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Neurofibromin 1 (*NF1*) Defects Are Common in Human Ovarian Serous Carcinomas and Co-occur with *TP53* Mutations[1,2]

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Abstract

Ovarian serous carcinoma (OSC) is the most common and lethal histologic type of ovarian epithelial malignancy. Mutations of *TP53* and dysfunction of the Brca1 and/or Brca2 tumor-suppressor proteins have been implicated in the molecular pathogenesis of a large fraction of OSCs, but frequent somatic mutations in other well-established tumor-suppressor genes have not been identified. Using a genome-wide screen of DNA copy number alterations in 36 primary OSCs, we identified two tumors with apparent homozygous deletions of the *NF1* gene. Subsequently, 18 ovarian carcinoma–derived cell lines and 41 primary OSCs were evaluated for *NF1* alterations. Markedly reduced or absent expression of Nf1 protein was observed in 6 of the 18 cell lines, and using the protein truncation test and sequencing of cDNA and genomic DNA, *NF1* mutations resulting in deletion of exons and/or aberrant splicing of *NF1* transcripts were detected in 5 of the 6 cell lines with loss of *NF1* expression. Similarly, *NF1* alterations including homozygous deletions and splicing mutations were identified in 9 (22%) of 41 primary OSCs. As expected, tumors and cell lines with *NF1* defects lacked mutations in *KRAS* or *BRAF* but showed Ras pathway activation based on immunohistochemical detection of phosphorylated MAPK (primary tumors) or increased levels of GTP-bound Ras (cell lines). The *TP53* tumor-suppressor gene was mutated in all OSCs with documented *NF1* mutation, suggesting that the pathways regulated by these two tumor-suppressor proteins often cooperate in the development of ovarian carcinomas with serous differentiation.

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Introduction

Ovarian epithelial cancer (OvCa) is the most lethal type of gynecologic cancer in much of the industrialized world. It is a morphologically and biologically heterogeneous disease. Morphological criteria define four major types of primary ovarian adenocarcinomas—serous, mucinous, endometrioid, and clear cell. Molecular studies have offered support for the notion that the different histologic types of OvCas likely represent distinct disease entities. Ovarian serous carcinoma (OSC) is the most common histologic type of epithelial OvCa, comprising roughly 70% of diagnoses and almost always presents at advanced stage [1]. Mutations of *TP53* and dysfunction of the Brca1 and/or

Abbreviations: OSC, ovarian serous carcinoma; OvCa, ovarian epithelial cancer; PTT, protein truncation test

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[2]This article refers to a supplementary material, which is designated by Table W1 and is available online at www.neoplasia.com.

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Brca2 tumor-suppressor proteins have been implicated in the molecular pathogenesis of a large fraction of OSCs [2–7].

However, relatively little is known about other somatic genetic alterations that are present in a substantial fraction of OSCs. Recent comprehensive sequence-based analyses of somatic mutations in potentially oncogenic kinases in OvCa suggest that a number of genes encoding kinases may each be mutated at low frequency in OSCs, such as the *LRRK2*, *STK36*, *ALPK2*, and *AKT1* genes (see http://www.sanger.ac.uk/genetics/CGP/Studies/Kinases/) [8,9]. In addition, despite the fact that the *TP53* gene is somatically mutated in 60% or more of OSCs and the *BRCA1* and/or *BRCA2* genes are inactivated by genetic and/or epigenetic mechanisms in more than of 80% of OSCs, prior studies have not offered supporting evidence for the notion that somatic mutations in other well-established tumor-suppressor genes, such as the retinoblastoma (*RB1*), neurofibromatosis type 1 or type 2 (*NF1* and *NF2*, respectively), Wilms tumor 1 (*WT1*), phosphatase and tensin homolog (*PTEN*), or the adenomatous polyposis coli (*APC*) genes, are common in OSCs.

Our initial genome-wide analysis of DNA copy number alterations in 36 primary OSCs identified two tumors with apparent homozygous deletions of the *NF1* gene and several additional tumors with suspected hemizygous loss of *NF1*. Prompted by this finding, we screened 18 OvCa cell lines and 41 primary OSCs for *NF1* alterations. We report here that mutational defects leading to reduced or absent Nf1 expression are common in the OvCa primary tumor specimens and cell lines analyzed and lead to Ras pathway activation. In addition, we found that all tumors with documented *NF1* alterations also harbored mutations of the *TP53* tumor-suppressor gene, suggesting the pathways regulated by these two tumor-suppressor proteins often cooperate in OSC pathogenesis.

Materials and Methods

Tumor Samples

Forty-one OSC samples were analyzed in this study, including 18 tumors from the Cooperative Human Tissue Network/Gynecologic Oncology Group Tissue Bank (Columbus, OH), 2 tumors from the New York Presbyterian Hospital (Weill Medical College of Cornell University), 11 tumors from the University of Michigan Health System, and 10 tumors from the Kumamoto University Hospital (Kumamoto, Japan). Primary tumor tissues were manually dissected with microscope guidance to ensure that each tumor sample contained a minimum of 70% tumor cells. Analysis of tissues from human subjects was approved by the University of Michigan's Institutional Review Board (IRB-MED 2001-0568 and 1999-0428).

Cell Lines

The OSC-derived cell lines SKOV-3 and CAOV3 [10] and TOV-21G (derived from a clear cell carcinoma) and the colorectal carcinoma cell line HCT116 were obtained from the American Type Culture Collection (Manassas, VA). Ovarian serous carcinoma cell lines HOC-1, HOC-7, HOC-8, and HEY [11,12] were a gift from L. Dubeau (USC School of Medicine, Los Angeles, CA). Ovarian carcinoma cell lines (histologic type not specified) A1847, A2780, OVCAR-4, OVCAR-5, OVCAR-8, and OVCAR-10 and OSC-derived cell lines PEO1 and PEO4 [13] were a gift from T. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Ovarian serous carcinoma cell lines OVCA420, OVCA429, OVCA432, and DOV13 [10] were a gift from D. Fishman (Northwestern University, Chicago, IL). IOSE-80 ovarian surface epithelial cells immortalized with SV40 large T antigen were a gift from N. Auersperg (University of British Columbia, Vancouver, Canada). All cell lines were maintained in DMEM with 10% FBS.

DNA, RNA, and cDNA Preparation

DNA, RNA, and cDNA were prepared using standard techniques. Briefly, genomic DNA was isolated from cultured cells or frozen tissue sections using SDS/proteinase K digestion followed by phenol/chloroform extraction. Total RNA was extracted from cultured cells or frozen tissue sections with Trizol (Invitrogen, San Diego, CA) according to the manufacturer's protocol. First-strand cDNA was synthesized from DNaseI-treated mRNA samples using random hexamer primers (Amersham Biosciences, Piscataway, NJ) and Superscript II (Invitrogen).

Representational Oligonucleotide Microarray Analysis and Data Analysis

Microarrays bearing 42,000 oligonucleotides designed to hybridize *Bgl*-II restriction fragments of human genome were custom-synthesized by Nimblegen Systems (Madison, WI) [14]. Briefly, tumor and normal human/male genomic DNA (1 μg each) were digested by *Bgl*-II enzyme (New England Biolabs, Ipswich, MA), and purified by QIAquick polymerase chain reaction (PCR) purification kit (Qiagen, Valencia, CA), followed by overnight ligation with the adapters (Bgl-12: gatctgctgcgt, Bgl-24: tcagcatcgagactgaacgcagca) to provide an anchoring site for subsequent PCR amplification to generate genome representations. Subsequent steps of genome representation amplification, array hybridization, and data acquisition/analysis were performed essentially as previously described [15]. The hybridized microarrays were scanned with an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA) with pixel size set to 5 μm.

