Tumorigenesis and Neoplastic Progression

The Nonreceptor-Type Tyrosine Phosphatase PTPN13 Is a Tumor Suppressor Gene in Non–Small Cell Lung Cancer

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The aim of the present work was to identify protein tyrosine phosphatases (PTPs) as novel, candidate tumor suppressor genes in lung cancer. Among the 38 PTPs in the human genome that show specificity for phosphotyrosine, we identified six PTPs by quantitative RT-PCR whose mRNA expression levels were significantly down-regulated in lung cancer–derived cell lines (ie, PTPRE, PTPRF, PTPRU, PTPRK, PTPRD, and PTPN13). After validation in primary samples of non-small cell lung cancer (NSCLC), we selected *PTPN13* for further studies. The results presented here demonstrate that *PTPN13* is a candidate tumor suppressor gene that is frequently inactivated in NSCLC through the loss of either mRNA and protein expression (64/87, 73%) or somatic mutation (approximately 8%). Loss of *PTPN13* expression was apparently due to the loss of one or both copies of the *PTPN13* locus at 4q (approximately 26% double deletion and approximately 37% single deletion) but not to promoter methylation. Finally, the manipulation of *PTPN13* expression in lung cancer cells (ie, NCI-H292, A549) demonstrated that *PTPN13* negatively regulates anchorage-dependent and anchorage-independent growth *in vitro* and restrains tumorigenicity *in vivo*, possibly through the control of the tyrosine phosphorylation of both EGFR and HER2. In conclusion, the expression screening of PTPs in lung cancer reported here has identified *PTPN13* as a novel candidate tumor suppressor in NSCLC whose loss increases signaling from epidermal growth factor receptor and HER2 tyrosine kin-

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Lung cancer is the leading cause of cancer deaths worldwide.^{1,2} Epithelial lung cancer is classified into two main groups: small cell lung cancer (approximately 15% of all lung cancers) and non-small cell lung cancer (NSCLC) (approximately 85% of all lung cancers).³ NSCLC comprises squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large cell cancer (LCC).³ Despite advances in early detection and standard treatment, NSCLC is often diagnosed at an advanced stage, and patients often have poor prognosis, with a 5-year survival rate of <15%.^{4,5} For this reason, a better understanding of the molecular origins of the disease will contribute to improve therapeutic treatment of patients with lung cancer.

Aberrant activation of protein tyrosine kinase (PTK) receptor signaling contributes to many types of human cancer, including NSCLC.^{6–9} Perturbation of PTK activity by mutations or overexpression results in malignant transformation,⁶ and PTK inhibitors are now established anticancer drugs.^{10–12} NSCLC is characterized by specific molecular alterations in PTKs^{5,12}; somatic activating mutations have been detected in the genes encoding the epidermal growth factor receptor (*EGFR*)/*HER1*,^{13,14} *ErbB2*/HER2,¹⁵ *ErbB4*/HER4,¹⁶ and the fibroblast growth factor receptor 4.¹⁷ Moreover, gene amplification or polysomy in *EGFR*, *HER2*, and *MET* receptors has been reported in NSCLC.^{18–20}

Tyrosine phosphorylation is reversible and governed by the balanced action of PTKs and protein tyrosine phosphatases (PTPs).²¹ However, although much progress has been made during the last 20 years in elucidating the significance of the numerous PTKs in signal transduction,²² less is known about PTPs, and only recently

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the complexity of the PTP family has become evident.^{23,24} A recent survey found that a total of 107 PTPs are encoded by the human genome.²⁵ Of these, 38 belong to the subgroup of "classic PTPs" that show specificity for phosphotyrosine.

The aim of this study was to identify PTPs whose expression was significantly reduced in NSCLC and that, therefore, may act as candidate tumor suppressors. To this aim, we performed a preliminary analysis of the Gene Expression Atlas (ArrayExpress Atlas) and selected 22 PTPs for their expression in normal lung. Among the 22 preselected PTPs, we identified, by quantitative RT-PCR, six PTPs whose mRNA expression was significantly down-regulated in lung cancer-derived cell lines (PTPRE, PTPRF, PTPRU, PTPRK, PTPRD, and PTPN13).

In this study, we report the results for *PTPN13*, a nonreceptor PTP (GenBank no. 5783) with a calculated molecular mass of approximately 270 kDa.^{26–29} The role of *PTPN13* in cancer is controversial, appearing to be endowed with oncogenic and tumor suppressive potential.³⁰ However, *PTPN13* is frequently mutated or down-regulated in colorectal, liver, or head and neck carcinomas,^{31–36} thus raising the possibility that it may act as a tumor suppressor gene in NSCLC.

Materials and Methods

Patients

Surgical samples from 105 patients diagnosed as having NSCLC were obtained from INT Fondazione Pascale (Naples, Italy). The median patient age was 64 years (range, 28 to 82 years); 21 women and 84 men were studied. Stage was known for 92 patients: 71 patients had stage I to II disease and 21 had stage III to IV disease. Grade was known for 94 patients: 7 had grade 1 disease, 32 had grade 2 disease, and 55 had grade 3 disease. Patient accrual was conducted according to the internal review board of the INT Fondazione Pascale (Internal Ethical Committee 556/10 dated December 3, 2010). The study was approved by the internal review board of the University Hospital Mater Domini/University Magna Graecia (Catanzaro, Italy) in a meeting on March 16, 2011.

All animal work was conducted according to the relevant Italian guidelines and was approved by the Internal Committee for Animal Study of the Institute for Genetic Research "Gaetano Salvatore" on April 7, 2008.

Tissue Microarrays and Immunohistochemistry

TMAbs were constructed in collaboration with the Unit of Immunostaining at the Centro Nacional de Investigaciones Oncológicas (Madrid, Spain). Microarray recipient block containing paraffin-embedded NSCLC tissue samples from 105 archival patient specimens was constructed according to established methods using a Tissue Arrayer (Beecher Instruments, Gene Micro-Array Technologies, Silver Spring, MD).³⁷ One cylinder of normal tissue adjacent to each tumor was also transferred to the recipient block. Finally, sections of the resulting tumor TMA block (4 μ m thick) were transferred to glass slides.

