RESEARCH ARTICLE

Synaptic Vesicle Protein 2 (SV2) Isoforms

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Abstract

New molecular markers of cancer had emerged with novel applications in cancer prevention and therapeutics, including for breast cancer of unknown causes, which has a high impact on the health of women worldwide. The purpose of this research was to detemine protein and mRNA expression of synaptic vesicle 2 (SV2) isoforms A, B and C in breast cancer cell lines. Cultured cell lines MDA-MB-231, SKBR3, T47D were lysed and their protein and mRNA expression analyzed by real-time PCR and western blot technique, respectively. SV2A, B proteins were identified in non-tumor (MCF-10A) and tumor cell lines (MDA-MB-231 and T47D) while SV2C only was found in the T47D cell line. Furthermore, the genomic expression was consistent with protein expression for a such cell line, but in MDA-MB-231 there was no SV2B genomic expression, and the SV2C mRNA and protein were not found in the non tumoral cell line. These findings suggest a possible cellular transdifferentiation to neural character in breast cancer, of possible relevance to cancer development, and point to possible use of SV2 as molecular marker and a vehicle for cancer treatment with botulinum toxin.

Keywords: SV2A - SV2B - SV2C - breast-cancer - protein/gene expression - BoNTA

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Introduction

Breast cancer (BCa) in women is the most prevalent oncologic disease worldwide. In Mexico, BCa presents an increase in mortality ratio without plenty knows of cause (McPherson et al., 2000). In the mechanisms that lead to development and immunologic evasion of the cancer, the modification of molecular expression pattern is important where the vesicle traffic can play an important role in superficial molecular expression and transduction regulation that result in apoptosis evasion (Chan and Giaccia, 2007; Choi et al., 2007; Gillies and Gatenby, 2007; Lukashev et al., 2007; Coelho et al., 2010).

The presence of secretory vesicles is characteristic in synaptic (de Groot et al., 2010; 2011), gastric (Bumming et al., 2007), pancreatic (Jakobsen et al., 2002), adrenal (Li et al., 1999) and prostatic (Karsenty et al., 2009) cells which synaptic vesicular receptor (SV) play an important role in exocytosis and secretory process of synaptic (de Groot et al., 2010) and endocrine cells, (Dong et al., 2006; Coelho et al., 2010), but when cancer occurs this protein tends to overexpress in brain (de Groot et al., 2010), neuroendocrine (Portela-Gomes et al., 2000), gastrointestinal (Bumming et al., 2007) and prostate (Karsenty et al., 2009) tumors. The paper of SV in cancer is not clear neither it has been reported in breast cells, but its role as molecular marker of cancer in others organs and tissues has been increasing in recent years (Portela-Gomes et al., 2000; Zhang et al., 2010).

The importance of SV receptors is more evident when this cellular membrane receptor is bound to neurotransmissors, drugs and neurotoxins, such as tetanus toxin and botulinium neurotoxin (BoNTA), especially the type A (BoNTA), that in therapeutic doses and for endocytosis lets to improve conditions as epilepsy and cancer in a more specific manner (Arnon et al., 2001; Karsenty et al., 2009; de Groot et al., 2010; 2011; Montal, 2010; Blum et al., 2012; Shi et al., 2011) and lead to apoptosis (Choi et al., 2007). BoNTA has been successfully used in the treatment of refractory detrusor overactivity. The toxin is internalized after binding a high-affinity receptor, synaptic vesicle protein 2 (SV2), which is exposed in the cell membrane during the exocytosis process. In the cytoplasm, BoNTA cleaves specific sites of synaptosomal-associated protein 25 (SNAP-25), preventing the assembly of the synaptic fusion complex SNARE and blocking exocytosis (Simpson, 1979; Chancellor et al., 2008; Coelho et al., 2010). Thus, BoNTA inhibits the growth of LNCaP human prostate cancer cells in vitro and in vivo (Karsenty et al., 2009).

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Three SV2 isoforms had been described principally in brain. These isoforms are known as A, B and C, and they exhibit differential expression. SV2A is widely distributed in the nervous system, in virtually all neurons. SV2B is also widely expressed in SV2A-containing neurons, although not as extensively as SV2A, whereas SV2C is only observed in a small number of neurons in a few brain areas (Bajjalieh et al., 1993; 1994; Janz et al., 1998; Janz and Sudhof, 1999). The study of these isoforms would aid in the understanding of the role of this protein in secretions of mammary glands and breast cancer development in order to detect opportunely breast tumors and to treat them with novel and specific therapies. We propose to identify and to evaluate the expression of SV2 receptor isoforms in T47D and MDA-MB-231 BCa cell lines as these not are yet reported in the literature and we think these proteins can play an important paper in secretory function in breast, and cellular immortality and transdifferentiation, which may derivate in understanding cancer phenomenon, obtaining breast cancer-novel molecular markers to opportune detection, and its helpfully in new therapies.