Southern Blot Analysis

For Southern blot analysis, 10 μg of DNA was digested with *Eco*RI for 12 to 18 hours at 37°C. The digested DNA was separated in 0.8% agarose gels and transferred overnight to positively charged nylon membranes (Hybond; Amersham Biosciences). Hybridization was carried out in Rapid-hyb buffer (Amersham Biosciences) to [α-^{32}P]dCTP-labeled probes using standard procedures as described previously [16]. Three probes located at different positions within the *NF1* locus and a control probe on the same chromosomal arm (*DHX40* at 17q23.1) were used. The oligonucleotide sequences used to generate each probe are provided in Table W1.

Northern Blot Analysis

Ten micrograms of total RNA was separated in 0.9% agarose gels containing formaldehyde and transferred to Hybond-N+ membranes (Amersham Biosciences). *NF1* and *GAPDH* probes were generated by PCR and labeled with ^{32}P-dCTP with a random primer kit (Invitrogen). The *NF1*-specific probe corresponding to nucleotides 5999 to 6658 (NM_001042492) of the human *NF1* cDNA and the *GAPDH* control probe were amplified using the primer sequences indicated in Table W1. Northern blot hybridization to ^{32}P-labeled probes was carried out by standard methods. Membranes were exposed to phosphor-imager screens and scanned by phosphorimager

(Molecular Dynamics, Sunnyvale, CA). The arbitrary units for intensity of photon emissions represent the level of *NF1* mRNA normalized to *GAPDH*.

Protein Truncation Test

The protein truncation test (PTT) assay was performed as described by Heim et al. [17]. Briefly, 2 to 5 μg of mRNA was reverse-transcribed, and the entire *NF1* cDNA was analyzed using the TNT Quick Coupled Transcription/Translation system (Promega Corp., Madison, WI) in overlapping segments using five pairs of primers (Table W1). The RT-PCR products were transcribed *in vitro*, and then *in vitro* translation was performed in the presence of ^{35}S-methionine. The PTT samples were separated on 4% to 20% SDS-PAGE gels, which were dried and subjected to autoradiography. For each sample with aberrant PTT product(s), the corresponding regions of cDNA and, in most cases, genomic DNA, were amplified and sequenced. The primers used for amplification and sequencing of individual exons were those described by Schirinzi et al. [18].

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS] containing complete protease inhibitor cocktail (Roche, Indianapolis, IN). The protein concentration was analyzed by bicinchoninic acid assay (Pierce, Rockford, IL). Subsequently, 100 μg of each cell lysate was separated on a 4% to 20% SDS-PAGE gel and then transferred to Immobilon P membranes (Millipore, Bedford, MA) by semidry electroblotting. Western blot analysis was carried out using anti-Nf1 antibody at a 1:1000 dilution (sc-67; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Expression of β-actin was used as a loading control and was detected with anti-actin polyclonal antibody (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:1000. The horseradish peroxidase–conjugated goat anti–rabbit immunoglobulin (Pierce) was used at a 1:10,000 dilution. Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposure to X-Omat film (Kodak, Rochester, NY). The cell line HCT116 is known to have frameshift mutations in both *NF1* alleles and hence serves as a control for extracts lacking Nf1 expression [19].

Ras Activation Assay

Levels of activated Ras in selected cell lines were detected with a Ras activation assay kit (Upstate USA, Inc., Charlottesville, VA) per the manufacturer's protocol. Briefly, cells were starved in serum-free DMEM for 3 hours at 37°C. Cells were lysed, and the protein concentration was determined by the bicinchoninic acid method. A total of 250 μg of cellular lysate was incubated with Raf-1 RBD agarose (10 μl) at 4°C for 45 minutes. The agarose pull-downs were washed three times with lysis buffer, boiled with 2× Laemmli sample buffer, and separated on SDS-PAGE gels, followed by Western blot analysis using a monoclonal anti-Ras antibody at a 1:3000 dilution (Upstate USA, Inc.). To determine the levels of total Ras protein, 15 μg of total cellular lysates was electrophoresed on polyacrylamide gels followed by immunoblot analysis with the monoclonal anti-Ras antibody. Subsequently, the blot was reprobed with anti-actin antibody (Sigma-Aldrich) at a dilution of 1:1000.

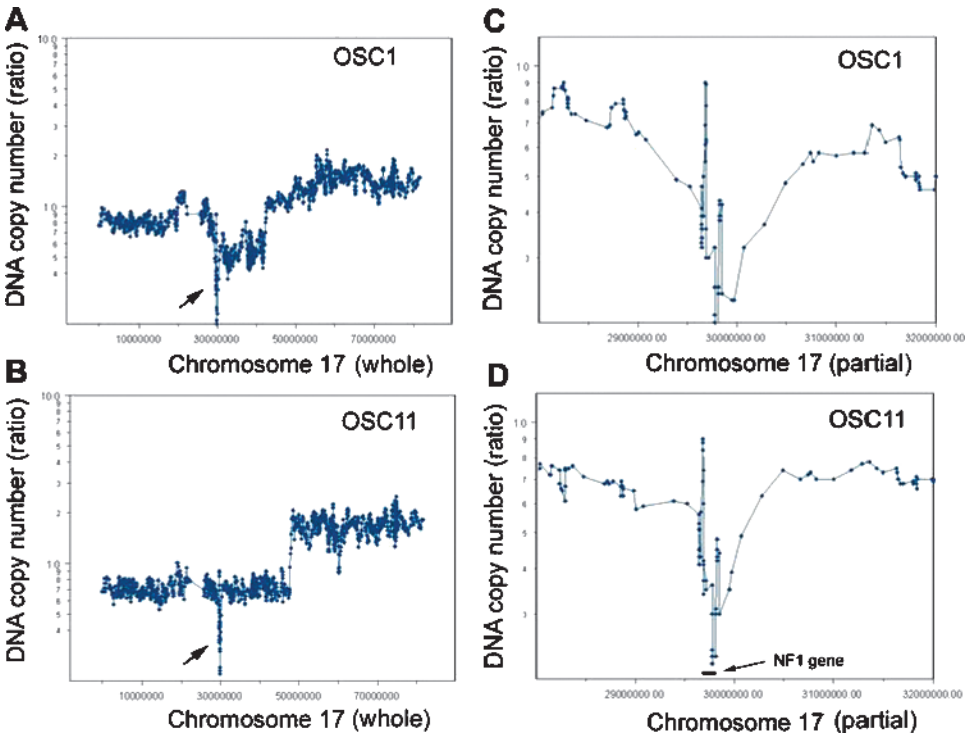

Figure 1. ROMA detects homozygous deletions including *NF1* in primary OSCs. Using a moving average algorithm of the window of five data points, the raw DNA copy number ratio of tumor over normal reference was smoothened and plotted against the chromosomal position of all the oligonucleotide probes on chromosome 17 for tumor samples OSC-1 (A) and OSC-11 (B). The arrows point at the apparent homozygous deletions including the *NF1* locus. To provide greater resolution of the deleted region, an area of 4 Mb across the *NF1* deletion is shown for OSC-1 (C) and OSC-11 (D). Location of *NF1* is as indicated in panel (D). Chromosomal positions of ROMA oligonucleotide probes were based on the NCBI build 34 (hg16 assembly).

