From plasma membrane to cytoskeleton: a novel function for semaphorin 6A

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Abstract

Class III β-tubulin (TUBB3) overexpression has been reported in ovary, lung, breast, and gastric cancer patients. Currently, no clinical drugs are available for a specific targeting of TUBB3, whereas the investigational drug IDN5390 specifically interacts with TUBB3. To gain insight into the pathways leading to TUBB3 up-regulation, we did a human genome microarray analysis in A2780 cells made resistant to IDN5390 to identify selected pathways specifically disrupted in resistant cells. Using this approach, we discovered that semaphorin 6A (SEMA6A) is down-regulated not only in IDN5390-resistant cells but also in cells made resistant to cisplatin, topotecan, and doxorubicin, whereas no changes were noticed in paclitaxel- and gemcitabine-resistant cells. Acute treatment with IDN5390 was able to down-regulate SEMA6A in cells unselected for drug resistance. TUBB3 expression was assessed in A2780 clones with stable overexpression of SEMA6A and in a panel of clones in which silencing of the protein was obtained. Quantitative PCR was then used to check the modulation of SEMA6A as well as to assess the expression of TUBB3. TUBB3 was increased (median value, 5.4) and reduced (median value, 0.47) in cells with overexpression and silencing of SEMA6A, respectively. Thus, the findings indicate a correlation between the expression of SEMA6A and TUBB3. Then, we found that a form of 83 kDa of SEMA6A is expressed in the cytoskeleton in association with β-actin. These findings suggest for SEMA6A a novel function in the cytoskeleton and a role in modulating tubulin isotype composition and microtubule dynamics. [Mol Cancer Ther 2008;7(1):233–41]

Introduction

Microtubule interacting drugs are widely used in the treatment of solid tumors, such as advanced ovary, breast, and lung cancers, in which they represent a mainstay in the medical management of these diseases. Unfortunately, after an initial response in the first line, the vast majority of patients relapse and they do not further respond, with the consequent disease progression and poor outcome. Study of resistance mechanisms has shown that there is not a unique factor of drug resistance but that several genes are involved in the generation of the resistant phenotype, thereby precluding a successful response to chemotherapy (1).

Within the factors associated to drug resistance, increased expression of TUBB3 (class III β-tubulin) seems to play a prominent role in several tissues. In fact, TUBB3 has been reported overexpressed in several human cancers, such as lung (2), ovary (3, 4), gastric (5), and breast (6) cancers. A direct role in drug resistance is suggested by the finding that TUBB3 is able to reduce sensitivity to bound paclitaxel and decreased ability by the drug to suppress microtubule dynamics (7, 8). Therefore, TUBB3 appears an attractive target and TUBB3 targeting agents could improve our therapeutic arsenal against drug resistance. A first example of this is represented by the seco-taxane IDN5390, a compound able to selectively target TUBB3 (9). This drug exhibits unique properties of cytotoxic, antiangiogenic agent and inhibitor of cell motility (10) and is currently undergoing preclinical development.

Study of the role of TUBB3 is complicated by the fact that this, as all the tubulin isotypes, cannot be easily overexpressed or silenced because it is tightly regulated in the cells. Therefore, to get insights into the TUBB3-dependent pathways, we used IDN5390 as a tool to investigate the genes downstream regulated by TUBB3.

To this end, we generated two human ovarian adenocarcinoma cell lines (A2780CG1 and A2780PTX) resistant to IDN5390 and paclitaxel, respectively. Then, through a full-genome microarray screening, genes diversely regulated in the two drug-resistant cells were analyzed. Using this approach, we discovered that semaphorin 6A (SEMA6A) gene expression is dramatically down-regulated in A2780CG1 cells.

Semaphorins are secreted or transmembrane proteins initially identified through their role in axon guidance and later involved in regulation of cell motility, immune response, angiogenesis, and tumor progression (reviewed in refs. 11–13). Semaphorins contain a conserved extracellular

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domain of ~500 amino acids (Sema domain) that is shared with their receptors plexins and the two tyrosine kinase receptors MET and RON (11). The transmembrane protein SEMA6A exhibited elevated levels of mRNA expression in several renal tumor tissue samples and in renal clear cell carcinoma cell lines, whereas its recombinant soluble extracellular domain inhibited *in vivo* the angiogenesis induced by growth factor and tumor cell line (14). Here, we describe that SEMA6A is involved in drug resistance and is an inducer of TUBB3 expression. The detection of a novel cytoskeletal form of the protein supports its role in intracellular signaling.

Materials and Methods

Drugs

IDN5390 and paclitaxel were kindly provided by Indena and were diluted in absolute DMSO. These solutions were further diluted at each experimental day to achieve a 0.1% final DMSO concentration. All the other chemicals were purchased from Sigma unless otherwise specified.

Cell Cultures

A2780 human ovarian cancer cells were purchased from the European Collection of Cell Cultures. Culture media were selected according to the suggestions of European Collection of Cell Cultures. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2/95% air. A2780 cells resistant to paclitaxel (A2780PTX) and IDN5390 (A2780CG1) were obtained through stepwise increase drug concentration. To prevent the expression of P-glycoprotein as a factor of drug resistance in the continuous presence of the P-glycoprotein inhibitor SB-RA (10 μmol/L; kindly given by Iwao Ojima), A2780PTX were then grown in the presence of 20 nmol/L paclitaxel and SB-RA and A2780CG1 in the presence of 50 nmol/L IDN5390 and SB-RA to maintain the drug-resistant phenotype. However, these cell lines do not depend on drug presence for growth.