Materials and Methods

Cell culture

The T47D and MDA-MB-231 cell lines were obtained from ATCC (Manassa, VA). MCF-10A cell line was a gift from Ph.D. Juan Pedro Luna Arias, CINVESTAV. T47D were cultured in RPMI-1640 (GIBCO). MCF-10A was cultured in DMEM F12 (GIBCO) supplemented with 4.18 μg/ml insulin, 10 μg/ml HEGF, 0.4 μg/ml Hydrocortisone. MDA-MB-231 and SKBR3 cell line was cultured in DMEM high glucose (Hyclon, Logan, Utah). All cells were supplemented with 10% FBS (GIBCO) and 1% penicillin/streptomycin (SIGMA). Cells were grown as monolayers under standard conditions at 37° in humidified atmosphere containing 5%CO_2 and 95% air.

Western blot analysis

The cells grown to 80-90% confluence and they were washed three times with PBS and then harvested with 5 mM EDTA on PBS. The cells were pelleted by centrifugation. The cell pellets were resuspended on Laemmli buffer without bromophenol blue and the cells were sonicated with 3 pulses at 100 W for 10 s and centrifuged. The supernatant was collected and a small aliquot was taken and it was precipitated and protein concentration was determined by the Lowry method. Equal amount of protein was taken and boiled for 2 min. The proteins were resolved by 12% SDS-PAGE. After the SDS-PAGE the gel was transferred onto nitrocellulose membrane by using a Bio-Rad Trans-Blot® Turbo™ Transfer System and followed by the addition of a blocking solution of powdered nonfat dried milk. The primary hybridization was performed with rabbit polyclonal anti-SV2A, SV2B or SV2C antibodies (Santa Cruz Biotechnologies) in a 1:1000 dilution. The secondary hybridization was realized with goat anti-rabbit secondary IgG antibodies, peroxidase-conjugated (Vector Laboratories). The autoradiography were scanned and quantified by densitometry using the ImageJ version 1.4 software (http://rsbweb.nih.gov/ij/index.html). The results were expressed as ratio of SV2 protein expression to β-actin protein expression.

RNA extraction

Total RNA from the cell lines was isolated using TRIzol Reagent according to the technical sheet protocol (Life Technologies, USA). Isolated RNA was quantified using the GENESYS 10 Series spectrophotometer (Thermo Scientific, USA) and 5 μg of RNA were separated on a 1.0 % agarose gel containing ethidium bromide in MOPS buffer. Running buffer and gel contained 0.2 M formaldehyde. To avoid trace amounts of DNA contamination, RNA samples were treated with amplification grade DNase I (Invitrogen) before reverse transcription. All RNA samples were stored at –70°C in RNA elution solution until further use.

RT-PCR assays

We used 0.5 μg of RNA for reverse-transcription with random hexamers in 20-μl reaction volume using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). The reactions were performed in Eppendorf Mastercycler thermocycler (Eppendorf, Mexico). The amplified cDNA was quantified on a photometer at 260 nm. RT-PCR reactions were carried out using the Human Universal ProbeLibrary (Roche Diagnostics). Specific oligonucleotide primers were originally designed by using the online assay design software (ProbeFinder: http://www.universalprobelibrary.com) and the primer sequence for each gene fragment is shown in Table 1. The 20 μl-reaction mixture contained 1x LightCycler TaqMan Master reaction mixture (Roche Diagnostics), 200 nM of each primer, 100 nM of Universal ProbeLibrary probe, 0.5 U LightCycler Uracil-DNAGlycosylase and 2 μl of standard DNA in appropriate dilution. The amplification was performed on borosilicate glass capillaries (Roche Diagnostics). The RT-PCR assay included a standard curve of four serial dilution points for each gene and samples were normalized with the endogenous control 18S gene. Data were analyzed by LightCycler software.

Statistics

In order to compare protein and mRNA expression we used One Way Analysis of Variance or Kruskal-Wallis One Way Analysis of Variance on Ranks according to the case. The difference between groups was determined with Tukey Test or Holm-Sidak method depending on the case. Statistical analysis was performed with SPSS v17 for Windows XP (SPSS, UK, Ltd, Woking, UK) and SigmaPlot for Windows v11.0. $P<0.05$ was regarded as significant.

Table 1. Primer Sets Used in Real-Time Pcr. The Design was Based on Ensemble Transcript Id of the Human ProbeLibrary

Gene	Nucleotide sequence accession number	Left primer 5'–3'	Right primer 5'–3'
SV2A	NM_014849.3	agatcggcaggctcagaat	gacaggaagaagcaggagaca
SV2B	NM_001167580.1	tggagcatcatcccacacta	tccagctatggaagtggtaattg
SV2C	NM_014979.1	tggcttctttctcttctgtcg	ccaggacttcagcaaagtacg

Results

SV2 Receptors were expressed highly on breast cancer cell lines.