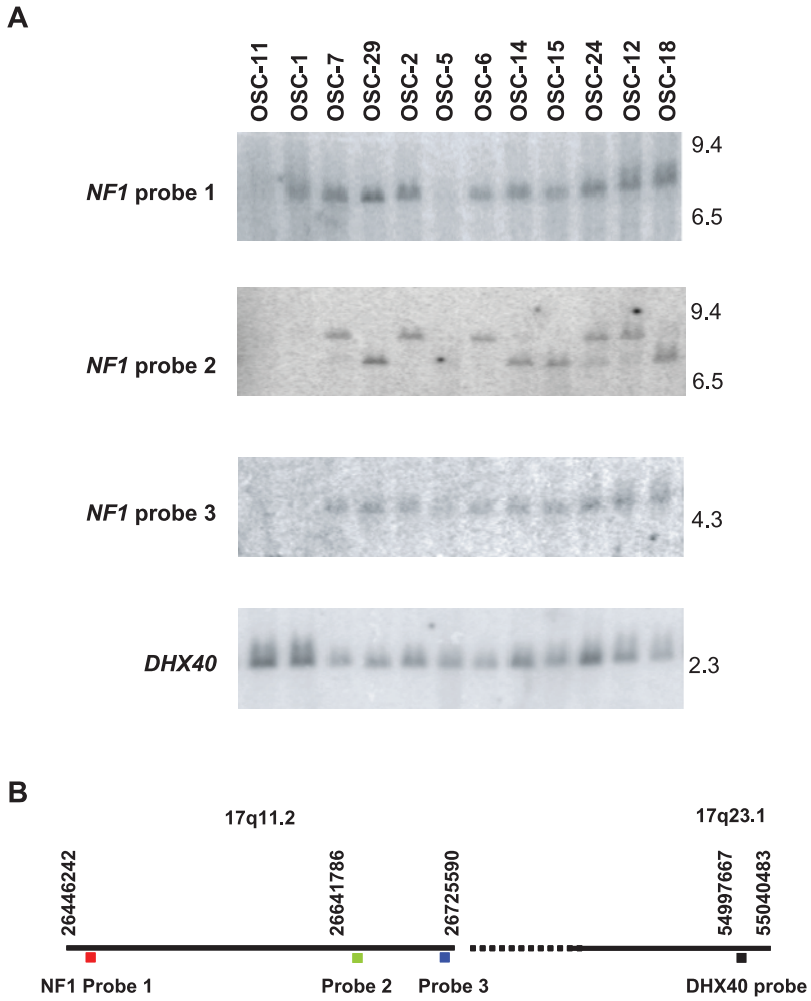

Figure 2. Southern blot analysis reveals homozygous deletions of *NF1* in primary OSCs. (A) Southern blot analysis of *Eco*RI-digested OSC DNA samples using three probes at different locations within the *NF1* gene. To control for loading, the blots were rehybridized with a probe for *DHX40*, which lies telomeric to *NF1* on chromosome 17q. (B) The relative location of each probe on chromosome 17 is shown in the diagram.

Immunohistochemical Analysis of p53 and pMAPK

Five-micrometer sections were cut from blocks of formalin-fixed paraffin-embedded tissue for routine staining with hematoxylin and eosin. Immunohistochemical staining was conducted using the avidin-biotin-peroxidase method. Sections were immunostained using rabbit polyclonal anti–active MAPK Pab at a 1:200 dilution (pTEpY; Promega Corp.) or with mouse monoclonal anti–TP53 antibody (1:100 dilution, clone D0-7; Invitrogen). Immunostaining for nuclear and/or cytoplasmic pMAPK was scored on a four-tiered scale for intensity (–, absent; +, weak; ++, moderate; and +++, strong). Immunostaining for nuclear TP53 was scored as negative (absent, weak, or focal) or positive (strong and diffuse).

Sequencing of TP53, KRAS, *and* BRAF

Mutational analyses of the *TP53* (exons 3-10), *KRAS* (exon 2), and *BRAF* (exons 11 and 15) genes were performed in the OSC samples using published and custom primer sequences as indicated in Table W1. Pearson's product moment correlation analysis (using the S-Plus statistical package) was used to measure the correlation between *TP53* and *NF1* mutations in the primary OSCs, and the Fisher's exact test was used to determine the significance of the association between mutations of these genes.

Results

A Subset of Primary OSCs Harbor Homozygous Deletions of the NF1 *Gene*

We pursued comprehensive analyses of DNA copy number alterations in 78 primary OvCas using a representational oligonucleotide microarray analysis (ROMA) approach. The full analysis of DNA copy number changes in OvCas will be presented elsewhere. However, 2 of 36 OSCs studied (OSC-1 and OSC-11) were found to show copy number changes consistent with potential homozygous deletions involving the *NF1* locus at chromosome band 17q11.2. Figure 1 shows moving average plots depicting the homozygous deletions identified by ROMA in these two tumors. To generate these plots, a moving window of five data points was used to average the raw DNA copy number along the whole chromosome 17. On the basis of the ROMA data, four additional tumors (OSC-2, OSC-5, OSC-24, and OSC-25) showed evidence for loss of one *NF1* allele. To confirm the presence of *NF1* homozygous deletions, we performed Southern blot studies of selected primary OSCs, including the tumors with apparent homozygous or hemizygous deletions of the *NF1* gene. The *NF1* gene is very large, spanning nearly 300 kb, with 57 common exons and at least 3 alternatively spliced exons. Three DNA sequence probes derived from widely spaced regions of the *NF1* locus were used for the Southern blot

analysis, and the *NF1*-specific hybridization intensities were compared to those for a control probe (*DHX40*), located telomeric to *NF1* on chromosome 17q. Genomic DNA from tumor OSC-11 did not show a detectable hybridization signal with any of the three *NF1* probes (Figure 2), suggesting that both chromosome 17q deletions in this tumor affected the entire *NF1* locus. OSC-1 showed no hybridization signal for probe 2 or 3 but showed hybridization to probe 1, whereas DNA from OSC-5 did not hybridize with probe 1 or 2 but retained *NF1* sequences detected by probe 3. These results indicate that, in tumor OSC-1, there was a homozygous deletion involving a telomeric region of the *NF1* gene (i.e., the 5′ end of the *NF1* gene), and in tumor OSC-5, there was a homozygous deletion of a more centromeric (3′) portion of the *NF1* gene. OSC-5 is one of the primary tumors suspected to have at least hemizygous deletion of *NF1* based on ROMA.