A2780CIS and A2780ADR resistant to cisplatin and doxorubicin were also purchased from European Collection of Cell Cultures. A2780TOP and A2780GEM, resistant to topotecan and gemcitabine, respectively, were obtained in our laboratory through stepwise increase drug concentration.

Growth Experiments

Cells were seeded in black 96-well flat-bottomed plates (Packard). After 24 h, media were replaced, and after one washing, media containing the drugs were added. A dose-response logarithmic curve was established in quadruplicates for each plate, starting from 0.1 to 10,000 nmol/L. Each experiment was done thrice. After 72 h of culture in the presence of the tested compounds, plates were harvested and the number of viable cells was estimated using the ATPlite kit (Perkin Elmer Life Science) and the automated luminometer Topcount (Perkin Elmer Life Science). The kit was employed according to the manufacturer's suggestions. For each drug/cell line, a dose-response curve was plotted and the IC_{50}s were then calculated by fitting the dose-effect curve data obtained in the three independent experiments with the sigmoid-Emax model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect.

Real-time Quantitative PCR

Total RNA was obtained from cultured cells using RNeasy plus mini kit (Qiagen) according to the manufacturer's directions. cDNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR was done using the iCycler iQ System (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad) in a final volume of 25 μL, starting with a 3-min template denaturation step at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The following primers were used: TUBB3 forward 5′-GCGAGATGTACGAAGACGAC-3′ and reverse 5′-TTTAGACACTGCTGGCTTCG-3′, primers 1 forward 5′-CCCTTCTCCGCTCGTCATTGG -3′ and reverse 5′-CCTTCGCAAGCCTTTGTCATTCC-3′, primers 2 forward 5′-AGGGAGTGATTCGGGAAAGTTACC-3′ and reverse 5′-ACAGACGCAGTAGACGGTGATG-3′, and primers 3 forward 5′-CACACATGCACACAACACATACAC-3′ and reverse 5′-GTATTTGTGTTTTGCAGGTTGGAAC-3′. To normalize the possible variation in sample concentration, in each experiment, quantitative PCR was done on glyceraldehyde-3-phosphate dehydrogenase mRNA with primers glyceraldehyde-3-phosphate dehydrogenase forward 5′-CTGACCTGCCGTCTAGAAA-3′ and reverse 5′-CCACCATGGAGAAGGGTGG-3′. The experiments were done two or more times and each time the samples were run in triplicate. The results were analyzed as described previously (9) using the Excel spreadsheet RelQuant (Bio-Rad) and the SD among the different experiments was calculated.

Northern Blot

Total RNA was obtained from cultured cells using TRI-Reagent solution (Molecular Research Center) according to the manufacturer's directions. mRNAs (2 μg) purified with Oligotex mRNA Kit (Qiagen) were run on 1% agarose formaldehyde gel and blotted on Hybond$^+$ nylon membrane (Amersham Pharmacia). Digoxin-labeled probes were obtained by PCR using 0.7 mmol/L alkali-labile DIG-11-dUTP (Roche) according to the manufacturer's directions. For the SEMA6A probe, a fragment corresponding to nucleotides 1 to 1045 of SEMA6A NM_020796 sequence was amplified using the plasmid pUSE-NMC (see below) as template. A 536-bp glyceraldehyde-3-phosphate dehydrogenase probe was obtained by PCR on A2780 cDNA. Hybridizations were done with DIG Easy Hyb solution (Roche) at 50°C and the chemiluminescent detection was carried out with Anti-Digoxigenin-AP and CDP-Star (Roche).

Western Blot Analysis

Total cellular proteins were obtained lysing the cells with EB buffer [20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100] in the presence of proteases and phosphatase inhibitors. Protein concentration was determined by spectrophotometer and proteins were run on 8% SDS denaturing gels. Western blots were done as described previously[5] on 50 μg total lysates, unless specified, with the following antibodies:

Table 1. IC_{50} (nmol/L) obtained in A2780, A2780PTX, and A2780CG1 after 72 h of drug exposure

	A2780	A2780PTX	A2780CG1	Fold resistance (A2780PTX)	Fold resistance (A2780CG1)
Paclitaxel	2.7 ± 1.9	24 ± 3.1	57.1 ± 9.3	8.9	21.1
IDN5390	27.4 ± 9.1	53.6 ± 1.9	458 ± 0.2	2.0	16.7
Cisplatin	614 ± 119	1011 ± 426	347 ± 44.4	1.6	0.6
Topotecan	8.5 ± 3.3	9.9 ± 1	7.5 ± 2.7	1.2	0.9
Doxorubicin	25.9 ± 7	11.4 ± 5.7	88.2 ± 20.3	0.4	3.4
Gemcitabine	11.6 ± 3.9	12.5 ± 1.3	3.3 ± 1.9	1.1	0.3

NOTE: Fold resistance was calculated by dividing IC_{50} obtained in resistant cells over the values noticed in parental A2780.

anti-human SEMA6A polyclonal antibody (1:500; R&D), anti-TUBB3 polyclonal antibody (1:5,000; Covance), anti-α-tubulin monoclonal antibody (1:5,000; Calbiochem), and anti-β-actin monoclonal antibody (1:10,000; Sigma). Blots were visualized by enhanced chemiluminescence procedures (Amersham, GE-Healthcare, or Pierce) as described by the manufacturers.