The expression levels of SV2A, B and C receptor isoforms in breast cancer cell lines were analyzed for western blot assays (Figure 1). The expression of the three isoforms was found in MDA-MB-231 and T47D cancer cell lines. SV2A protein was expressed notably in T47D and MDA-MB-231 cancer cell lines in comparison with MCF-10A non-tumor cell line (P≤0.001), furthermore, SV2A was expressed more in T47D than MDA-MB-231 cell line (P=0.050, Figure 1A and B). SV2B protein was only higher than the control line in MDA-MB-231 cell line (P=0.004), although there was a notable basal expression in control line in comparison with each other two isoforms (Figure. 1 A and C). And the expression of SV2C was slightly higher T47D and MDA-MB-231 tumor cell lines, although it was higher in MDA-MB-231 (P=0.001) than in T47D and MCF-10A lines (Figure 1A and D).

Genic expressions of SV2 in breast cancer and non-tumor cell lines

Differences in the protein profile were compared with the mRNA levels in this study. Here, mRNA expression of SV2 isoforms was analyzed in three cancer cell lines and compared with an epithelial cell line of breast, non-transformed derived from human fibrocystic mammary tissue (Figure 2). The expression of different isoforms was not equal in each cell line. SV2A mRNA showed higher expression in cancer cell lines T47D and SRBK3 than in MCF-10A non-transformed cell line (P<0.05); in the same way, T47D showed more protein expression than MDA-MB-231, although MDA-MB-231 was not significantly different to MCF 10A (Figure 2A). SV2B isoform mRNA level, consistently with the protein expression, T47D had not higher mRNA level than MCF-10A cells, inclusive its expression is lower than control cell line and MDA-MB-231 did not express detectable mRNA, only SRBK3 breast cancer line (P<0.05) expressed a high level of mRNA (Figure 2B). On the other hand, SV2C isoform was only expressed in the T47D cell line, it was not detectable in control cell line, hence the relative expression was not calculated (data no show).

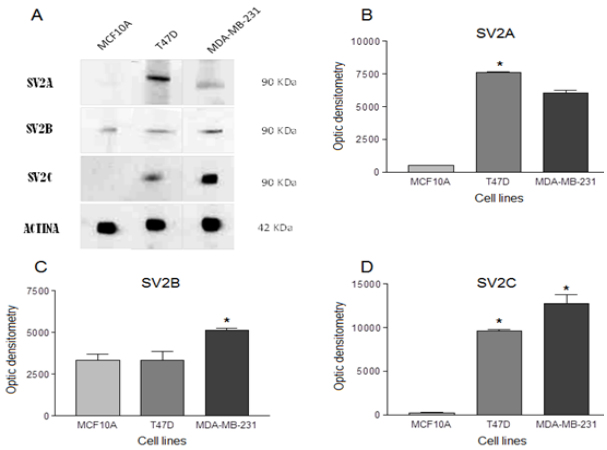

Figure 1. SV2 Protein is Expressed in Breast Cell Lines. A, different SV2 isoforms western blot; first lane MCF10A non-tumor cell line, second lane, T47D tumor line and third lane MDA-MB-231 tumor line. B, C and D, optic densitometry of the western blot. Data represent the mean±SD (n=3) for triplicate, *P vs. control cell line

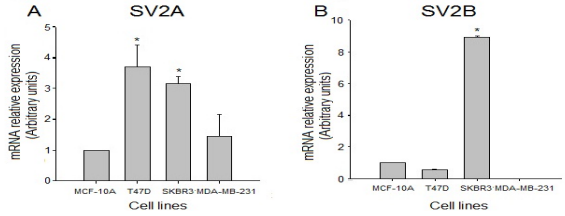

Figure 2. Genic Expression in Breast Cell Lines. Quantitative real-time PCR assays. Total RNA was prepared from cells as described in the Material and Methods section. MCF-10A human mammary epithelial cells as control. The relative expression level of genes SV2A and SV2B (A, B) was determined after normalization against the 18S internal control for each sample. Data represent the mean±SD (n=3) for triplicate, *P vs. control cell line