Immunostaining was performed using the avidin-biotin-peroxidase method (LSAB kit; Dako, Glostrup, Denmark) as described previously.³⁸ All sections were counterstained with hematoxylin. Two pathologists reviewed all slides (R.F. and G.V.). Antibodies used for immunostaining were selected according to previously published work and were from Cell Signaling Technology (Danvers, MA) [anti-pS473 (no. 9277), anti-AKT1 (no. 2938), anti-pEGFR (Y1068, no. 3777), anti-pMAPK (no. 4370)], AbCam (Cambridge, UK) [anti-PTPN13 (AC21)], and R&D Systems (Minneapolis, MN) [anti-pHER2 (Y1248, no. AF1768)].

The immunohistochemical score (IS) of PTPN13 used in this study was selected on the basis of previously reported criteria where IS was calculated as immunointensity (0 to 3) $\times$ (percentage of immunoreactive cells).³³ PTPN13 was classified high when IS was >40 , low when IS was between 10 and 40, and absent when IS was <10 .

Cell Culture

The following lung cancer cell lines were purchased from ATCC-LGC Promochem (South West London, England): SCC (BEN-1, NCI-H292, NCI-H226, and CALU-1), ADC (A549, NCI-H596, NCI-H2009, NCI-H23, NCI-H522, and NCI-H1975), and LCC (NCI-H460). Cells were maintained in RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA), supplemented with 10% of fetal bovine serum (FBS) and 100 U/mL of penicillin-streptomycin (Life Technologies Inc.). Normal human bronchial epithelial cells (NHBECS) and human small airway epithelial cells (SAECs) were purchased from Cambrex (Milan, Italy) and grown as suggested by the manufacturer.³⁹

Fluorescence in Situ Hybridization

Fluorescence *in situ* hybridization (FISH) analysis was performed on TMAs. BAC clones were designed according to the Ensembl database. TMA slides were deparaffinized, heated in a pressure cooker with 1 mmol/L EDTA (pH 8.0) for 10 minutes, and incubated with pepsin at 37°C for 30 minutes. The slides were then dehydrated in increasing ethanol concentrations and air-dried. The probes were denatured at 96°C for 5 minutes; a hybridization solution was applied on each slide and incubated at 75°C for 1 minute. After overnight incubation at 37°C in a humid chamber, slides were washed with 0.4 $\times$ standard saline citrate and 0.3% NP40 for 2 minutes at 75°C, air-dried in darkness, and counterstained with DAPI and then coverslips were applied.

The BAC clone covering the *PTPN13* gene (RP11-64A1) was labeled with dUTP-Spectrum Orange (Vysis Inc., Downers Grove, IL). Control BAC probes covering the chromosome 4 centromeric region was labeled green (KRETECH, Poseidon FISH probes SE4 KBI-20004G D4Z1). Two different investigators who had no previous knowledge of the genetic, clinical, and immunohistochemistry (IHC) results evaluated FISH analysis (R.F. and M.S.). All FISH was scored on an average of 130 nuclei (range, 60 to 210). For evaluation of copy number of *PTPN13*, samples containing a signal to centromere ratio

of 1.0 were classified as disomy, samples containing a signal to centromere ratio of 1.0 but <2 signal spots in at least 50% of cells were classified as monosomy, and samples containing a signal to centromere ratio of <0.75 in at least 50% of cells were considered to carry a loss. This allowed the classification of patients into three groups: FISH disomic, FISH monosomic, and FISH loss.

Quantitative RT-PCR and Quantitative Real-Time PCR

Total RNA and genomic DNA were prepared as previously described.^{40,41} Quantitative RT-PCR and quantitative real-time PCR were performed using the Power SYBR Green PCR Master Mix in an ABI Prism 7300 thermocycler (Applied Biosystems, Foster City, CA) according to the manufacturers. cDNA was synthesized from 1 μ g of total RNA using QuantiTect Reverse Transcription (Qiagen, Venlo, The Netherlands).

The sequence of the primers used in the screening of receptor PTPs (PTPRs, O, E, F, A, M, U, K, J, G and D) and nonreceptor PTPs (PTPNs 4, 14, 6, 9, 21, 23, 4A1, 18, 3, 4A2, and 1) is available on request.

For RT-PCR analysis of PTPN13, the primers used were designed on NM_080685.2: forward: 5'-TGG-CTCTCCAGGCTGAGTATG-3' (nt 2114–2134, exon 14) and reverse: 5'-CGGGCAAATAGTGCTCCATT-3' (nt 2174–2194, exon 15). For gene dosage analysis, primers were designed to amplify 81-bp spanning exon 14 and intron 14 of the *PTPN13* gene and subsequently normalized by β -actin: *PTPN13* forward: 5'-TGGCTCTCCAGGCTGAGTATG-3', *PTPN13* reverse: 5'-AACACCAAACAAAATGGTCCTG-3', β -actin forward: 5'-TGC GTGACATTAAGGAGAAG-3', and β -actin reverse: 5'-GCTC-GTAGCTCTTCTCC-3'. Each sample was run three times, and each PCR experiment included two nontemplate control wells. The relative amounts of mRNA or DNA were calculated by the comparative C_T method.⁴²

Mutation Analysis

Mutation analysis for *PTPN13* using direct sequencing performed using the BigDye v3.03 cycle sequencing kit (Applied Biosystems) in a capillary automatic sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems). Protocols and primers for quantitative real-time PCR and sequencing *PTPN13* (exons 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 44, 45, 46, 47, and 48) are available on request.

Methylation Analysis

Bisulfite modification of 1 μ g of DNA was performed by using the Epitect Bisulfite Kit (Qiagen) according to the manufacturer protocol. Subsequently, bisulfite-treated genomic DNA was amplified using primers designed to amplify nucleotides –214 to –1 of the *PTPN13* promoter region: forward: 5'-TTAAGGTATTGGTTGTTATGGT-AAT-3' and reverse: 5'-CACC ACTAAA ACTCCACCTC-3'. After amplification, the PCR products were directly sequenced.