SEMA6A Expression Vector

SEMA6A cDNA was obtained from total RNA of human placenta using SuperScript II reverse transcriptase (Invitrogen) and a SEMA6A specific primer (SEM-R 5′-AAGCGGCCGCTTATGTACACGCATCATTGGGCT-3′). The coding sequence was PCR amplified with primers SEM-R and SEM-F (5′-CCGGATCCACTATGAGGTCAGAAGCCTTGCT-3′) and T/A cloned in pCR2.1 vector (Invitrogen). The sequence was verified to match to SEMA6A mRNA sequence (GenBank accession no. NM_020796). To add the HIS tag, the coding sequence without stop codon was then amplified with primers SEM-F and C-SEMR (5′-AAGCGGCCGCTGTACACGCATCATTGGGCTT-3′) and cloned in *Bam*HI/*Not*I sites of pET28a(+) vector (Novagen). The fragment containing the SEMA6A sequence fused to the sequence codifying 6XHIS tag at the COOH terminus was amplified with primers SEM-F and SEMR-6H (5′-AAGGGCCCTTTGTTAGCAGCCGGATCTCAATGG-3′) and cloned *Bam*HI/*Apa*I in pUSE plasmid (Upstate Biotechnology) obtaining the expression construct pUSE-NMC.

SEMA6A Interfering Vector

GeneScript software allowed to identify a small interfering RNA target site in SEMA6A mRNA (nucleotides 3004-3022) and to design a small DNA insert coding for a specific short hairpin RNA. This insert (obtained by the annealing of the oligonucleotides si1-F 5′-GATCCCGTTTGTGCAGGCATTGATGAGGTTGATATCCGCCTCATCAATGCCTGCACAAATTTTTTCCAAA-3′and si1-R 5′-AGCTTTTGGAAAAAATTTGTGCAGGCATTGATGAGGCGGATATCAACCTCATCAATGCCTGCACAAACGG-3′) was subsequently cloned in *Bam*HI/*Hind*III sites of the small interfering RNA expression vector pRNAT-U6.1/Neo (GeneScript).

As a control, the construct siC was built, expressing a small interfering RNA that targets a scramble sequence. For siC, the annealed oligonucleotides were si1C-F 5′-GATCCCGTTAGTCTGATATGGGATGGGCTTGATATCCGGCCCATCCCATATCAGACTAATTTTTTCCAAA-3′ and si1C-R 5′-AGCTTTTGGAAAAAATTAGTCTGATATGGGATGGGCCGGATATCAAGCCCATCCCATATCAGACTAACGG-3′.

Stable Transfection of A2780 Cell Line

A2780 cells were transfected with the described vectors using 2 μg DNA per 1×10^6 cells. DNA was electroporated using a Gene Pulser (Bio-Rad) at 350 V, 500 μF. Cells were allowed to recover for 48 h, after which the medium was

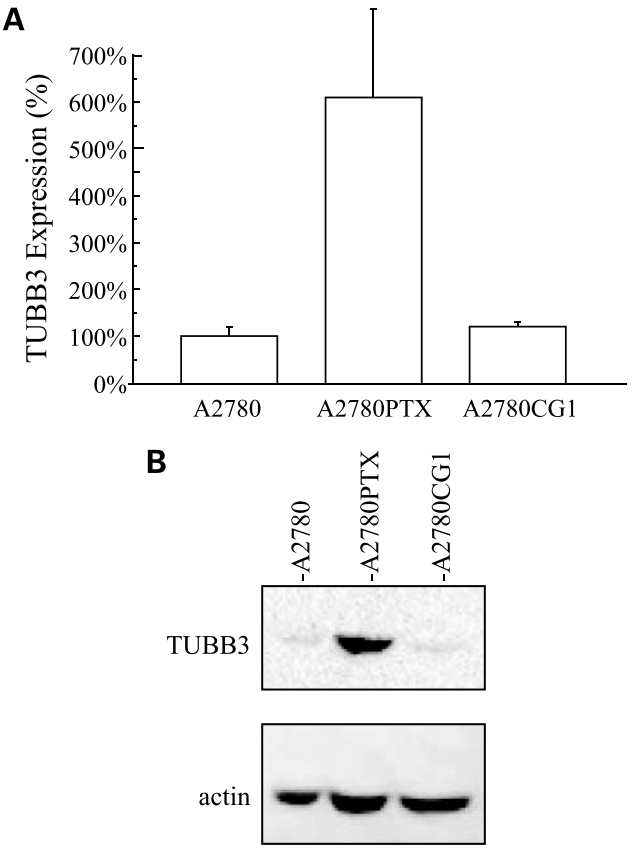

Figure 1. **A,** real-time PCR analysis of TUBB3 mRNA expression on A2780PTX and A2780CG1 cell lines compared with A2780 cells. *Y axis,* relative fold expression of TUBB3 mRNA. **B,** Western blot for TUBB3 protein on the A2780-resistant cell lines. The incubation with anti-β-actin was used as loading control.

removed and replaced with fresh medium containing 1.5 mg/mL G418 (Sigma). Fresh G418-containing medium was added every 3 days until visible colonies appeared. After three to four passages, cells were collected and real-time PCR analysis was done to evaluate the mRNA expression of SEMA6A and TUBB3 genes (primers 3 and TUBB3 described above). PCR analysis confirmed the expression of exogenous SEMA6A mRNA in SEMA6A(+) clones obtained by transfection with pUSE-NMC vector: HIS-F2 and HIS-R2 primers (5′-CCACCACCACCACCA-CTGAG-3′ and 5′-TGGCTGGCAACTAGAAGGCAC-3′, respectively) were used in standard PCRs on cDNAs obtained using iScript cDNA Synthesis Kit (Bio-Rad).