Discussion

SV2 receptor is a glycoprotein whose expression is characteristic of synaptic and secretory vesicles of glandular tissues as prostate, thyroid, parathyroid, pancreas, pituitary and adrenal medulla (Portela-Gomes et al., 2000), however it has been not clear the SV2 expression in normal breast neither in BCa. We are showing the evidence of expression of both SV2 protein and mRNA in non-tumor and transformed breast cell lines; we found SV2 A, B and C isoforms expression in breast cell lines. A recent research indicates that cells of the luminal epithelium of ducts and lobules of human mammary glands express neuroendocrine markers such as vesicular monoamine transporter 2 and chromogranin B, as well as the regulatory peptides obestatin, ghrelin, adrenomedullin and apelin (Gronberg et al., 2010), furthermore neuronal/glial markers (nestin, TUBB3 and GFAP) have been identified in MCF-10A, SKBR3 and MBA-MB-231 cell lines, although they are overexpressed in tumor-cell lines, these breast cancer lines are showing a multi-lineage differentiation potential of breast cancer cell lines to express multiple neuronal/glial lineage-specific markers as well as mammary epithelial and melanocytic-specific markers (Zhang et al., 2010). Furthermore, in the same study ectoderm multi-lineage transdifferentiation was also found in human melanoma (Ul-MeL) and glioblastoma cell lines (U87 and D54). These observations indicate that aberrant multi-lineage transdifferentiation or lineage infidelity may be a wide spread phenomenon in cancer. This is consistent with others findings of a neuroendocrine phenotype in gastrointestinal stromal tumors which regularly express synaptic vesicle proteins too (Bumming et al., 2007). All these suggest that mammary glands may have neuroendocrine functions, however, SV2 presence had not already been reported in tumor and non-tumor breast cells, and this is in part the importance of our work.

The SV2 protein expression is consistent with SV2 mRNA expression, thus both molecules keep a direct relation and SV2 control depends on other stimulus.

SV2B expression tends to be constitutive at simple sight because the protein expression is similar in non-tumor and MDA-MB-231 and T47D cell lines, despite SKBR3 cell line mRNA expression is higher. However, the A and B isoforms present superior expression in tumor cell lines than in non-tumor cells.

The role of SV2 in breast cells could be associated to secretory nature of mammary glands, and may interact with other vesicle proteins such as synaptobrevin, which is essential for secretion but not for the development of synaptic process (Ahnert-Hilger et al., 1996), however, the SV2 role in cancer is not clear, although it is already reported in others types of cancer, for instance, brain (de Groot et al., 2010; 2011), pancreas (Jakobsen et al., 2002), gastrointestinal tract (Jakobsen et al., 2002; Bumming et al., 2007), liver (Hanoun et al., 2010), bladder (Coelho et al., 2010), prostate (Karsenty et al., 2009) and adrenals (Li et al., 1999) tumors among others, where SV2 has already been proposed as molecular and transdifferentiation marker of neural nature (Nilsson et al., 2004; Zhang et al., 2010). This is possible because in the cancerous state the terminal differentiation to the anticipated cellular type is altered and the phenomena of lineage infidelity that is associated with the ability of cancer cells to transdifferentiate, occurs in different cancer types and occur in breast cancer (Zhang et al., 2010); thus, it is a commune phenomenon that cancer cell turns-off/-on non-habitual genes changing the ontogeny to evade the immune system and hold the linage independence.

Thus the SV2 role in cancer may be involved in regulation of membrane markers and transdifferentiation (Zhang et al., 2010), immune evasion (Lukashev, 2007), tumor angiogenesis and apoptosis inhibition (Choi et al., 2007), where exocytosis play an important role regulating external messenger interactions and internal signalizing.

SV2 expression can have a double importance as cancer molecular marker (Zhang et al., 2010) and therapeutic use in malignity due SV2 leads the entry of BoNTA, which has anti-proliferative, pro-apoptotic and quimiodenervation functions in prostate cancer (Arnon et al., 2001; Dong et al., 2006; Choi et al., 2007; Karsenty et al., 2009; Hanoun et al., 2010; Zhenzhen et al., 2012) that could be extrapolate to breast tumors. BoNTA is recognized by SV2 receptors in neurons. Once internalized by nerve terminals, BoNTA undergoes a pH-dependent conformational change that causes the dissociation of its heavy and light chains. After translocation to the cytosol, the light chain enzymatically cleaves specific sites of the synaptosomal-associated protein 25 (SNAP-25), preventing the SNARE mediated fusion of synaptic vesicles with the cellular membrane and, thus, blocking secretion release (Simpson, 1979; Chancellor et al., 2008) for secretory vesicle exocytosis (Schiavo et al., 2000). The antiproliferative role of BoNTA in prostate cancer (Karsenty et al., 2009) possibly is due to neuropeptides exocytosis inhibition. So, the ability of BoNT to block vesicle release through SV2 also provides the basis for their medical applications in therapy for breast cancer with this toxin or specifically-directed chimeric pharmaceutics drugs, and this is the relevance of our research; however, we believe this study will be complemented with histological and population studies in order to acquire more accurate knowledge of SV2 profile and behavior and to find a correlation between its distribution and prevalence.

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