Reduced or Absent NF1 *Gene and Protein Expression in OvCa Cell Lines*

To explore the possibility that the *NF1* gene might be inactivated in a significant fraction of OSCs, we pursued analysis of *NF1* transcript and protein levels in a panel of 18 human OvCa cell lines. Twelve of the ovarian cancer cell lines are known to have been derived from OSCs [10–13]. The other six lines were derived from ovarian carcinomas of unspecified histologic subtype. However, given the frequency of OSC as a fraction of all OvCa diagnoses, many of the lines of unspecified type are likely to have been derived from serous tumors. *NF1* transcripts range in size from 11 to 13 kb owing to alternative splicing and differences in the extent of 3′ untranslated sequences [20,21]. On the basis of Northern blot analysis, *NF1* transcript levels varied significantly among the ovarian carcinoma cell lines, with high expression seen in OVCA420 and OVCA432 and minimal *NF1* gene expression seen in several other cell lines including DOV13, HOC-7, HOC-8, OVCA429, A1847, A2780, and CAOV3 (Figure 3*A*). To assess expression of the Nf1 protein in the OvCa lines, Western blot analysis with an antibody directed against a carboxyl-terminal region of the Nf1 protein was pursued (Figure 3*B*). The HCT116 colon carcinoma cell line is known to have frameshift mutations in both *NF1* alleles and hence serves as a negative control for Nf1 expression [19]. Of the 18 OvCa lines

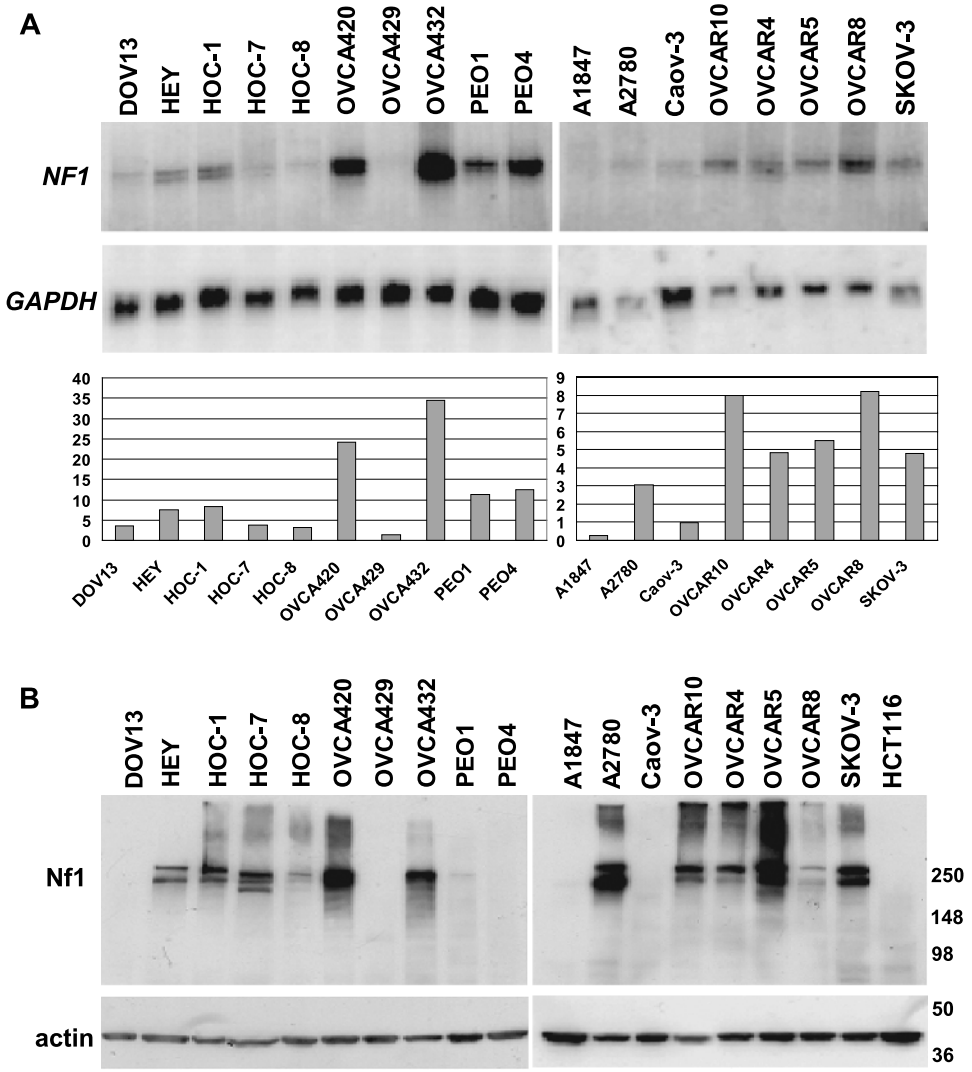

Figure 3. Expression of *NF1* is reduced or absent in several ovarian carcinoma cell lines. (A) *NF1* transcripts in the indicated cell lines were detected by Northern blot analysis, using expression of *GAPDH* as a loading control. Relative expression of *NF1* in each cell line normalized to *GAPDH* (arbitrary units) is indicated. (B) Nf1 protein levels in the same cell lines were determined by Western blot analysis using an anti–Nf1 antibody. Detection of actin was used as a loading control.

examined, five cell lines (DOV13, OVCA429, PEO4, A1847, and CAOV3) had essentially undetectable Nf1 protein expression. One additional cell line, PEO1, expressed only very minimal levels of Nf1. The findings suggest that perhaps more than one-third of OSCs might have mutational or epigenetic defects leading to *NF1* inactivation.

NF1 *Mutations Are Present in OvCa Lines with Reduced or Absent Nf1 Expression*

Protein truncation test assays were performed on the six OvCa lines (OVCA429, CAOV3, DOV13, PEO4, PEO1, and A1847) with low or absent Nf1 protein expression to ascertain a molecular basis for altered expression in the lines. cDNA was prepared from mRNA extracted after cells were cultured in the presence of puromycin, which inhibits nonsense-mediated decay of mRNA [22]. The entire *NF1* coding region was examined by *in vitro* transcription and translation in five overlapping fragments (segments I-V). We were able to detect aberrant PTT products in five of the six cell lines, either as altered sized RT-PCR fragments (data not shown) or as truncated peptide fragments in segments I, II, or III in the PTT assay (Figure 4*A*). The corresponding cDNA and genomic DNA fragments from each cell line were cloned and sequenced (data summarized in Table 1). The cell line OVCA429 shows an aberrant PTT product in segment II, and the corresponding cDNA and genomic DNA both show a 17 base pair (bp) deletion in exon 17. The CAOV3 line has an aberrant PTT product in segment III, and sequencing of the corresponding cDNA revealed deletion of exons 22 and 23-1 and inclusion of 102 bp from the intron downstream of exon 23-2. We were unable to amplify these exons from genomic DNA by PCR, but amplification using adjacent intron primers revealed deletion of 1448 bp from this region. The A1847 line also showed an aberrant peptide in segment III, and the cDNA had an insertion of 101 bp from the intron downstream of exon 23-2. Sequencing of genomic DNA from A1847 revealed a single nucleotide change (C to G) within this cryptic exon, but the mechanism by which this change may lead to inclusion of intronic sequences in the *NF1* transcript is not clear. DOV13 has a splicing mutation at the +1 position of exon 23-1, resulting in deletion of exon 23-1 from the cDNA. A germ line *NF1* mutation identical to the presumed somatic mutation in DOV13 has been reported earlier [23]. In the cell line PEO4, PCR amplification of cDNA segment I yielded a RT-PCR product that was smaller than expected (data not shown). Our inability to detect a corresponding truncated protein fragment in the PTT assay is likely caused by the very small size of the predicted protein product (≈12 kDa). Sequencing of the cDNA revealed deletion of the entirety of exon 4a, and at the genomic level, mutation at the splice junction of exon 4a (–2, acceptor site, A to T), which leads to the skipping of this exon. Mutation at the same site in this splice junction (A to G) has also been reported previously [24]. The PTT assay failed to detect any mutations in PEO1 cells. This result is perhaps not unexpected, because a previous study that screened for *NF1* germ line mutations using PTT only had a combined mutation detection rate of 56% [25]. Overall, our studies provide evidence for Nf1 inactivation in 6 (33%) of 18 OvCa lines evaluated, with definitive inactivating mutations identified in 5 of the 6 lines.

