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Analysis of *SEMA6B* gene expression in breast cancer: Identification of a new isoform

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ABSTRACT

Background: SEMA6B is a member of the semaphorins axon-guidance family. A growing body of evidence has been accumulated describing the role of semaphorin molecules in cancer development and the involvement of *SEMA6B* in cancer progression has recently been proposed.
Methods: Our analysis, based on real-time PCR, focused on the expression of *SEMA6B* in a panel of breast cancer tissues, compared to the normal counterpart.
Results: In cancer tissues we found a significantly strong down-modulation of this transcript. Moreover we identified and characterized a novel *SEMA6B* isoform, named *SEMA6B*a. This isoform has a novel splice junction, created by the usage of alternative donor and acceptor splice sites internal to the exon 17. By *in silico* analysis we found that the new transcript 3′ UTR lacks some highly-conserved miRNA binding sites, suggesting possible consequences on both spatial and temporal expression of *SEMA6Ba.* The translated sequence of *SEMA6Ba* lacks the cytoplasmic tail, crucial for triggering the reverse signaling described for the transmembrane semaphorins. We also demonstrated, by immunofluorescence analysis of endogenous and overexpressed *SEMA6Ba*, that the protein clearly localized to the endoplasmic reticulum and plasma membrane. In conclusion, *SEMA6B* gene products are strongly down modulated in breast cancer tissues and a new isoform named *SEMA6Ba* has been described and characterized.
General significance: Our work states a clear relation among breast cancer and *SEMA6B* expression; moreover we describe for the first time the *SEMA6Ba* protein and report here the analysis of *SEMA6Ba* RNA messenger, the protein expression and the cellular localization.

1. Introduction

Semaphorins were initially identified as evolutionarily conserved axon-guidance cues in the assembly of the neural circuitry [1,2]. However, it is now clear that they are widely expressed outside the nervous system, and are implicated in a range of processes, including regulation of cell survival, apoptosis, cell–substrate adhesion and directional cell migration [3–5]. In addition to their function in a plethora of basic cellular processes, several studies have shown that semaphorin-mediated signals might also play important regulatory functions in cancer [6,7]. Semaphorins are secreted or membrane-associated glycoproteins characterized by an N-terminal 500 aminoacid sema domain [8,9]. They consist of more than twenty family members and are grouped into eight classes, based on their structural elements and the amino acid sequence similarity. Invertebrate semaphorins mainly fall into classes 1 and 2, whereas classes 3 to 7 comprise vertebrate semaphorins and the final group (class V) contains semaphorins encoded by viral genomes.

Class 3 semaphorins are the only secreted vertebrate semaphorins, while class 4–7 semaphorins are cell membrane-anchored proteins. Such semaphorins can be further processed into soluble forms through proteolytic degradation, as observed for SEMA4D [10,11]. Membrane-bound semaphorins that do not appear to be processed in soluble forms, such as semaphorin 6 (from A to D), are involved in reverse signaling mechanisms [12–14].

The human *SEMA6B* gene was first identified in 2001 by EST sequencing from a cDNA pool of breast cancer donors, and its expression was described in MCF-7 cell line, a widely used breast cancer-derived cell line [15]. Three spliced isoforms were described by *in silico* analysis

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although, to date, two of them have not been validated and have been removed from databases.

In 2007 Kuznetsova and colleagues [16] demonstrated the *SEMA6B* promoter to be abnormally methylated in breast cancer tissue and its expression to be down-modulated in 44% of analyzed samples. On the other hand it has been more recently reported that *SEMA6B* RNAi in U87MG cells induces a significant impairment of cell proliferation ability and *in vivo* tumor growth [17].

In this paper we describe a quantitative analysis of *SEMA6B* expression in a panel of breast cancer bioptic samples, classified for the estrogen receptor (ER) expression and the metastatic distribution. Along with these information, and given the increasing number of newly identified alternative splice isoforms for many human genes, as well as their role in cancer and other genetic diseases, we also provide evidence for the expression of *SEMA6B* alternative isoforms. In particular, herein we describe the identification and characterization of a novel transcript, referred as *SEMA6B*a, encoding a new protein isoform which lacks the C-terminus domains still retaining its N-terminus SEMA domain.

2. Methods

2.1. Cell lines and tissue samples

MCF-7, MDA-MB-231 and HK293T cells were cultued in DMEM (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (Lonza), antibiotics and 10 mM L-glutamine (Sigma-Aldrich S.r.l. Milan, Italy). HeLa cells were grown in MEM supplemented with 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin, 10% FBS and 100 μM MEM non-essential amino acids solution containing glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-serine. All of these products were purchased from Gibco/BRL (NY, USA). Peripheral blood mononuclear cells (PBMCs) and dendritic cells were derived as reported in D'Apice [18], Jurkat, EBV derived B cells and 1512 cells were grown in RPMI 1640 (Lonza) supplemented with 10% (v/v) fetal calf serum (Lonza), antibiotics and 10 mM L-glutamine (Sigma). Specimens from normal breast and cancer tissue from 33 women who had undergone surgery at the Salvatore Maugeri Foundation in Pavia were collected and snap frozen. Breast cancer ($n = 33$) and normal matched healthy tissues ($n = 16$) were collected immediately after excision during surgery, and stored at −80 °C until use. A consultant pathologist examined hematoxylin and eosin stained frozen sections to verify the presence of tumor cells in the collected samples. Normal tissue was derived from the background breast parenchyma of breast cancer patients within the study group. The study was approved by the local Independent Ethics Committee and the patients entered the protocol after signed the informed consent. The specimens were fully characterized and collected at the Institutional Biobank. The characteristic of patients' tumor samples was reported in Supplementary Material Table 1.

