250 K Array according to the manufacturer’s protocol (Affymetrix). Data analysis was performed in Genotyping Console v. 4.0 using a reference of 48 CEU HapMap samples provided by Affymetrix. The BRLMM and CNAT algorithms with default settings were used for genotyping and copy number analysis, respectively. Copy number data were viewed and evaluated in the Genotyping Console Browser. A copy number alteration was called when ≥10 genetically adjacent markers showed the same copy number (≠2) and genetic positions of markers surrounding familial disease genes were double-checked using the Ensembl database. The presence of alterations too small for detection by this methodology cannot be excluded.

Quantitative mRNA expression analysis

For each sample, RNA integrity was validated by microfluidic analysis on a 2100 Bioanalyzer (Agilent Technologies) and RNA integrity numbers were between 6.6 and 9.4 (mean 7.9). Five hundred nano grams of total RNA was transcribed into cDNA with the Maxima First Strand cDNA synthesis kit (Thermo Scientific). Expression of RET, VHL, NF1, SDHB, SDHD, SDHAF2, KIF1Bβ and MAX mRNA was investigated by reverse transcription quantitative real-time PCR (RT-qPCR) on the 7900HT fast real-time PCR System (Applied Biosystems), using pre-developed exon-junction spanning primer-probe mixes (Supplementary Material, Table S3) and TaqMan® Fast Universal PCR Master mix (Applied Biosystems). GUSB and HPRT1 were used as reference genes and were analyzed simultaneously with the other genes for all samples. GUSB and HPRT1 were selected because they are stably expressed in several tumor forms (47) and had expression levels comparable with the other analyzed genes in our samples. In each reaction, we used cDNA transcribed from 20 ng of total RNA and all reactions were performed in duplicates. The normalized relative expression of each gene was calculated according to the established methods (48) as follows: First, the mean threshold cycle (Ct) value was calculated for each sample and gene by taking the mean of the PCR replicates. A normalized value (∆Ct) was calculated by subtracting the mean of Ct (GUSB) and Ct (HPRT1) from Ct (investigated gene), and finally the relative expression was obtained as 2^(-∆Ct). NF1 gene expression was analyzed in all 41 tumors where RNA was available (40 sporadic and one with a germline NF1 mutation), whereas the other genes were analyzed in 26 (25 sporadic and one with NF1) tumor samples.

cDNA sequencing of NF1

The coding region of NF1 cDNA, with parts of the 5′- and 3′-UTR, was divided into nine overlapping amplicons, for which the primers were designed (Supplementary Material, Table S2). Care was taken to avoid amplification of known pseudogenes and the primers were tested for specificity in silico using BiSearch (49). cDNA synthesis was performed as described above. Amplification was performed using HotStar Taq polymerase (Qiagen) with a touchdown PCR program (Supplementary Material, Table S2) and Sanger sequencing was performed as described above. All samples with NF1 loss and 20 samples without NF1 loss (the 20 samples without loss that were first included in the study) were sequenced. Mutations were confirmed by targeted sequencing of the involved exons in the corresponding genomic tumor DNA. Also, these primers were tested for specificity in silico to avoid amplification of pseudogenes. Normal DNA from blood (samples 9, 12, 25 and 44) or normal tissue/cells (samples 3, 10, 48, 51, and 54), was also sequenced. In two cases (47 and 53), germline NF1 mutations had already been excluded by analysis of blood of the patients (unpublished data).

Several transcript variants of NF1 have been described, of which type I (encoding a 2818 amino acid protein) and type II (encoding a 2839 amino acid protein, chosen as the canonical isofrom in Uniprot) are the two most well-characterized (50). When sequencing cDNA, we mainly detected type I. The type II sequence, containing an alternative exon of 63 nucleotides, was also detectable, to different extent, in all tumor samples but with a weaker intensity than the shorter variant (Supplementary Material, Fig. S9). Both sequences, which were detected as a mixed sequence in the fifth cDNA amplicon (Supplementary Material, Table S2), were read and blasted separately in the forward and reverse direction. Due to uncertainty in the mixed sequence, the alternative exon (exon 31) was also sequenced in gDNA in all samples (Supplementary Material, Fig. S9). No other splice variants were detected. The canonical variant (Ensembl accession ENSP00000358273) was used for all exon and mutation nomenclature.