Antibodies and Western Blot

Western blot analysis was performed by standard methods.⁴¹ Whole cell extracts were prepared by homogenizing cells in radioimmunoprecipitation assay buffer (9.1 mmol/L dibasic sodium phosphate, 1.7 mmol/L monobasic sodium phosphate, 150 mmol/L NaCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μ g/mL of phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate) containing protease inhibitors. Lysates were cleared by centrifugation, and proteins were separated by SDS-PAGE. Antibodies used were from Cell Signaling Technology [anti-pEGFR (no. 3777), anti-EGFR (no. 2232), anti-pHER2 (Y1228, no. 2247), anti-HER2 (no. 2242), anti-pMAPK (no. 4370), anti-MAPK (no. 4695), anti-AKT1 (no. 2938), and anti-pAkt (S473, no. 9277)], Santa Cruz Biotechnology (Santa Cruz, CA) [anti-PTPN13 (H300)], or Novus Biologicals (Littleton, CO) [anti-pHER2 (Y877, no. NB100-92549)]. Mouse and rabbit monoclonal special secondary antibodies were from Santa Cruz Biotechnology.

Virus Generation and Infection

The human *PTPN13* (NM_006264) MISSION short hairpin RNA (shRNA) set (Sigma-Aldrich, St. Louis, MO) and the mission nontarget control transduction virus (SHC002V; Sigma-Aldrich) were used to generate lentiviral particles in HEK293T packaging cells. Subconfluent HEK293T cells were co-transfected with 13 μ g of *PTPN13* MISSION shRNA set, 18 μ g of pCMV-deltaR8.91, and 12 μ g of pCMV-VSVG.36 After transfection, supernatants were collected at 8-hour intervals, filtered, and used for three rounds of transduction of NCI-H292 cells in the presence of 8 μ g/mL of polybrene (Sigma-Aldrich). Transduced cells underwent three rounds of infection (8 hours each round) and were selected in medium containing 1 μ g/mL of puromycin.

DNA Constructs and Transfection

The *PTPN13* expression plasmid was a generous gift from Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden). The pcDNA3-HER2 and pcDNA3-EGFR plasmids were purchased at Addgene. A549 cells were transfected with *Trans*-LT1 transfection reagent (Mirus, Madison, WI), according to the manufacturer's protocol. In most experiments transfection efficiency was approximately 50% to 60%, as demonstrated by parallel transfection with plasmid-enhanced green fluorescent protein (Clontech Laboratories, Mountain View, CA).

In Vitro Proliferation Assay

Cell proliferation was assayed by MTT (Sigma-Aldrich) reduction. Cells were plated in 96-well, flat-bottomed microtiter plates (200- μ L cell suspensions, 2×10^3 per well for NCI-H292) and incubated with MTT substrate (5 mg/mL) for 4 hours. Every 24 hours, the culture me-

Figure 1. Quantitative RT-PCR expression of selected PTPs in NSCLC-derived cell lines. Relative mRNA expression of selected receptor type ($n = 10$) and nonreceptor type ($n = 12$) PTPs in NSCLC-derived cell lines. The cell lines used were as follows: NHBECs, ADC-derived cell lines (A549, NCI-H522, NCI-H2009, NCI-H23, NCI-H460, and NCI-H596), and SCC-derived cell lines (NCI-H292, NCI-H226, CALU1, and BEN1). The value of mRNA expression of the different PTPs in NHBECs was set arbitrarily at one (**black line**); **diamonds** report the mRNA expression levels of the different PTPs in the 10 NSCLC-derived cells. Black and gray lines report the mean of mRNA expression level.

dium was removed and anhydrous 2-propanol was added. Optical density was measured at 570 nm.

Soft Agar Assay

NCI-H292 cells (5×10^3) were resuspended in RPMI plus 10% FBS, containing 0.35% low-melting agarose (type VII; Sigma-Aldrich) and seeded onto 0.5% low-melting agarose in Dulbecco's modified Eagle's medium containing 10% FBS. Colonies were scored 3 weeks after preparation and stained with 0.1% crystal violet (Sigma-Aldrich). Quantification (mean and SD) was from three independent experiments.

Tumorigenic Assays

NCI-H292 cells (1×10^6) were resuspended in 100 μ L of 10% FBS and 100 μ L of Matrigel (BD Biosciences, Franklin Lakes, NJ) and subcutaneously injected into the right flank of 6-week-old, athymic, nude mice (Charles River, Wilmington, MA) in triplicate. Every 7 days tumor size was measured with a caliper.

Colony Formation Assay

Cell were transfected with pBABE (1 μ g) in the presence of *PTPN13* or control empty vector (4 μ g) by *Trans*-LT1 transfection reagent (Mirus), according to the manufacturer. After 48 hours, cells were selected with 1 μ g/mL of puromycin (Sigma) for approximately 20 days, stained with crystal violet solution for 10 minutes, and counted.

Results

Analysis of PTP Expression in Lung Cancer-Derived Cell Lines and Tissues

The aim of the study was to identify tyrosine phosphatases that contribute to the development of NSCLC. A recent survey found that 107 PTPs are encoded by the human genome.²⁵ Of these, 38 belong to the subgroup of "classic PTPs" that show specificity for phosphotyrosine. By analysis of the Gene Expression Atlas (ArrayExpress Atlas), a meta-analysis-based database of the ArrayExpress Archive, we selected 22 PTPs that are expressed in normal lung among the subgroup of classic PTPs: 10 receptor PTPs (PTPRs, O, E, F, A, M, U, K, J, G and D) and 12 nonreceptor PTPs (PTPNs 13, 4, 14, 6, 9, 21, 23, 4A1, 18, 3, 4A2, and 1). Subsequently, we performed a preliminary quantitative RT-PCR screening of the mRNA expression of the 22 selected PTPs in ADC- or SCC-derived cell lines compared with NHBECs (Figure 1). The cell lines used were as follows: ADC-derived cell lines, A549, NCI-H522, NCI-H2009, NCI-H23, NCI-H460, and NCI-H596; and SCC-derived cell lines, NCI-H292, NCI-H226, CALU1, and BEN1. The quantitative RT-PCR analysis of NSCLC-derived cell lines identified six different PTPs with significant down-regulated expression: five receptor PTPs [PTPRE ($P = 0.01$), PTPRF ($P = 0.001$), PTPRU ($P = 0.02$), PTPRK ($P = 0.0005$), and PTPRD ($P = 0.039$)] and one nonreceptor PTP [PTPN13 ($P = 0.013$)]. Statistical analysis was performed with Student's *t*-test.