NF1 *Mutations Are Present in Primary OSCs*

We screened a collection of 41 OSCs (including 29 of the 36 OSCs evaluated by ROMA) for *NF1* mutations using the PTT assay. Aberrant PTT products affecting segment III, IV, or V were detected in six tumors (Figure 4*B* and Table 1). Sequencing of the cDNA corresponding to the aberrant PTT products identified aberrant transcripts in all six cases. cDNA from two of the tumors, OSC-2 and OSC-35, showed duplications of exons 24 to 27b (662 bp), whereas OSC-25 has deletion of exons 22 and 23-1. OSC-24 and OSC-32 showed deletions of exons 30 and 31, respectively. Finally, OSC-18 had an insertion of 178 bp corresponding to the entire intron between exons 44 and 45. Chromatograms of *NFI* cDNA sequence alterations in representative cell lines and primary tumors are shown in Figure 5. Genomic DNA isolated from OSC-24 revealed a deletion of 34 nucleotides

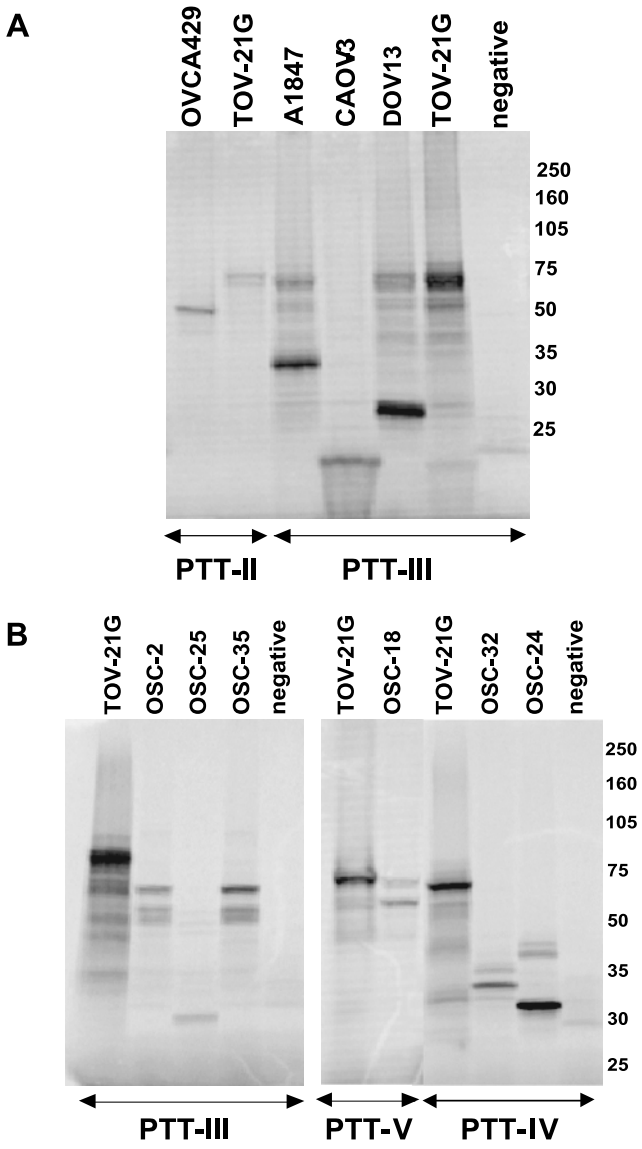

Figure 4. The PTT assay shows truncated Nf1 peptides in ovarian carcinoma cell lines and primary tumors. (A) PTT was performed using five overlapping cDNA segments encompassing the entire *NF1* coding region. Representative examples of truncated peptides in segments II and III from the indicated cell lines are shown. The TOV21G cell line expresses normal levels of full-length *NF1*, and it was used to determine the size of the expected (normal) PTT products in each segment. (B) Representative examples of truncated peptides in Segments III, IV and V from the indicated OSC samples are shown. The TOV21G cell line expresses normal levels of *NF1*, and it was used to determine the size of the expected (normal) PTT products in each segment.

Table 1. *NF1* Mutations Detected in OvCa Cell Lines and Primary Tumors by PTT.

ID	cDNA Sequencing (NM_001042492, ATG as +1)	Genomic DNA Sequencing	Protein Change	Mutation Type
PEO4	Del* c[†]291-481 (exon 4a)	A478T (first nucleotide of exon 4b, NM_001042492)	aa 97, PTC[‡] at 109	Splicing
CAOV3	Del c3711-3977 (exons 22 and 23-1), c4110ins[§]102 bp	Del 1448 bps (exons 22 and 23-2 and partial introns 21 to 23-2)	aa1237, PTC 1245	deletion/splicing
OVCA429	Del c2943-2959 (exon 17)	Del 2943-2959 (NM_001042492)	aa 981, PTC 981	deletion
A1847	c4110 Ins101 bp from intron 23-2	C-G intron 23a (at 304314067 NT_010799)	aa1371, PTC 1379	Splicing
DOV13	del c3873 -3977 (exon 23-1)	3974 (exon 23-1) + 1 (G to T) (NM_001042492)	aa1291, PTC 1298	splicing
OSC-2	Duplication 662 bp (exons 24-27b)	No mutation detected	aa 1370, PTC 1379	splicing
OSC-18	Ins 178 bp (exons 44-45)	No mutation detected	aa 2602, PTC 2609	splicing
OSC-24	Del c5549-5751 (exon 30)	Del 34 nucleotides 5′ exon 30, Ins ATG	aa 1849, PTC 18451	deletion/splicing
OSC-25	Del c3711-3977 (exons 22 & 23-1)	No mutation detected	aa 1237, PTC 1245	splicing
OSC-32	Del exon 31	No mutation detected	aa 1916, PTC 1921	splicing
OSC-35	Duplication 662 bp (exons 24-27b)	No mutation detected	aa 1370, PTC 1379	splicing

*Del = deletion.
[†]Sequence changes identified in cDNA are indicated by "c" before the number.
[‡]PTC = premature termination codon.
[§]Ins = insertion.

encompassing the 5′ splice site of exon 30, with an insertion of ATG in its place. Genomic DNA sequencing of the corresponding *NF1* exons and exon-intron boundaries in the other tumors failed to reveal mutations. This might reflect the likelihood that a significant fraction of mutations that alter appropriate splicing of *NF1* transcripts are distant from the intron-exon boundaries; these mutations presumably create novel splice sites or activate cryptic splice sites within exonic or intronic sequences [26]. Alternatively, splicing errors have also been shown to occur in the absence of identifiable sequence alterations in the *NF1* gene, with tumors showing nearly twice the amount of aberrant transcript as normal tissues [27]. Overall, we were able to document *NF1* alterations in 9 (22%) of 41 primary OSCs, including three tumors with homozygous deletions of sizable portions of the *NF1* gene and six tumors with localized mutations leading to premature truncation of the Nf1 protein. Three of these six tumors also showed evidence for hemizygous loss of *NF1* based on ROMA.

The Ras Pathway Is Activated in Ovarian Carcinoma Cell Lines and Primary OSCs with NF1 *Mutations*

The Nf1 (neurofibromin) protein is a 2839 amino acid polypeptide, with a domain homologous to the catalytic domain of GTPase activating proteins (GAPs) [28]. This domain, called the GAP-related domain (GRD), regulates Ras activity by accelerating the conversion of GTP-bound "active" Ras to its inactive GDP-bound form [29]. Previous studies have shown that reduced or absent Nf1 protein expression in neurofibrosarcoma cell lines results in high levels of the active GTP-bound form of Ras (Ras-GTP) [30]. To determine whether *NF1* mutations and/or concomitant reduction of Nf1 expression are associated with increased levels of active Ras-GTP in OSCs, an assay for activated Ras was performed in representative cell lines. Cell lines with markedly reduced or absent Nf1 expression (PEO4, A1847, CAOV3, DOV13, and OVCA429) had higher Ras-GTP levels compared to the cell lines with robust expression of Nf1 protein (Figure 6*A*). One OvCa line, HEY, which showed readily detectable levels of Nf1 protein (Figure 3*B*), also manifested high levels of active Ras-GTP.