Cytoskeleton Isolation

Cell pellets were homogenated in PB buffer [0.1 mol/L K-Pipes (pH 6.8), 0.5 mmol/L $MgCl_2$, 2 mmol/L EGTA, 0.1 mmol/L EDTA, 1 mmol/L ATP, protease and phosphatase inhibitors] and centrifuged at 100,000 × *g* for 60 min at 4°C. Cytosolic supernatants were collected and mixed with half-volume of 100% glycerol preheated at 37°C, and GTP, ATP, and $MgCl_2$ were added at the concentrations of 0.1, 1, and 3.5 mmol/L, respectively, and incubated at 37°C for 60 min to allow tubulin polymerization. After centrifugation at 100,000 × *g* for 45 min at 37°C, pellets were collected, resuspended with ice-cold EB buffer with proteases and phosphatase inhibitors. Protein concentration was determined by spectrophotometry and proteins were run on SDS denaturing gel.

Two-Dimensional Gel Electrophoresis

Proteins were dissolved in the isoelectric focusing solution containing 8 mol/L urea, 4% CHAPS, and 2% carrier ampholyte (pH 3-10). The IPG strips (pH 3-10 nonlinear, 7 cm long; GE-Healthcare) were rehydrated with isoelectric focusing solution with 0.002% bromophenol blue containing protein sample to carry out analytical analysis in Ettan IPGphor II (GE-Healthcare) for 12 h at 20°C. The

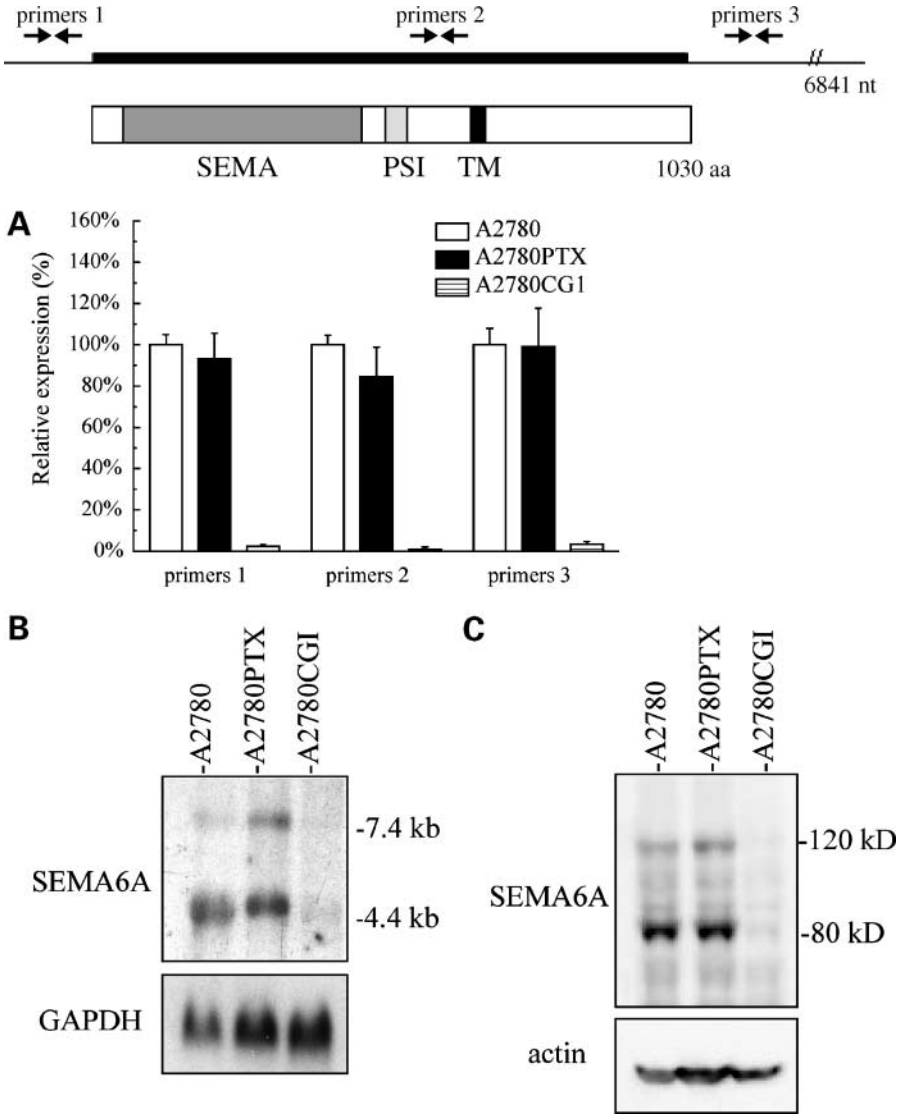

Figure 2. SEMA6A is down-regulated in A2780CG1 cell line. **A,** real-time PCR analysis of SEMA6A mRNA expression on A2780PTX and A2780CG1 cells compared with A2780 cells. The position of the primers used is shown on SEMA6A mRNA in the scheme above (*thin bar*). In the representation of SEMA6A protein (*thick bar*), the Sema domain (*SEMA*), the PSI domain (*PSI*), and the transmembrane domain (*TM*) are indicated. **B,** Northern blot analysis of SEMA6A mRNA expression on A2780, A2780PTX, and A2780CG1 cells. The hybridization with the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe allows the normalization of the samples. **C,** Western blot for SEMA6A protein expression analysis on the A2780, A2780PTX, and A2780CG1 cell lines. The incubation with anti-β-actin was used as loading control.