To validate the screening performed in cancer cell lines, we analyzed the mRNA expression of a representative set of PTP genes (PTPRE, PTPRF, PTPRD, and PTPN13) in a panel of normal ($n = 8$) or neoplastic ($n = 23$; 9 ASCs, 11 SCCs, and 3 ASCs) lung tumor tissues (Figure 2). We found that the expression of all tested PTPs in NSCLC was significantly decreased in most pri-

Figure 2. Quantitative RT-PCR expression of selected PTPs in primary NSCLCs. Relative mRNA expression of PTPRE, PTPRF, PTPRD, and PTPN13 in primary NSCLCs by quantitative RT-PCR. **Diamonds** and **square** represent, respectively, mRNA expression levels in normal and lung tumor tissues.

mary tumors (PTPN13, $P = 0.0003$; PTPRF, $P = 0.024$; PTPRE, $P = 0.0001$; PTPRD, $P = 0.05$). These findings confirmed the validity of screening performed in the cell lines system and, on the other hand, allowed the identification of four potential tumor suppressor genes in NSCLC. For the aim of this study, we selected the non-receptor PTPN13.

Analysis of PTPN13 Expression in Lung Cancer-Derived Cell Lines

Figure 3 shows the protein levels of PTPN13 in 10 cell lines derived from human NSCLCs. Primary human normal SAEs were used as control of normal lung for ADCs, and NHBEs were used as controls for normal lung for SCCs. Immunoblot analysis demonstrated that the levels of PTPN13 protein were significantly decreased in most ADC-derived cell lines compared with SAEs used as control normal cells, except for NCI-H522 cells (Figure 3A). Subsequent mRNA analysis of PTPN13 expression by quantitative RT-PCR (Figure 3B) showed a good correlation between levels of PTPN13 mRNA and protein in most cell lines analyzed: cells with low levels of PTPN13 protein (ie, NCI-H23, NCI-1975) also presented a low amount of mRNA, and cells with high PTPN13 protein levels showed a high amount of PTPN13 mRNA (ie, SAE, NCI-H522). Similarly, PTPN13 expression was decreased in two of four SCC-derived cell lines (CALU-1 and BEN) compared with SAEs (Figure 3A). Subsequent mRNA analysis of PTPN13 expression in SCC-derived cell lines by quantitative RT-PCR showed a good correlation between levels of PTPN13 mRNA and protein in all cell lines analyzed (Figure 3B).

Analysis of PTPN13 Expression in Primary NSCLCs

The study of PTPN13 expression was performed on TMAs containing duplicated core biopsy specimens of

105 NSCLC samples. Patients' clinicopathologic characteristics have been described previously.³⁸ PTPN13 staining could be evaluated for 87 NSCLCs [35 SCCs, 42 ADCs, six adenosquamous carcinomas (ASCs), and 4 LCCs]. As controls, 35 matched normal samples were analyzed. The criteria for the score of PTPN13 in NSCLC are described in *Materials and Methods*. Briefly, PTPN13 expression was classified high when normal or tumor cells retained strong diffuse cytoplasmic staining in several cells per high-power field (IS >40), low when the IS was 10 to 40, and negative when the IS for PTPN13 was 0 to 10.³³ The results obtained from PTPN13 staining in NSCLC are summarized in Table 1. Figure 4 shows representative stains. PTPN13 presented strong expression in the cytoplasm of epithelial cells from normal alveolar epithelium and from upper airways 25/35 (Figure 4A). In contrast, PTPN13 expression was significantly decreased in a total of 64 of 87 NSCLCs analyzed (73%) (41/87 cases with IS in the range of 10 to 40 were considered low expressors, and 23 of 87 cases with IS in the range of 0 to 10 were considered completely negative) (Figure 4, B and C) ($P < 0.001$; Fisher's exact test).

Among the different types of NSCLCs, PTPN13 expression was considered low in 23 of 42 ADCs (55%) and negative in 13 of 42 ADCs (31%), low in 14 of 35 SCCs (40%) and negative in 8 of 35 SCCs (23%), low in 2 of 6 and negative in 2 of 6 ASCs, and low in 2 of 4 LCCs (Table 1). Finally, the examination of the immunoscore data failed to reveal a significant correlation of PTPN13 immunostaining with patient age, tumor size, node involvement, and clinical stage or grade (not shown).

Mechanisms Responsible for PTPN13 Loss in NSCLC

Quantitative RT-PCR and FISH analysis of PTPN13

Figure 3 demonstrates the existence of a significant relationship between PTPN13 mRNA and protein in cell lines. We used quantitative RT-PCR analysis of normal

Figure 3. Analysis of PTPN13 expression in NSCLC-derived cell lines. **A:** Immunoblot analysis of PTPN13 in human NSCLC-derived cell lines: SAEs, NHBEs, ADC-derived cell lines (NCI-H522, NCI-H23, A549, NCI-H1975, NCI-H460, and NCI-H209), and SCC-derived cell lines (NCI-H596, NCI-H292, Calu1, and Ben). **B:** PTPN13 relative mRNA expression by quantitative RT-PCR in human NSCLC-derived cell lines.