A major consequence of Ras pathway activation is the phosphorylation of MAPK [31]. Hence, we examined our collection of primary OSCs for the presence of active MAPK by immunohistochemistry using an antibody directed against the active phosphorylated form of p44/42 MAPK. As shown in Figure 6*B* and summarized in Table 2, eight of nine primary OSCs with documented *NF1* alterations showed strong (++ or +++) pMAPK expression. Among the remaining OSCs studied, ~41% (12/29) also stained positively for pMAPK. In these tumors, *NF1* mutations may have been missed by our detection approach or MAPK may be activated by an alternative mechanism, such as activating mutations of upstream signaling components, including

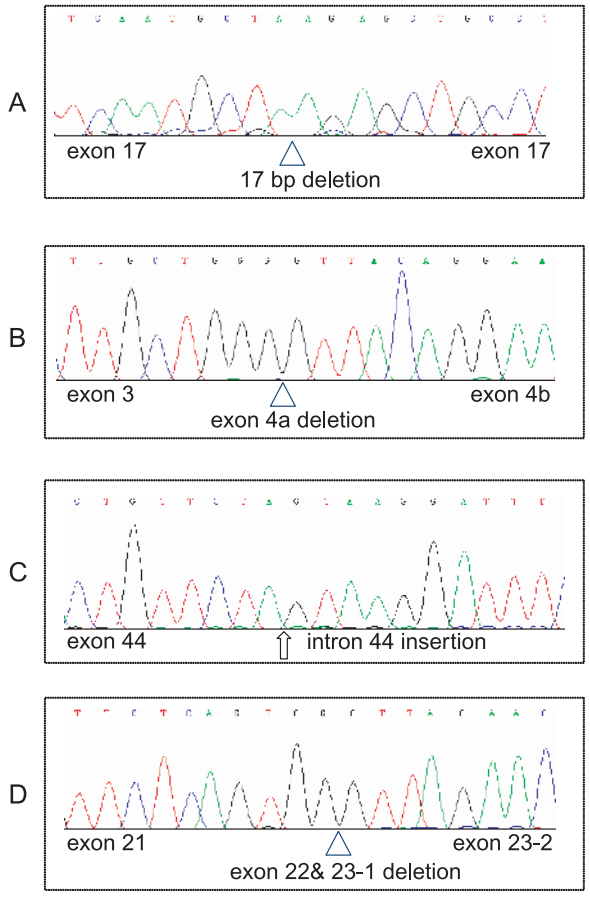

Figure 5. *NF1* cDNA sequence alterations in representative cell lines and primary tumors. Chromatograms showing (A) absence of 17 bp of exon 17 from the cDNA of cell line OVCA429, (B) deletion of exon 4a in cDNA from PEO4, (C) portion of 178 bp insertion from intron 44 between exons 44 and 45 in tumor OSC-18, and (D) deletion of exons 22 and 23-1 in cDNA from tumor OSC-25.

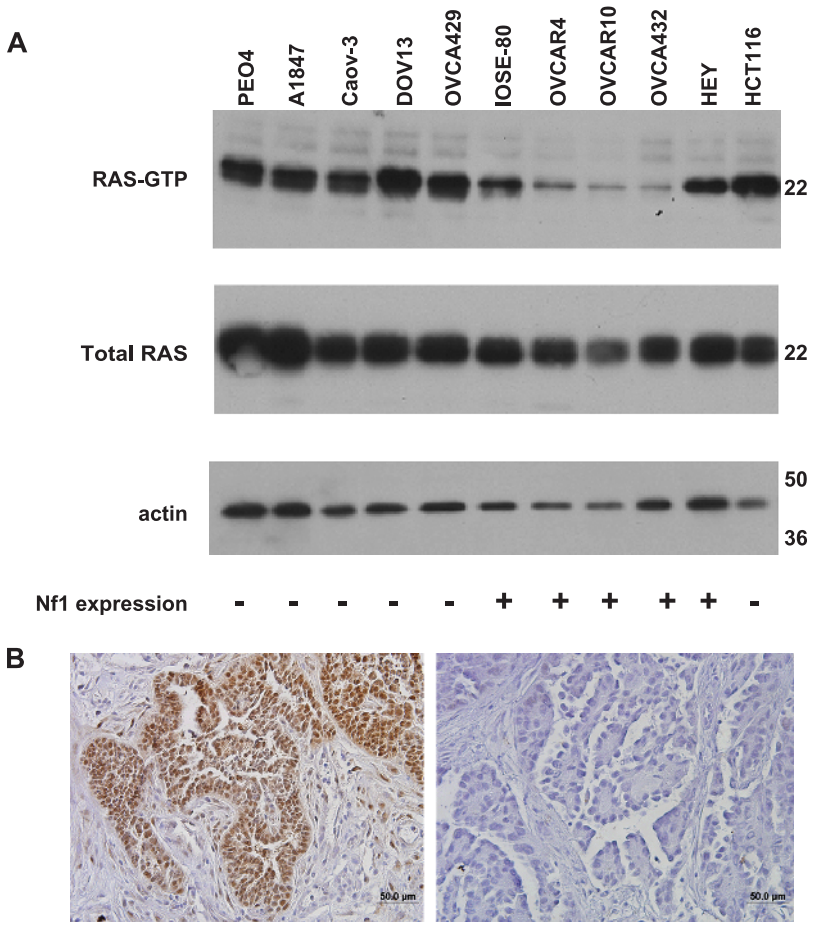

Figure 6. The Ras pathway is activated in ovarian carcinoma cell lines and primary OSCs. (A) Ras-GTP and total Ras levels in the ovarian carcinoma cell lines were detected using a Ras activation assay kit as described in the Materials and Methods section. Representative data are shown. The blot was reprobed with antiactin as a loading control. HCT116, which does not express *NF1*, and HEY, which expresses mutant KRas (G12D), were used as a positive controls for Ras-GTP levels. Expression of Nf1 in each cell line [low or absent (−) *versus* readily detectable (+)] is indicated below each lane. (B) Immunohistochemical analysis of pMAPK in primary OSCs; representative examples are shown. OSC-32 (left panel) has a *NF1* mutation and shows strong nuclear staining for pMAPK in the tumor cells, with absence of staining in the non-neoplastic stromal cells. No *NF1* alterations were detected in OSC-33, which is negative for pMAPK expression (right panel).

the transmembrane receptor tyrosine kinase epidermal growth factor receptor (EGFR), or HER-2/Neu.