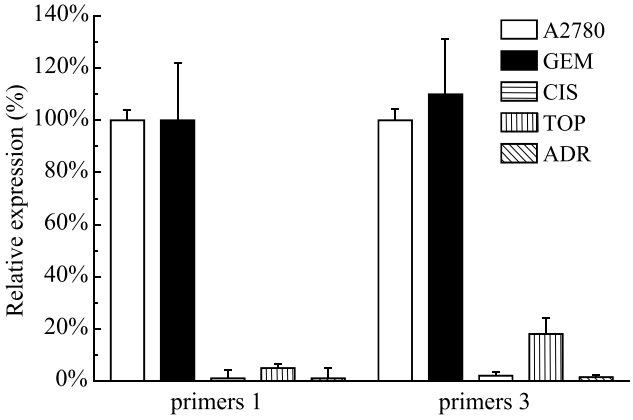

Figure 3. SEMA6A is down-regulated in different drug-resistant cell lines. Real-time PCR analysis of SEMA6A mRNA expression on A2780 cell lines made resistant to gemcitabine (*GEM*), cisplatin (*CIS*), topotecan (*TOP*), and doxorubicin (*ADR*) compared with the expression of the parental A2780 cells. The experiments were done with primers 1 and primers 3.

rehydrated strips were focused at 20°C using the following running conditions: 300 V for 4 h, voltage gradient from 300 to 1,000 V in 1 h, voltage gradient from 1,000 to 5,000 V in 2 h, and 5,000 V for 40 min. Before running the second dimension, the IPG strips were equilibrated for 15 min in equilibration buffer [6 mol/L urea, 2% (w/v) SDS, 50 mmol/L Tris-HCl buffer (pH 8.8), 30% (v/v) glycerol] with 10 mg/mL DTT and for 15 min with equilibration buffer containing 25 mg/ml IAA. Electrophoresis in the second dimension was carried out in 10% polyacrylamide gels. The IPG strips were embedded with 0.5% (w/v) melted agarose solution before running on the SDS-PAGE slabs. Standard blotting was then done (3 h at 380 mA, with TE62 Transphor; Amersham-Biosciences).

Immunoprecipitation Analysis

Cytoskeleton proteins were resuspended in RIP buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% NP-40] for the depolymerization step. Extract for each sample (100 μg) was precleared with 30 μL protein A/G Plus-Agarose suspension (Santa Cruz) in RIP buffer for 2 h at 4°C. After centrifugation, the beads were discarded and the extract was incubated with 4 μg β-actin (clone AC-15; Sigma), α-tubulin (clone DM1A; Calbiochem), γ-actin (Chemicon), or rabbit anti-goat IgG horseradish peroxidase (Bio-Rad) as control, overnight at 4°C. Protein A/G Plus-Agarose suspension was added followed by incubation 2 h at 4°C. Following centrifugation, the proteins of the supernatant fraction were recovered, and the collected beads were washed thrice with RIP buffer; the last wash was done by adding 1% NP-40. The precipitated proteins were resuspended in SDS loading buffer, boiled, and run on 10% SDS-denaturing gel for Western blot analysis.

Results

To get functional insights into the pathways dependent on TUBB3, we generated from A2780 human ovarian cancer cells two cell lines, A2780CG1 and A2780PTX, resistant to IDN5390 and paclitaxel, respectively. Growth inhibition effects obtained with the most commonly used chemotherapeutics are reported in Table 1. As a first approach, we looked at TUBB3 expression in both cells lines (Fig. 1A and B). As expected, TUBB3 was up-regulated in A2780PTX, whereas in A2780CG1 the up-regulation was not present at either mRNA or protein level, confirming the targeting of TUBB3 by IDN5390.

With the aim to identify genes differentially regulated in the two cell lines, we did a full-genome microarray screening (data not shown). From this analysis, we found that SEMA6A (NM_020796) was down-regulated in A2780CG1. Results of microarray analysis were then validated using quantitative PCR and a set of three primers designed at the 5′, 3′, and coding regions of the gene (Fig. 2). In A2780CG1 cells, SEMA6A was dramatically down-regulated as measured with all the three set of primers, whereas no significant changes in the expression were observable in A2780PTX. By Northern blot analysis (Fig. 2B), two transcripts of 5 and 7 kb in length were detected as already described in normal adult tissues (15). The almost undetectable mRNA expression of SEMA6A in A2780CG1 cells was confirmed. To assess the expression at the protein level, Western blot analysis was done with an anti-SEMA6A antibody detecting the Sema domain of the protein (Fig. 2C). In addition to the full-length SEMA6A protein [114.4 kDa predicted, probably glycosylated (12) to give a 120-kDa form], an ~83-kDa form was present in A2780 and A2780PTX cell lines. Both forms are down-regulated in A2780CG1 cell line, indicating a correlation of SEMA6A expression at the mRNA and protein levels.