Table 1. Expression of PTPN13 in NSCLCs

Histologic type	No. of samples	PTPN13 expression*			P value versus normal
		Positive	Low	Negative	
Normal	35	25	5	5	
ADC	42	6	23	13	<0.001
SCC	35	13	14	8	0.013
ASC	6	2	2	2	0.192
LCC	4	0	2	0	0.011
Total	87	21	41	23	<0.001

*The IS of PTPN13 used in this study was selected on the basis of previously reported criteria where IS is calculated as the immunointensity (0 to 3) $\times$ (percentage of immunoreactive cells). PTPN13 was classified as positive when the IS was >40 , low when the IS was 10 to 40, and absent when the IS was <10 .

lungs ($n = 4$) and 20 representative primary NSCLCs (6 ADCs, 11 SCCs, and 3 ASCs) that have been analyzed by immunostaining to investigate whether the loss of PTPN13 protein observed in the TMA studies occurred through mechanisms that dysregulate its mRNA transcription (Figure 5A). By matching the quantitative RT-PCR analysis of PTPN13 mRNA with the protein expression analysis performed on TMAs, we found a significant correspondence between loss of protein and loss of mRNA. All tumors ($n = 19$) that showed loss of PTPN13 protein also showed consistently reduced mRNA levels; only one standard saline citrate sample that showed mRNA expression comparable with normal tissue had lost protein expression in IHC.

We also determined whether the observed decreased expression of PTPN13 in cancer samples could be ascribed to monoallelic or biallelic deletion of the gene encoding *PTPN13* or to loss of heterozygosity at *4q21.3* because previous cytogenetic studies on NSCLCs have shown that the chromosome 4q, where the *PTPN13* gene has been assigned (*4q21.3*), is frequently lost in tumors.^{36,43}

To this aim, we examined the gene copy number of *PTPN13* by quantitative real-time PCR in the same 19 NSCLCs (seven ADCs, nine SCCs, and three ASCs) and corresponding normal tissues ($n = 3$) (Figure 5B). Normal human diploid peripheral blood lymphocytes (PBLs) were used as control (copy number = 2). We found that although normal tissues presented, on average, a gene copy number that was similar to the PBLs used as control, several NSCLCs presented reduced values for *PTPN13* quantitative real-time PCR. In particular, two ADCs presented almost complete loss of the locus (<0.2), three ADCs presented one copy of the *PTPN13* gene, and two ADCs presented two copies. Similarly, one SCC presented almost complete loss of the locus (<0.2), three SCCs presented values encompassing one copy of the *PTPN13* gene (approximately one), and five SCCs presented no alteration. For ASCs, two samples presented complete loss of the locus (<0.2) and one sample presented one copy of the *PTPN13* gene. Overall, monoallelic loss was observed in 7 of 19 (approximately 37%), and complete loss was observed in 5 of 19 NSCLC samples (approximately 26%). The results obtained by quantitative real-time PCR were confirmed by FISH analysis of *PTPN13*, in which we found that seven of eight samples that apparently presented one *PTPN13* copy by quantitative real-time PCR were monoallelic by FISH and that

six of six samples that presented normal DNA content of *PTPN13* by quantitative real-time PCR locus were diploid for the *PTPN13* locus also by FISH (Figure 5C). None of the samples, which had apparently lost both copies of *PTPN13* by quantitative real-time PCR, could be evaluated by FISH.

Expectedly, all NSCLCs with loss of *PTPN13* as detected by quantitative real-time PCR and/or FISH were negative for PTPN13 expression by RT-PCR and/or immunostaining, suggesting that the loss of one or two copies of chromosome 4q accounts for the decrease in PTPN13 expression in a large fraction of NSCLCs and, for this reason, may contribute to the pathogenesis of NSCLC.

Mutation and Methylation Analysis of *PTPN13*

An important genetic characteristic of tumor suppressor genes is the occurrence of genetic mutations that affect the normal gene function. Notably, the *PTPN13* somatic mutations have been identified previously in 10% of colorectal cancers.^{44,45} To investigate whether genetic mutations in *PTPN13* also occur in NSCLCs, we analyzed five cell lines (NCI-H292, A549, NCI-H23, NCI-H460, and BEN) and 25 primary NSCLC samples (nine ADCs, 10 SCCs, four ASCs, and two other histotypes) by direct DNA sequencing of exons 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 44, 45, 46, 47, and 48. We found missense mutations in 1 cell line (NCI-H23) and in 2 primary NSCLC samples of 25 (1 SCC and 1 ASC, respectively) (8%). The genetic changes [single-nucleotide polymorphisms (SNPs) and mutations] identified in the samples analyzed are summarized in Table 2. We also included the SNPs of PTPN13 available from a public SNP database (dbSNP BUILD 121). See Figure 6A for representative chromatograms. The mutation identified in NCI-H23 was C268G, leading to L90V in the kind domain of exon 3. The mutations identified in the primary tumors were A808C, leading to N270H in exon 7, and G1925A, leading to R482Q in exon 10. The mutations identified in this study had not been described before and thus have not been associated with loss of function. However, they represent drastic amino acid changes in the middle of the protein and thus are likely to be pathogenetic. Moreover, it is to be underlined that in both primary tumor samples, the mutations were somatic, having not been detected in the noncancer tissues adjacent to the corresponding cancer (Figure 6A).

Figure 4. TMA immunostaining analysis of PTPN13 expression in primary NSCLCs. Expression of PTPN13 in NSCLCs: a representative immunostaining of PTPN13 in normal and pathological lung tissues. Serial formalin-fixed, paraffin-embedded sections from normal lungs were analyzed for PTPN13 expression. **A:** Epithelial cells from normal lung tissue are strongly positive for PTPN13 expression. **B:** Cancer samples showed intense (IS >40; **upper row**), moderate (IS of 10 to 40; **middle row**), or negative (IS of 0 to 10; **lower row**) staining in SCCs. **C:** Cancer samples showed intense (IS >40; **upper row**), moderate (IS of 10 to 40; **middle row**), or negative (IS of 0 to 10; **lower row**) staining in ADCs. Original magnification, $\times 10$ (left) and $\times 40$ (right).