OSCs with NF1 *Defects Harbor Frequent Mutations in* TP53 *But Not* KRAS *or* BRAF

Previous studies provide evidence for two major pathways in the pathogenesis of OSCs [32,33]. Low-grade OSCs are less common but have a high prevalence of activating mutations of the *KRAS* or *BRAF* proto-oncogenes and low prevalence of inactivating mutations of the *TP53* tumor-suppressor gene. Indeed, nearly 70% of low-grade OSCs and their putative precursor lesions (serous borderline tumors) have either *KRAS* or *BRAF* mutations [34,35]. In contrast, high-grade OSCs comprise most OSCs, and in these tumors, *KRAS* and *BRAF* mutations are rare, whereas *TP53* mutations are common [35,36]. We analyzed all 41 OSCs for mutations of *TP53* (exons 3-10), *KRAS* (codons 12 and 13), and *BRAF* (exons 11 and 15). None of the OSCs had *BRAF* mutations, and only one tumor had a *KRAS* mutation (Table 2). Immunostaining for p53 protein was also performed in all but 1 of the 41 tumors (Table 2). Strong and diffuse nuclear p53 expression of the type seen in tumors with missense *TP53* mutations was identified in 20 (50%) of 40 OSCs (data not shown). To confirm the presence of missense mutations and to detect mutations leading to loss or truncation of the p53 protein, *TP53* exons 3-10 were sequenced. Overall, we identified *TP53* mutations in 30 (73%) of 41 tumors. Of these, 21 were missense, 4 were frameshift, and 5 were nonsense mutations. Notably, all nine tumors with inactivated *NF1* had documented *TP53* mutations (Table 2). The association between *NF1* and *TP53* mutations was statistically significant ($P = .041$, 1-tail Fisher's exact test) and there was a positive correlation between mutations of *NF1* and *TP53* ($r = 0.3211308$, $P = .0406$; Pearson's product moment correlation).

Discussion

Although progress has been made in defining genetic alterations underlying the pathogenesis of OvCa, including the identification of specific mutations and gene expression patterns characteristic of the various morphological subtypes of OvCa, much work would seem to remain before we will have a full accounting of the key gene defects contributing to the development of OvCa. Because genome-wide analysis of DNA copy number alterations in primary OvCas

Table 2. Summary of the Mutational Analysis and Immunohistochemistry in OSC Tissues.

Tumor ID	Clinical Data			ROMA-*NF1* Deletions	*NF1* Mutations Type	*pMAPK* (IHC)	*p53* (IHC)	*TP53* Mutations (Exon 3-10)				*KRAS* Exon 2,3	*BRAF* Exon 11,15
	Age	Stage	Grade					Exon	Nucleotide	Codon	Type		
OSC-1	83	3C	3	homo	Deletion	+++	+	8	G824A	C275Y	Missense	WT	WT
OSC-2	61	4	3	hemi	Splicing	++	+	5	A395G	K132R	Missense	WT	WT
OSC-3	43	3C	3		WT	–	+	7	A701G	Y234C	Missense	WT	WT
OSC-4	66	3C	3		WT	–	–				WT	WT	WT
OSC-5	58	3B	2	hemi	Deletion	+++	+	5	C380T	S127F	Missense	WT	WT
OSC-6	57	3C	2		WT	++	–				WT	WT	WT
OSC-7	44	3C	3		WT	–	–				WT	WT	WT
OSC-8	57	3C	3		WT	+++	+	5	A491G	K164E	Missense	WT	WT
OSC-9	67	1C	3		WT	+++	+	5	T537G	H179Q	Missense	WT	WT
OSC-10	53	2C	2		WT	ND	+	5	A395G	K132R	Missense	WT	WT
OSC-11	60	4	2	homo	Deletion	+++	+	4	C215G	P72R	Missense	WT	WT
OSC-12	44	3D	3		WT	–	+	9	G976T	E326stop	Nonsense	WT	WT
OSC-13	67	4	1		WT	–	+	5	A395G	K132R	Missense	WT	WT
OSC-14	40	3C	2		WT	++	–	5	C499T	Q167stop	Nonsense	WT	WT
OSC-15	74	3C	3		WT	+	–	5	C497G	S166stop	Nonsense	WT	WT
OSC-16	67	3C	3		WT	++	+	6	C569T	P190L	Missense	WT	WT
OSC-17	62	3C	3		WT	+	–				WT	WT	WT
OSC-18	59	1A	2		Splicing	+++	–	7	746delG	344stop	Frameshift	WT	WT
OSC-19	52	3	3		WT	ND	ND	7	C742T	R248W	Missense	WT	WT
OSC-20	65	2	3		WT	+++	+	7	C725T	C242F	Missense	WT	WT
OSC-21	19	4	1		WT	–	–				WT	WT	WT
OSC-22	30	4	1		WT	–	–				WT	WT	WT
OSC-23	58	3C	2		WT	–	–	8	C916T	R306Stop	Nonsense	WT	WT
OSC-24	53	3C	2	hemi	Deletion/splicing	–	+	8	C817G	R273G	Missense	WT	WT
OSC-25	58	3C	3	hemi	Splicing	+++	+	8	C844T	R282W	Missense	WT	WT
OSC-26	73	3C	2		WT	–	+	8	C844T	R282W	Missense	WT	WT
OSC-27	41	1C	2		WT	ND	–	7	689delC	246stop	Frameshift	WT	WT
OSC-28	61	2C	1		WT	–	–	6	C637T	R213Stop	Nonsense	WT	WT
OSC-29	49	3C	1		WT	++	–				WT	G12R	WT
OSC-30	42	3C	3		WT	+++	–				WT	WT	WT
OSC-31	50	3C	2		WT	–	–				WT	WT	WT
OSC-32	54	3C	3		Splicing	+++	+	5	G517A	V173M	Missense	WT	WT
OSC-33	60	3C	3		WT	–	–	6	C637T	R213Stop	Nonsense	WT	WT
OSC-34	61	3	1		WT	+++	–				WT	WT	WT
OSC-35	43	3C	3		Splicing	+++	+	6	A659G	Y220C	Missense	WT	WT
OSC-36	56	4	3		WT	–	+	6	A659G	Y220C	Missense	WT	ND
OSC-37	48	3C	3		WT	++	+	6	T581G	L194R	Missense	WT	WT
OSC-38	68	1A	3		WT	–	–	7	689delC	246stop	Frameshift	WT	WT
OSC-39	32	1B	1		WT	++	–				WT	WT	WT
OSC-40	68	3C	3		WT	++	–	5	455delC	169stop	Frameshift	WT	WT
OSC-41	73	3C	3		WT	–	+	8	G796A	G266R	Missense	WT	WT

suggested the possibility of homozygous deletions of the *NF1* gene in a subset of OSCs, we pursued in-depth molecular analyses to assess the frequency and mechanisms underlying *NF1* inactivation in OSCs. We provide data here that mutational defects leading to reduced or absent Nf1 expression were found in 5 of 18 OvCa cell lines and 9 (22%) of 41 primary OSCs. In addition, given what is known about the role of the Nf1 protein in negatively regulating the activity of Ras proteins, we found, not unexpectedly, that OSCs with *NF1* mutations lacked *KRAS* or *BRAF* mutations. All tumors with documented *NF1* alterations were found to harbor mutations of the *TP53* tumor-suppressor gene.

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder affecting approximately 1 in 3000 individuals [37,38]. The most common manifestations are café-au-lait macules, neurofibromas, Lisch nodules, skin-fold freckling, bony dysplasia and learning disabilities [38]. In addition to neurofibromas, those with neurofibromatosis type 1 are at increased risk for development of other neoplasms, including malignant peripheral nerve sheath tumors, gliomas, and gastrointestinal stromal tumors. Interestingly, coinactivation of *TP53* by deletion or by point mutation in conjunction with *NF1* inactivation has been shown to be a negative prognostic marker in *NF1* patients with malignant peripheral nerve sheath tumors [39,40]. A number of studies have characterized germ line *NF1* mutations in individuals with neurofibromatosis type 1 and identified a diverse spectrum of mutations that includes small deletions and insertions, missense and nonsense point mutations, and mutations that affect splicing [17,23,24,41–44]. There are no clear mutational hotspots. Because more than 70% of *NF1* mutations are predicted to result in truncation of the gene product [25], PTT has been widely used to screen for germ line (constitutional) *NF1* mutations.