To determine whether the modulation in SEMA6A expression occurs also in other drug-resistant cell lines, quantitative PCR analysis was done on A2780GEM, A2780CIS, A2780TOP, and A2780ADR cell lines made resistant to chemotherapeutics gemcitabine, cisplatin, topotecan, and doxorubicin, respectively (Fig. 3). Interestingly, a relevant down-regulation was noticeable in all the cell

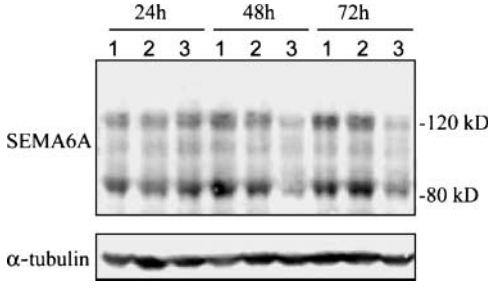

Figure 4. SEMA6A is down-regulated by treatment with IDN5390 in parental A2780 cells. Western blot analysis of SEMA6A protein without treatment (*lane 1*) or after a treatment with 5 nmol/L paclitaxel (*lane 2*) or 25 nmol/L IDN5390 (*lane 3*). The analysis was done after 24, 48, and 72 h of treatment. The incubation with anti-α-tubulin was used as loading control.

lines, with the exception of A2780GEM, suggesting that SEMA6A expression is altered in different drug-resistant pathways.

To determine the effects of acute drug exposure in A2780 parental cells, we investigated the protein expression after treatment with equitoxic doses of paclitaxel and IDN5390 (Fig. 4). Only IDN5390 treatment was able to induce after 48 h a significant decrease of expression of both SEMA6A forms, whereas paclitaxel did not affect the levels of the protein. In this short exposure, TUBB3 levels remain unmodified by both drug treatments (data not shown). Acute treatment with other chemotherapeutics, such as doxorubicin, cisplatin, topotecan, and gemcitabine, did not affect SEMA6A expression (Fig. 5). This experiment suggests that only IDN5390 exerts its effects by a direct down-regulation of SEMA6A expression, linked later to the absence of TUBB3 overexpression in resistant cells (A2780CG1).

Because IDN5390 is able to preferentially interact with TUBB3, we investigated the correlation between SEMA6A and TUBB3 gene expression. Stable cell clones were obtained either overexpressing SEMA6A coding region [SEMA6A(+) cells] or transfected with empty vector (pUSE cells). In addition, SEMA6A(−) stable clones were selected, where the SEMA6A gene was interfered using a small interfering RNA expression vector (pRNAT-U6.1/Neo); as control, we used the same vector, in which the targeted SEMA6A sequence was scrambled [SEMA6A(C) cell clones]. Representative Western blot analysis of few clones is shown in Fig. 6A: the experiment shows an increase in TUBB3 expression in SEMA6A(+) cells. A wider and quantitative analysis of SEMA6A and TUBB3 expression was done on mRNA by real-time PCR on a panel of 20 cell

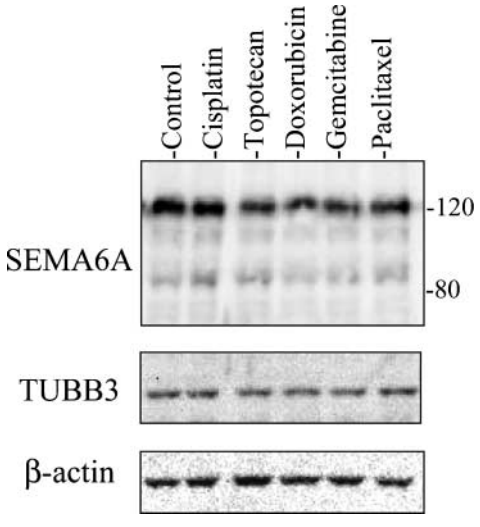

Figure 5. SEMA6A expression in parental A2780 cells is not modulated on acute exposure to chemotherapeutics. Western blot showing expression levels of TUBB3 and SEMA6A in parental A2780 cells on 48 h of acute drug exposure at equitoxic doses (IC_{50}) of cisplatin, topotecan, doxorubicin, gemcitabine, and paclitaxel.

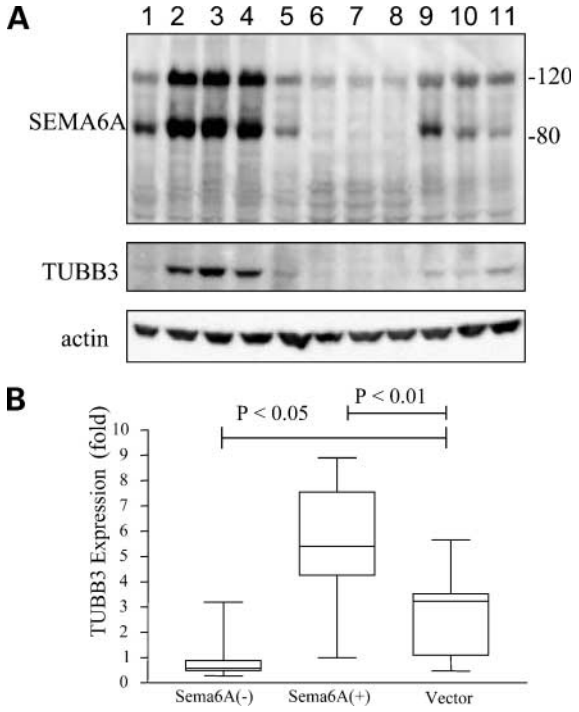

Figure 6. Modulation of SEMA6A expression correlates with TUBB3 expression modulation. **A,** Western blot analysis of A2780 cell clones either overexpressing SEMA6A coding region (*lanes 2-4*), transfected with empty vector (*lane 5*), interfered for SEMA6A expression (*lanes 6-8*), or transfected with a vector of control for interference (*lanes 9-11*). *Lane 1,* A2780 control. Lysates were immunoblotted with antibodies to SEMA6A, TUBB3, and lastly to actin. **B,** real-time PCR analysis of TUBB3 mRNA expression on SEMA6A(+) A2780 cell clones overexpressing SEMA6A coding region, SEMA6A(−) clones interfered for SEMA6A expression, and A2780 cell clones transfected with control vectors. The TUBB3 fold expression values were compared with A2780 cells (=1).