2.2. RNA extraction

Total RNA from cells was extracted using TRI reagent (Sigma) according to the manufacturer's instructions; for biopsy, tissue samples were cut in small pieces and homogenized in TRI reagent with the use of a polytron. 300 μg of proteinase K (Sigma) was added during the extraction according to Egyházi [19]. RNA concentration was measured using nanodrop (Thermo Fisher Scientific, Illkirch France scientific); 1 μg of RNA was used in reverse transcription reaction in a 20 μl reaction mixture using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas).

2.3. Plasmids, end point PCR cloning and sequencing

End point PCR reactions were performed in 20 μl using 0.5 μl cDNA, 0.4 μM for each primer, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs) and 1 U Taq DNA polymerase (High fidelity Taq Invitrogen). All the primers used are listed in Supplementary Material Table 2. The temperature cycling protocol was 94 °C 30 s, 55–60 °C 30 s (adapted for each primer pair) and 72 °C 45–90 s (adapted to amplicon). The cycling was repeated 35 times. Each PCR started with a denaturation step of 5 min and ended with 10 min of extention at 72 °C. PCR products were analyzed on agarose electrophoresis gel and purified using the Quiagen PCR cloning kit (Quiagen, Milan, Italy) according to the manufacturer's guidelines, and sent to a commercial sequencing facility (Primm, Milan, Italy) for DNA sequencing.

For *SEMA6Ba*-GFP the PCR product, obtained using the primers 5′ Eco*SEMA*, 3′Sal*SEMA6Ba* (Supplementary material Table 3), was cloned into the pEGFPN3 vector (Clontech) using the EcoRI-SalI restriction enzimes (New England Biolabs) producing the pSEMA6Ba-GFP plasmid. All the primers used were synthesized by Primm (Milan, Italy).

For *SEMA6Ba*-Myc construct, the *SEMA6Ba* construct was digested with EcoRI-Hind III restriction enzymes from pSEMA6Ba-GFP plasmid and ligated with the EcoRI-Hind III cutted pCDNA4/Myc-His vector (Invitrogen).

2.4. Real-time quantitative PCR

Real-time quantitative PCR was performed using the iCycler iQ System (Bio-Rad, Milan, Italy) and the iQ SYBR Green Supermix (Bio-Rad) in a final volume of 25 μl, starting with a 10 min template denaturation step at 95 °C followed by 40 cycles of 20 s at 95 °C, 20 s at 62 °C and 25 s at 72 °C. Melting curves were generated after amplification. All the primers used are listed in Supplementary material Table 3.

The primers used were 5′*SEMA6B*F and 5′*SEMA6B*R, designed into the exons 7–8 with >95% reaction efficiency. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [20].

To normalize the possible variation in sample concentration, hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) was used as reference gene, using the primers HPRT1-FWand HPRT1-REV. As calibrator the normal breast tissue samples were used. All samples were run as triplicates.

The absolute quantitation of *SEMA6B* messenger copy number was obtained using the primer pairs 5′*SEMA6B*F–5′*SEMA6B*R and 3′*SEMA6B*F–3′*SEMA6B*R (designed into the exon 17 and with >95% reaction efficiency). We produced a standard curve for each reaction using serial dilution of a pDrive (Quiagen) plasmid where the two amplicons corresponding to the product of the 5′*SEMA6B*F–5′*SEMA6B*R and 3′*SEMA6B*F–3′*SEMA6B*R were cloned, and derived the copy number of each sample by interpolation on the calibration curve. Each reaction was performed in triplicate and mean ± sd values were used in the data analysis.

2.5. Transfection

Lipofectamin 2000 (Invitrogen) was used according to the manufacturer's instructions; for immunofluorescence analysis, HeLa and MCF-7 cells were seeded at 20,000 cells/well in 24 well plates onto glass coverslips and the day after were transfected; cells where fixed 24 h after the transfections. For biochemical assays, HeLa and MCF-7 cells were seeded at 1.5×10^6/plate and the day after were transfected. 24 h after, cell culture supernatants were collected and cells were lysed.

2.6. RNA interference

Hela and MCF-7 cells were transfected with 100 and 150 nM Smart Pool of the SEMA6B SiRNA (Dharmacon; see Supplementary Table 4 for sequences) for 48 h using Lipofectamine 2000, according to the manufacturer's instructions. The efficiency of interference was assessed by Western blotting.