The Ras proteins have central roles in the regulation of cell proliferation and differentiation, and mutational activation of Ras signaling contributes to the development of many types of cancer. Ras proteins function as molecular switches in signaling pathways that transmit signals from the cell membrane to the nucleus [31,45,46]. Ras cycles between the inactive GDP-bound and active GTP-bound forms, signaling to downstream effectors that regulate basic cellular functions including cell proliferation, differentiation, and apoptosis. Nf1 acts as a Ras-GTPase activating protein (Ras-GAP), which catalyzes hydrolysis of Ras-GTP to Ras-GDP, with resultant downregulation of downstream signaling through Raf, Ral/Cdc42, PLC, and PI3K. Loss of Nf1 function has been shown to deregulate Ras

signaling in many types of cells, including Schwann cells, astrocytes, hematopoietic cells, mast cells, and melanocytes [47–49]. Moreover, *NF1* mutations and/or loss of expression have been identified in several different types of tumors, including melanomas, colorectal carcinomas, small cell lung carcinomas, and transitional cell carcinomas [50–54]. Only a few studies in the published literature have addressed the role of *NF1* in ovarian cancer pathogenesis, and to the best of our knowledge, no studies have described comprehensive analysis of *NF1* mutations in ovarian cancers. Interestingly, Salud et al. [55] described a 29-year-old woman with neurofibromatosis type 1 who developed epithelial ovarian cancer. We believe ours is the first comprehensive study undertaken to determine the frequency of *NF1* mutations in a sizable collection of OSC primary tumors and ovarian cancer cell lines.

We identified *NF1* alterations in 5 (28%) of 18 ovarian carcinoma–derived cell lines and 9 (22%) of 41 primary OSCs. Evidence for biallelic inactivation of *NF1* was obtained for six of the nine primary tumors (three with homozygous deletion and three with hemizygous deletion and mutation). The actual prevalence of inactivating *NF1* mutations in OSCs is likely higher, because our mutation detection strategy was based entirely on the PTT assay, which fails to detect a third or more of *NF1* mutations [42]. The high frequency of splicing defects identified in our analysis is in keeping with other studies in the published literature, which reported high rates of splicing mutations in *NF1* [41,42]. Although absence of matched normal tissue precluded us from more definitively determining whether OSC tumors or cell lines with mutations of one *NF1* allele had allelic deletions of the other copy, frequent allelic losses at the *NF1* locus in ovarian cancers have been reported by others [56,57].

As mentioned previously, a major consequence of Ras pathway activation is the phosphorylation of MAPK. Notably, in their analysis of active MAPK in OSC, Hsu et al. [58] showed that 41% of high-grade OSCs expressed the active (phosphorylated) form of MAPK (pMAPK) by immunohistochemistry, although *KRAS* or *BRAF* mutations were not present in these tumors. Our findings confirm the paucity of *KRAS* and *BRAF* mutations and suggest that MAPK activation in OSCs may be largely attributable to Nf1 inactivation. All but one of our OSCs with documented *NF1* alterations expressed pMAPK. Several additional tumors without demonstrable *NF1* mutations also highly expressed pMAPK, suggesting that these tumors harbor *NF1* mutations missed by PTT or mutations of other genes upstream of MAPK, such as *HER-2/Neu* or *EGFR*.

Most studies have shown that ~50% to 80% of "typical" (i.e., high-grade, high-stage) OSCs have mutations in *TP53* [36,59,60]. Mutations of *KRAS* in these tumors are much less common (0-12%), and *BRAF* mutations are extremely rare [33]. Our mutational data are in agreement with these published reports. *TP53* mutations were detected in 30 (73%) of 41 primary OSCs. No *BRAF* mutations were found, and only one tumor had mutant *KRAS*. The co-occurrence of *TP53* and *NF1* mutations in our series of OSCs suggests the pathways regulated by these two tumor-suppressor proteins often cooperate in the development of ovarian carcinomas with serous differentiation. Additional studies, for example, in genetically engineered mice with conditionally mutant *P53* and *NF1* alleles, will be required to test this possibility.

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Table W1. Primer Sequences.

Use	Primer ID	Sequences
Southern	NF1 Probe 1-F	CCTTCTTTGACTGTTGATGTAGT
	NF1 Probe 1-R	TTATGCAGGAATGGGTTATGGA
	NF1 Probe 2-F	AGAGCCTATGCGTTTTGAGAAGTATGAC
	NF1 Probe 2-R	TATTGGTGTTGGTTGTTTGGTGTTGTAG
	NF1 Probe 3-F	ACCAAGGCCTTCTCACTAACT
	NF1 Probe 3-R	TAACCAAAAGCTTCAACACTATG
	DHX40-F	GGCAGTTTCCATTTTGTTTCTTGTCC
	DHX40-R	CATTACCACCACCACCCTCTCCA
Northern	NF1-F	AGGGCCAGTTACTAGAGACATCAG
	NF1-R	AAGTAACAACGTGGAAGAGGTAGG
	GAPDH-F	CAGACCACAGTCCATGCCATCAC
	GAPDH-R	CCATGAGGTCCACCACCCTGTTGC
Sequencing	Kras ex2-F	GCCTGCTGAAAATGACTGAAT
	Kras ex2-R	GGTCCTGCACCAGTAATATGC
	BRAF-ex11-F	TCTCTTCCTGTATCCCTCTCAGGCA
	BRAF-ex11-R	CTTGTCACAATGTCACCACATTACA
	BRAF-ex15-F	CCTAAACTCTTCATAATGCTTGCTCTG
	BRAF-ex15-R	TAACTCAGCAGCATCTCAGGGCCAAA
	TP53-5F	TTTATCTGTTCACTTGTGCCC
	TP53-5R	ACCCTGGGCAACCAGCCCTG
	TP53-6F	CACTGATTGCTCTTAGGTCT
	TP53-6R	AGTTGCAAACCAGACCTCAGG
	TP53-7F	GCCACAGGTCTCCCCAAGGC
	TP53-7R	CAGTGTGCAGGGTGGCAAGT
	TP53-8F	GACCTGATTTCCTTACTGCC
	TP53-8R	CCACCGCTTCTTGTCCTGCT
PTT	PTT-I-F	GGATCCTAATACGACTCACTATAGGGAGACCACCATGATGGCCGCGCACAGGCCGGTGGAAT
	PTT-I-R	TGACAGGAACTTCTATCTGCCTGCTTA
	PTT-II-F	GGATCCTAATACGACTCACTATAGGGAGACCACCATGATGGTGAAACTAATTCATGCAGAT
	PTT-II-R	TGTCAAATTCTGTGCCTTG
	PTT-III-F	GGATCCTAATACGACTCACTATAGGGAGACCACCATGATGGAAGCAGTAGTTTCACTT
	PTT-III-R	TAGGACTTTTGTTCGCTCTGCTGA
	PTT-IV-F	GGATCCTAATACGACTCACTATAGGGAGACCACCATGGAGTACACCAAGTATCATGAG
	PTT-IV-R	TATACGGAGACTATCTAAAGTATGCAG
	PTT-V-F	GGATCCTAATACGACTCACTATAGGGAGACCACCATGATGGAGGCATGCATGAGAGATATTC
	PTT-V-R	TCTGCACTTGGCTTGCGGAT