clones from SEMA6A(+) and SEMA6A(−) groups. In parallel, 10 clones from each vector group were analyzed (Fig. 6B). Compared with the expression levels measured in A2780 cells (=1), in SEMA6A(+) cells, a detectable up-regulation of TUBB3 was noticed (mean, 5.4-fold). On the other hand, silencing of the gene in SEMA6A(−) decreased TUBB3 expression up to 0.47-fold. Vector groups also exhibited an increase of TUBB3 expression with respect to A2780, but its extent was lower than that noticed in SEMA6A(+) cells. Compared with the levels noticed in the vector group, changes of TUBB3 expression in SEMA6A(+) and SEMA6A(−) cells were statistically significant at $P < 0.001$ and $P < 0.05$, respectively.

Due to the effect that SEMA6A has on TUBB3 expression, we analyzed if the protein was detectable in the cytoskeleton, which was isolated using ultracentrifugation. Cytoskeletal fraction was purified from A2780 cells and from SEMA6A(+) clones overexpressing the SEMA6A coding region. By Western blotting analysis, a signal was found in this fraction, corresponding to the 83-kDa form of SEMA6A protein, whereas the full-length 120-kDa form is almost undetectable (Fig. 7A). To better resolve

this band, two-dimensional gel analysis was done in a SEMA6A(+) cell clone (Fig. 7B). This analysis allowed to identify a pattern of acidic post-translational changes in the 83-kDa form, thereby indicating that cytoskeleton compartmentalization of SEMA6A is linked to specific post-translational changes.

It is known that human SEMA6A protein binds EVL protein (15), a member of the ENA/VASP family of protein involved in filament dynamics control and actin-based motility. To analyze if SEMA6A in the cytoskeletal fraction was associated with other proteins, we did coimmunoprecipitation experiments, employing antibodies specific for β-actin, γ-actin, and α-tubulin (Fig. 7C). These experiments revealed that the 83-kDa form of the SEMA6A protein is associated in a complex with β-actin and not with γ-actin and α-tubulin.

Discussion

Drug resistance represents a major obstacle to improve the overall response and survival of cancer patients. From *in vitro* models of resistant cells, multiple mechanisms responsible for the resistant phenotype have been studied. In particular, TUBB3 expression is associated with poor prognosis and resistance to chemotherapy in a variety of cancers, including ovarian (3, 4, 16), lung (2), gastric (5), breast (6), pancreas (17), and unknown site (18).

Several reports link semaphorins to the control of tumorigenesis and tumor progression both positively and negatively (reviewed in ref. 13). Among the semaphorins of class III, SEMA3A, SEMA3B, and SEMA3F may play a suppressive role in tumorigenesis, whereas overexpression of SEMA3E and SEMA3C are associated with the invasive behavior of tumor cells. Membrane-bound SEMA4D can trigger the activation of the oncogenic receptor MET, which is associated with plexin-B1 on the cell surface (19). Interestingly, SEMA6A gene was mapped to 5q21-22, which is known to be deleted in certain forms of lung cancer (20), and on the other hand, elevated levels of SEMA6A mRNA expression were shown in several renal tumor tissue samples and in renal clear cell carcinoma cell lines (14).

The present study describes that the expression of SEMA6A in A2780 human ovarian cancer cells and its relationship with TUBB3. Because the seco-taxane IDN5390 is able to target TUBB3 (9), the results suggest that SEMA6A and TUBB3 are involved in the same pathway of resistance. This hypothesis is supported by the experiments of correlation of the two genes: the overexpression of SEMA6A in A2780 cells correlated with the up-regulation of TUBB3, whereas in SEMA6A-interfered cells the expression of TUBB3 decreased. A significant down-regulation of the SEMA6A mRNA was also noticed in A2780 cell lines resistant to cisplatin, topotecan, and doxorubicin, thereby revealing that the mechanism is not specific for IDN5390 and suggesting a more general role in drug resistance. However, if in the case of IDN5390 there is a direct SEMA6A down-regulation when cells are acutely exposed to the drug, in the case of the other drug-resistant cell lines SEMA6A down-regulation is obtained only when cells acquire the drug-resistant phenotype associated to TUBB3 up-regulation. This finding enforces the recent discovery that TUBB3 overexpression occurs not only with microtubule interacting drugs but also for DNA-damaging drugs, such as cisplatin (21).