Another important genetic characteristic of tumor suppressor genes is the occurrence of aberrant methylation of the promoter, which affects the normal rate of transcription. The DNA promoter region that drives expression of the major PTPN13 RNA transcript has been identified in previous studies,⁴⁴ and a CpG-rich sequence embedded in this region just upstream of the transcription initiation site, containing 12 CpG dinucleotides, has been reported to be differentially methylated in hepatocellular carcinomas.³⁴ To analyze PTPN13 promoter methylation in NSCLC, a 213-bp DNA fragment from nucleotide -214 to nucleotide -1 of the PTPN13 promoter (containing 15 CpGs) from 5 cancer cell lines (NCI-H292, A549, NCI-H23, NCI-H460, and BEN) and 20 primary

Figure 5. Analysis of PTPN13 expression and genetic status in primary NSCLCs. **A:** PTPN13 relative mRNA expression by quantitative RT-PCR of representative primary NSCLCs analyzed by immunostaining. The mean PTPN13 expression of normal lung tissues was set as one. **B:** Relative gene copy number of PTPN13 by quantitative PCR of the same NSCLC samples. DNA copy number from normal PBLs was set arbitrarily as one. **C:** FISH analysis of primary NSCLCs: tumor with one PTPN13 and one centromeric signal (**top**) and tumor diploid for PTPN13 (**bottom**). Red, PTPN13 gene; green, chromosome 4 centromere. Nuclei were visualized under an Olympus BX61 microscope using a 60 $\times$ objective; a magnification of the inset is shown on the right side.

Table 2. Mutations of PTPN13 in NSCLCs

Exon no.	Intron no.	Contig position	Reference SNP	SNP		Mutations in primary NSCLC	
				bp	aa	bp	aa
2	5–6	12158097	rs10025433	G21A	E7E	A808C G1925A	N270H[†] R482Q[†]
3			rs78046638	C268G	L90V		
7			rs114810267	T/A			
10			rs61730646	G865A	G289S		
	44–45	12276397	rs17694112	A/C			
	47–48	12280050	rs12504551	T/C*			
				T/G			

The number in front of the genetic variations indicates the nucleotide or protein position of PTPN13 according to the accession no. NM_080685.2. The bolded entries indicate the genetic variations that resulted in the change of amino acid (aa) codon.

*The SNP is a newly identified SNP by this study.

[†]Mutation newly identified by this study.

NSCLCs (6 ADCs, 8 SCCs, 4 ASCs, and 2 others), selected for low PTPN13 mRNA expression, were PCR amplified and analyzed for the CpG methylation pattern by direct sequencing the DNA after sodium bisulfite modification. Surprisingly, sequence analysis of PCR fragments demonstrated no difference between normal and tumor tissues, suggesting that methylation of CpGs in this promoter region of *PTPN13* is not a major mechanism of PTPN13 down-regulation in NSCLC (Figure 6B).

In conclusion, the results presented here demonstrate that *PTPN13* is a candidate tumor suppressor gene that is frequently inactivated in NSCLC through the loss of mRNA and protein expression (64/87, 73%) or somatic mutation (8%). Loss of PTPN13 expression was apparently due to the loss of one or both copies of the *PTPN13* locus at 4q (26% double deletion and 37% single deletion) but not to silencing by promoter methylation as demonstrated in liver cancer.³⁴

PTPN13 Negatively Regulates Anchorage-Dependent and Anchorage-Independent Proliferation of NSCLC-Derived Cells in Vitro and Tumor Growth in Vivo

To understand the role of PTPN13 in the development of lung cancer, we determined the effects exerted by manipulation of PTPN13 in human lung cancer cells. To this aim, we interfered PTPN13 in NCI-H292 cells by transducing a lentiviral vector expressing shRNAs to PTPN13 or a scrambled control sequence (SCR). We used different shRNAs. Among the five different shRNAs to PTPN13, we found that three different shRNAs were able to efficiently down-regulate PTPN13: NM_006264.x-6679s1c1 with 90% efficiency and NM_006264.x-1731s1c1 and NM_006264.x-1307s1c1 with 50% efficiency. We used two different shRNAs for experimental purposes. The effects were apparently dose dependent because the NM_006264.x-6679s1c1 (the shRNA with 90% efficiency of PTPN13 down-regulation) was slightly more efficient in reducing anchorage-dependent and anchorage-independent growth than NM_006264.x-1731s1c1 (the shRNA with 50% efficiency of PTPN13 down-regulation).

For the sake of clarity, we present the results for shRNA NM_006264.x-6679s1c1.

Control NCI-H292 cells and derivatives were analyzed for anchorage-dependent growth by MTT (Figure 7, A and B) and trypan blue (not shown) assays. Control and PTPN13-interfered NCI-H292 cells were plated and measured every day for 7 days in the absence or presence of serum. We found that the growth rate of NSCLC cells interfered with anti-PTPN13 shRNA lentivirus was significantly increased compared with parental cells or cells transduced with control scrambled lentivirus during serum deprivation but not in complete medium (Figure 7, A and B, respectively).

Subsequently, we determined the effect of suppressing PTPN13 expression on the malignant potential of NSCLC cells *in vitro* (anchorage-independent growth assay) and *in vivo* (xenograft assay). Control, scrambled-transduced, or PTPN13-interfered NCI-H292 cells formed consistently more colonies than parental cells (approximately twofold) in soft agar, demonstrating that PTPN13 negatively regulates anchorage-independent growth of NSCLC cells (Figure 7C). Finally, we investigated the effects of PTPN13 on the tumorigenic potential of NCI-H292 cells *in vivo* by subcutaneously injecting into the flanks of athymic mice control and PTPN13-interfered NCI-H292 (1×10^6) ($n = 6$ for each group). Mice were monitored for tumor appearance for 6 to 12 weeks. After tumor explanting, we found that the suppression of PTPN13 expression in NCI-H292 cells markedly increased *in vivo* growth of NSCLC cells. In fact, after 7 weeks tumors generated by PTPN13-interfered NCI-H292 cells were more than threefold greater than tumors generated by prenatal NCI-H292 cells (Figure 7D, $P = 0.002$).