Splice-site strength prediction

The strength of natural splice sites as well as cryptic splice sites activated by point mutations was estimated in silico by calculation of splice-site scores using the Analyzer Splice Tool (http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm), based on an algorithm developed by Shapiro and Senapathy (51), according to which a perfect match to the consensus 5′ sequence CAG/gtaagt or 3′ sequence tttttttttcag/G would give a score of 100. Using this method, the de novo splice sites created by the mutations c.1466A>G and c.1885G>A (Table 1) yielded scores of 75.26 and 94.93, respectively, which could be compared with the corresponding natural splice sites that generated scores of 74.27 and 87.68, respectively. The de novo 5′ splice site created by a mutation in intron 13 could also be shown to be strong (score 82.91), as could the already existing cryptic 3′ splice site (score 86.06).

Laser-capture microdissection

For one sample where no blood or normal tissue was available (sample 10), microdissection was performed to separate blood vessel cells from a tissue section of the tumor. The formalin-fixed, paraffin-embedded section was deparaffinized and weakly stained by hematoxylin (Vector Laboratories) as previously described (52). About 35 blood vessel cells were captured
(Supplementary Material, Fig. S10) using an Olympus Arcturus laser microdissection system (Arcturus Bioscience) and Arcturus CapSure macro LCM caps (Applied Biosystems). Caps with captured cells were incubated with 20 μl Proteinase K solution (0.1 mg/ml Proteinase K, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% Tween-20) at 55°C overnight followed by centrifugation at 900 g for 5 min and enzyme inactivation at 95°C for 8 min. PCR (37 cycles, using 3.5 μl of the isolated DNA in a 20 μl reaction) and Sanger sequencing were performed as described above. About 35 tumor cells were captured and analyzed as a control and were confirmed to contain the NF1 mutation, and 35 captures outside the tissue area were used as a negative control.

**TA cloning**

In the case of a somatic intronic mutation, resulting in the inclusion of a cryptic exon, the cDNA sequencing yielded a mixed sequence. After amplification (using primers defined in Supplementary Material, Table S2), the two PCR products were separated by electrophoresis on a 1.5% agarose gel (Supplementary Material, Fig. S5) and the shorter (wild-type) product could be punched out and sequenced directly. The punch from the longer product was contaminated with the shorter product and was, therefore, cloned into a pCR2.1-TOPO plasmid vector which was transformed into TOP10F’ competent *Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen) according to the manufacturer’s protocol. After overnight incubation on ampicillin lysogeny broth agar plates at 37°C, colonies containing the insert were selected by blue white screening. PCR amplification (30 cycles) and sequencing (as described above) were performed directly on the colonies using M13 primers, and resulted in a clean sequence of the abnormal cDNA (Supplementary Material, Fig. S5).

**Methylation-specific PCR**

Primers specific for methylated and non-methylated DNA were designed for the promoter regions of *NF1*, *SDHB*, *SDHD*, *SDHAF2*, *KIF1B* and *MAX* (Supplementary Material, Table S2 and Supplementary Material, Fig. S11) using the software MethPrimer (53). Primer specificity was tested using BiSearch (49). For the *VHL* promoter, previously published primers were used (54). Bisulfate conversion of 400 ng of tumor DNA was performed for 28 tumor samples (27 sporadic and one NF1 control) using the EZ DNA methylation kit (Zymo Research). Amplification was performed using HotStar Taq polymerase (Qiagen) under the following conditions: 95°C for 15 min, 38 cycles of (94°C for 40s, 56–60°C for 40s, 72°C for 40s), 72°C for 5 min and hold at 4°C (annealing temperatures were optimized for specificity for each primer pair). The PCR products were separated in 1.5% agarose gels containing ethidium bromide and visualized under ultraviolet illumination. Methylated, non-methylated and non-converted DNA from the EpitTect PCR Control DNA set (Qiagen) were used as controls. The *HIC1* gene was analyzed as a control, using methylation-specific PCR with previously published primers (55).

**Statistical analysis**

The relative gene expression (2^ΔCT) was compared between tumors with and without an altered copy number. The difference in means was tested using a two-tailed Student’s *t*-test.

Demographical and clinical information such as age at diagnosis, tumor size and biochemical phenotype was compared between patients with and without somatic *NF1* mutations, using two-tailed Student’s *t*-tests. The difference in benign/malignant histopathology between the groups was accessed using a two-tailed Fisher’s exact test.

Allele frequencies of the *NF1* polymorphisms detected by cDNA sequencing were compared with the reported population frequencies in CEU HapMap individuals (31) using a two-tailed Fisher’s exact test.

Differences were regarded as significant when showing a *P*-value < 0.05. Graphs were generated using the GraphPad Prism software (http://www.graphpad.com/prism/Prism.htm).

**Multiple sequence alignment**

The existence of conserved regions was investigated by comparing the amino acid sequence of human neurofibromin (UniProt accession number P21359) with those of *Mus musculus* (Q04690), *Danio rerio* (E7FFY1) and *Drosophila melanogaster* (O01397) using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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