A common theme in the mechanisms of semaphorin function is that they alter the cytoskeleton and the organization of actin filaments and the microtubule network (12). Semaphorins exert the majority of their effects by serving as ligands and binding to other proteins through their extracellular domains (12), but intracellular signaling was also shown. A potential indirect linkage of transmembranous semaphorins to cytoskeletal element-binding proteins was firstly provided by the finding that SEMA6B binds the protooncogene c-Src, a protein kinase that phosphorylates ENA/VASP (22). Activation of SEMA6D leads to Abl kinase activation, phosphorylation of ENA, and its dissociation from the semaphorin cytoplasmatic tail (23). Klostermann et al. (15) have indirectly linked SEMA6A signaling to cytoskeletal elements, showing the

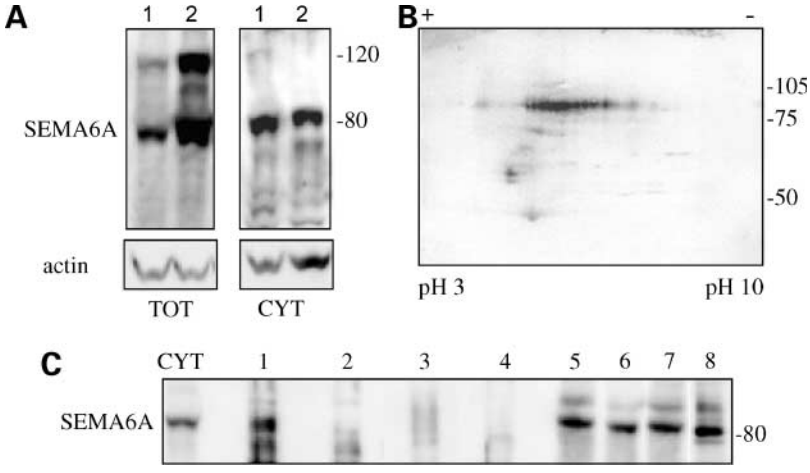

Figure 7. A SEMA6A protein form is linked to the cytoskeletal fraction. **A,** SEMA6A protein was analyzed on 50 μg total lysates (*lane TOT*) and 20 μg cyoskeletal extracts (*lane CYT*). Samples from an overexpressing SEMA6A clone (*lane 2*) were compared with samples of A2780 cells (*lane 1*). **B,** two-dimensional gel electrophoresis analysis of 50 μg cytoskeletal fraction of the same SEMA6A(+) clone as in **A. C,** SEMA6A protein of the A2780 cytoskeletal fraction (*lane CYT*) was assayed for coimmunoprecipitation with anti-β-actin (*lane 1*), anti-α-tubulin (*lane 2*), anti-γ-actin (*lane 3*), or a secondary antibody as control (*lane 4*). The proteins of the corresponding supernatant fraction were also loaded (*lanes 5-8*, respectively).

interaction of SEMA6A with EVL protein and thereby suggesting a role in retrograde signaling and cytoskeletal rearrangement. In this context, we show that a SEMA6A protein form is present in the cytoskeletal fraction. The cytoskeletal protein is a fragment of ~83 kDa, which includes the Sema domain, because it is recognized by the specific antibody used throughout this study. We did not yet characterize if this fragment is a product of an alternative mRNA splicing of the gene or a cleavage product of the full-length protein; indeed, both mechanisms were reported to amplify the repertoire of semaphorin functions. In fact, within class 6 semaphorins, different isoforms derived from alternative splicing were identified for SEMA6C and SEMA6D (24), with differentially regulated expression, as well as for SEMA6B (25). On the other hand, several secreted and transmembrane semaphorins undergo proteolytic processing, which correlates with different cellular functions (12).

Many reports illustrate that the Sema domain provides a common structural scaffold that can be adapted to mediate a diverse range of protein/protein interactions, such as dimerization, semaphorin/plexin binding, semaphorin/neuropilin binding, MET/HGF-SF and RON-HGF1/MSP (26), and possibly yet undiscovered targets. The presence of a SEMA6A form in the cytoskeleton opens a novel prospective for the study of the signals involving cytoskeleton-targeted agents. At the moment, the molecular mechanism remains to be identified, but for homology with that occurring at the plasma membrane we can hypothesize that SEMA6A could interact through the Sema domain with itself or maybe with other Sema domain proteins, so initiating or participating to a signaling cascade involved in the drug response and/or in the resistance process.

From a functional point of view, often actin and tubulin are considered as belonging to two independent systems. Actually, the two systems profoundly interact and recent studies have shown a complex network of interactions mediated by several molecular components (reviewed in ref. 27), which establish a crosstalk and a cross-regulation between the two systems. In particular, in leukemic cells and patients, where drug responsiveness occurs without involvement of TUBB3, it was recently reported that resistance to *Vinca* alkaloids was accompanied by mutations and decreased expression of γ-actin (28). In this work, we noticed that SEMA6A did not directly interact with γ-actin or α-tubulin but with β-actin. This finding suggests that the network of interactions within cytoskeleton may involve the actin and tubulin systems in a tissue-dependent way, with specific signals likely associated to a given tubulin isotype.

In summary, this work shows that a SEMA6A form of 83 kDa, including the Sema domain, is expressed in the cytoskeleton in a complex with β-actin. SEMA6A is able to regulate tubulin isotype composition, thereby indirectly affecting microtubule dynamics and drug sensitivity. This process seems to be disrupted not only in cells resistant to IDN5390 but also in cells exhibiting drug resistance to other chemotherapeutics, such as cisplatin, topotecan, and doxorubicin, drugs not having microtubules as a target. Comprehension of these signaling pathways could open novel therapeutic avenues and prompt innovative strategies to circumvent drug resistance in cancer cells.

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