A complementary approach to investigate the role played by PTPN13 in the development of lung cancer was to restore PTPN13 expression in the highly malignant human lung cancer cell line (A549). Cells were selected in puromycin-containing medium. The presence of the exogenous PTPN13 protein in transduced cells was detected by immunoblot (Figure 7D). We found that the constitutive expression of PTPN13 significantly reduced the colony formation efficiency of NSCLC-derived A549

Figure 6. Mutation and methylation status of PTPN13 gene. **A:** DNA sequence analysis of PTPN13 exon 3 in NCI-H23 cells; DNA sequence analysis of PTPN13 exon 7 in SCC sample and of exon 10 in ASC sample. **Arrows** indicate the mutated nucleotide. N, nontumor tissue; T, tumor tissue. **B:** CpG methylation pattern at the promoter region of PTPN13 in NSCLC. Primers were designed to amplify nucleotides -214 to -1 of the PTPN13 promoter region containing 15 CpGs. The PCR products were sequenced. Representative results of the direct sequencing analysis of NSCLCs. **C:** DNA sequence of the promoter region of PTPN13 before and after bisulfite treatment.

cells: compared with control mock transduced cells, PTPN13 overexpressing cells consistently formed a smaller number of colonies (approximately 25%), and above all, colonies formed by PTPN13-transfected cells were much smaller than those formed by parental cells (Figure 7D).

PTPN13 Negatively Regulates Tyrosine Phosphorylation of HER2 and EGFR in NSCLC Cells in Vitro and in Tumors in Vivo

Recently, it has been suggested that PTPN13 plays a role in attenuating invasiveness and metastasis of HER2-dependent tumors by dephosphorylating HER2 in HeLa cells.⁴⁵ Therefore, we investigated the effects exerted by PTPN13 on the phosphorylation status of HER2 and EGFR in NSCLC cells. Cells were selected in puromycin (1 μ g/mL for 3 to 5 days) and analyzed by immunoblot for PTPN13 expression (Figure 8A). We observed that

PTPN13-interfered NCI-H292 cells presented consistently increased phosphorylated HER2 and EGFR when cells were exposed to serum or 10 ng/mL of EGF for 15 minutes (Figure 8A). In parallel with the increased phosphorylation of EGFR (Y1068) and HER2 (Y1248), PTPN13-interfered NCI-H292 cells presented increased activation of Akt- and MAPK-dependent pathways, as determined by the increased phosphorylation of MAPK (T202/Y204) and Akt (S473) in the presence of EGF. Immunostaining of tumor explanted by SCR- or PTPN13-interfered NCI-H292 cells demonstrated that the large tumors induced in mice by injection of PTPN13-interfered NCI-H292 cells presented loss of PTPN13 staining and high-level staining for pHER2 (Y1248) and pEGFR (Y1068) (Figure 8B). See Figure 7E for the comparison of xenografts of NCI-H292 cells or of PTPN13-interfered NCI-H292 cells. On the other hand, co-expression of PTPN13 and HER2 into A549 cells induced a significant reduction of the activation of HER2 and of MAPK and AKT

Figure 7. PTPN13 negatively regulates anchorage-dependent and anchorage-independent proliferation of NSCLC-derived cells *in vitro* and tumor growth *in vivo*. **A:** MTT assay of SCR and shRNA PTPN13-transduced NCI-H292 cells in low serum. Cells (2.5×10^5) were seeded in 6-well plates, and the number of cells was measured every day for 7 days. $*P < 0.005$ for shRNA PTPN13 cells compared with control. **B:** MTT assay of SCR and shRNA PTPN13-transduced NCI-H292 cells in normal medium. Cells (2.5×10^5) were seeded in 6-well plates, and the number of cells was measured every day for 7 days. **C:** Soft agar assay of vector-transduced or shRNA PTPN13-transduced NCI-H292 cells. Experiments were performed in triplicate. $**P = 0.002$, compared with control. **D:** Colony formation assay of vector-transduced or PTPN13-transfected A549 cells. Experiments were performed in triplicate. $**P = 0.013$, compared with control. Immunoblot analysis of PTPN13 in A549 cells transfected with empty vector or PTPN13. **E:** Control scrambled- and shRNA PTPN13-transduced NCI-H292 cells were subcutaneously injected into the flank of athymic, nude mice, and the growth of xenotransplanted tumor was measured. Representative images of control and shRNA PTPN13-NCI-H292 tumors are shown in the inset. $**P = 0.008$, compared with control.

signaling as determined by a decrease in the corresponding phosphorylation levels (Figure 8C).

Above all, these shRNA experiments indicated that the loss of PTPN13 expression plays a significant role in the malignant behavior of NSCLC cells, regulating anchorage-dependent and anchorage-independent growth *in vitro* and restraining tumorigenicity *in vivo*, likely by modulation of the phosphorylation status of EGFR and HER2 receptors.

Discussion

In the search for novel tumor suppressor genes in NSCLC, we performed a quantitative RT-PCR screening of PTP expression in lung cancer cell lines and primary tumors among those that are expressed in normal lung as determined by analysis of the ArrayExpress Archive database. We report on the identification of *PTPN13* as candidate tumor suppressor gene in NSCLC.

This conclusion is supported by the findings described in this article: i) PTPN13 is frequently inactivated in

NSCLC through the loss of protein expression (approximately 73%) or somatic mutation (approximately 8%); ii) PTPN13 negatively regulates anchorage-dependent and anchorage-independent growth of NSCLC cell lines *in vitro*; iii) PTPN13 restrains tumorigenicity of NSCLC xenografts *in vivo*; and iv) PTPN13 negatively regulates, in the living cells, tyrosine phosphorylation of two oncogenic PTKs (EGFR and HER2) implicated in the development of NSCLC.

PTPN13 (also known as PTPL1, FAP-1, PTP-BAS, and PTP1E) is a nonreceptor type PTP of 270 kDa that is the largest phosphatase within this group.^{26–29} The role of PTPN13 in cancer is controversial, appearing to be endowed with oncogenic and tumor suppressive potential. Although there is no direct evidence that *PTPN13* is an oncogene per se, *PTPN13* has been implicated in tumorigenesis via modulation of cell growth and stress responses. The *PTPN13* gene is a direct transcriptional target of the Ewing's sarcoma family of tumors oncogene, EWS-FLI1, and the resulting overexpression of PTPN13 in Ewing's sarcoma cells supports cell growth and motil-

Figure 8. PTPN13 negatively regulates tyrosine phosphorylation of HER2 and EGFR in NSCLC-derived cells *in vitro* and tumor growth *in vivo*. **A:** Immunoblot analysis of PTPN13, pHER2, HER2, pEGFR, EGFR, pAKT, AKT, pMAPK, and MAPK in lysates obtained from NCI-H292 cells and from the corresponding cells interfered with shRNA to PTPN13, treated with solvent or EGF for 15 minutes. ACTB, actin used for normalization. **B:** Immunostaining analysis of PTPN13, pHER2, and pEGFR in tumor xenografts generated by injection of NCI-H292 cells and from the corresponding cells interfered with shRNA to PTPN13 (original magnification, $\times 40$). In particular, pHER2 staining was detected in $50\% \pm 3\%$ of NCI-H292 interfered with shRNA to PTPN13 compared with $20\% \pm 8\%$ of pHER2-positive NCI-H292 cells, and pEGFR staining was detected in $35\% \pm 7\%$ of NCI-H292 interfered with shRNA to PTPN13 compared with $10\% \pm 6\%$ of pEGFR-positive NCI-H292 cells. **C:** Immunoblot analysis of PTPN13, pHER2, HER2, pMAPK, MAPK, pAKT, and AKT in lysates obtained from A549 cells transfected with PTPN13 and activated HER2.

ity.⁴⁶ PTPN13 promotes survival in cancer cells inhibiting Fas-induced apoptosis or modulating the activity of transient receptor potential M2 and NF- κ B.^{47–49} Conversely, support for a tumor suppressive role of PTPN13 comes from mutation screens of the colon and in human papillomavirus-negative head and neck SCCs,^{31,32,50} expression analysis in breast and liver cancer,^{34,35} the finding that high-risk human papillomavirus protein E6 induced degradation of PTPN13 in squamous epithelial cells, and evidence that PTPN13 is involved in the proapoptotic effects of antiestrogens in breast cancer.^{51,52}

The findings reported in this article are consistent with the idea that PTPN13 is a tumor suppressor gene in NSCLC. TMA immunostaining analysis of PTPN13 expression demonstrated that PTPN13 is severely down-regulated in most NSCLC analyzed, implying that PTPN13 loss might represent an oncogenic event during cancer development in the lung. Moreover, the observation that there is no significant difference in PTPN13 levels with respect to histologic subtypes, grade, stage, or metastasis suggests that the loss of PTPN13 expression might occur at an early stage of the tumorigenic process.

As to the mechanism whereby PTPN13 expression is dysregulated in NSCLC, our data indicate that the loss of PTPN13 expression occurred at the transcriptional level because we found a good correlation between mRNA and protein both in cell lines and in a representative panel ($n = 20$) of primary NSCLC. Moreover, our results also indicate that the drastic reduction in the levels of

PTPN13 mRNA (and protein) observed in NSCLC was not due to promoter methylation as reported to occur in liver cancer.³⁴ On the contrary, we found that more than 60% of NSCLC showing a significant reduction or complete loss of PTPN13 mRNA (and protein) presented the loss of one or both copies of the *PTPN13* locus at chromosome 4q. In particular, approximately 26% of NSCLC analyzed presented deletion of both copies, whereas approximately 37% of NSCLC presented the deletion of a single allele, indicating that loss of the *PTPN13* gene at 4q21 may represent a major mechanism capable of silencing *PTPN13* expression in NSCLC. Our results confirm the results of previous reports, indicating that loss of part or the entire long arm of chromosome 4 (where the *PTPN13* gene maps are located) is recurrent in NSCLC.³⁶ Yet, because approximately 40% of NSCLC presents reduced PTPN13 expression in the absence of chromosome loss, other mechanisms must be involved in the dysregulation of PTPN13 expression during development of lung cancer.

Regarding the role of PTPN13 in pulmonary cells, we found that PTPN13 negatively regulates anchorage-dependent and anchorage-independent growth *in vitro* and restrains tumorigenicity *in vivo*. The silencing of PTPN13 by lentiviral-mediated transduction of shRNA of NCI-H292 cells increased proliferation in monolayer and growth in soft agar and significantly augmented the tumorigenicity of interfered cells. On the contrary, restora-

tion of PTPN13 expression in A549 cells efficiently reduced colony-forming efficiency of transfected cells.

Deregulated signaling from PTKs drives tumor initiation and progression in a variety of human tissues, including the lung transmitting oncogenic signals through phosphorylation of their cytosolic domains. Somatic mutations or overexpression of EGFR or HER2 contributes significantly to the development of NSCLC.^{7,12–16} Our results indicate that PTPN13 regulates critical PTK-dependent signaling pathways inside the cell. On one hand, we found that the silencing of PTPN13 in NCI-H292 cells increased EGF-stimulated EGFR and HER2 phosphorylation, leading to increased activation of the MAPK- and Akt-dependent pathways. On the other hand, restoring PTPN13 expression in A549 cells significantly decreased the phosphorylation status of a constitutively active HER2 mutant, strongly reducing MAPK- and Akt-dependent pathways. These results are in agreement with those of Zhu et al,⁴⁵ who showed that PTPN13 was essential for the dephosphorylation of HER2.

In conclusion, the expression screening of PTPs performed in lung cancer cell lines and tumors reported here identified PTPN13 as a novel candidate tumor suppressor in NSCLC that restrains anchorage-dependent and anchorage-independent growth *in vitro* and tumorigenicity *in vivo* of lung cancer cells. In this setting, we also demonstrate that loss or mutations of *PTPN13* contribute to the increase of signaling from EGFR and HER2 tyrosine kinase receptors in tumors.

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