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Functional Expression of Voltage-Gated Sodium Channels Nav1.5 in Human Breast Caner Cell Line MDA-MB-231

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Summary：Voltage-gated sodium channels (VGSCs) are known to be involved in the initiation and progression of many malignancies, and the different subtypes of VGSCs play important roles in the metastasis cascade of many tumors. This study investigated the functional expression of Nav1.5 and its effect on invasion behavior of human breast cancer cell line MDA-MB-231. The mRNA and protein expression of Nav1.5 was detected by real time PCR, Western Blot and immunofluorescence. The effects of Nav1.5 on cell proliferation, migration and invasion were respectively assessed by MTT and Transwell. The effects of Nav1.5 on the secretion of matrix metalloproteases (MMPs) by MDA-MB-231 were analyzed by RT-PCR. The over-expressed Nav1.5 was present on the membrane of MDA-MB-231 cells. The invasion ability *in vitro* and the MMP-9 mRNA expression were respectively decreased to (47.82±0.53)% and (43.97±0.64)% (P<0.05) respectively in MDA-MB-231 cells treated with VGSCs specific inhibitor tetrodotoxin (TTX) by blocking Nav1.5 activity. It was concluded that Nav1.5 functional expression potentiated the invasive behavior of human breast cancer cell line MDA-MB-231 by increasing the secretion of MMP-9.
Key words：voltage-gated sodium channels; Nav1.5; invasion; migration; breast cancer

Among women, breast cancer is the most common malignant tumor and the first cause of death in the world. Death occurs primarily after the development of metastasis[1]. Until recently, the studies on ionic channels and breast cancer focused mainly on potassium channels and their involvement in proliferation. Increasing evidence suggests that ion channels are involved carcinogenic process. Ionic channels are becoming the new targets of cancer therapy [2]. Among these channels, voltage-gated sodium channels (VGSCs) appear to be most promising targets in anti-cancer therapy [3].

VGSCs are membranes of panning proteins that expressed in a wide variety of excitable and non-excitable cells, where they are responsible for the rising phase and the propagation of action potentials, display a range of functional forms. VGSCs consist of a single heavily glycosylated α-subunits (VGSCαs) and a variable number of auxiliary β-subunits (VGSCβs). Until now, 10 genes encoding α-subunits have been identified. Nine of these constitute a single family named Nav1 according to their phylogeny and are noted Nav1.1 to Nav1.9[4].

In the present study, we investigated the expression of Nav1.5 and its possible roles in breast cancer cell line MDA-MB-231.

1 MATERIALS AND METHODS

1.1 Main Reagents

DMEM and fetal calf serum (FCS) were obtained from GIBCO (USA). Rever-Tra Ace and SYBR Green 1 were ordered from TOYOBO (Japan). Tetrodotoxin (TTX) was obtained from Qinhuangdao Shuichansuo (China). MTT was obtained from Sigma (USA). RIPA lysis buffer and BCA protein assay kit were the products of Beyotime Biotechnology (China). β-actin monoclonal antibody and Nav1.5 polyclonal antibody were respectively ordered from Santa Cruz (USA) and Alomone labs (Isr).

1.2 Cell Culture

Human breast cancer cell lines MDA-MB-231 (high metastasis) and MCF-7 (low metastasis) were obtained from the China Type Culture Collection (CTCC, China). In this study, MCF-7 cells served as negative controls [5]. Cells were cultured at 37°C with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 ng/L streptomycin and 0.3 ng/L glutamine.

1.3 Detection of NaV1.5 mRNA Expression in Breast Cancer Cell Lines by Real-time PCR

Total RNA was extracted using Trizol according to the manual. One microgram total RNA was generated for the reverse transcription reaction using ReverTra Ace kit. Real-time PCR with SYBR Green 1 was performed. β-actin was measured in each sample as a control gene and used to normalize the respectively measured Nav1.5 expression. The prime pairs were used as follows: TCA CCC ACA TGT GCC CAT CTA CGA/CAG CGG AAC CGC TCA TTG CCA ATG G (β-actin, 295 bp); TGG TGC AGA CAG ATG ACC AA/ACG TGG TGA TCT GGA AGA GG (NaV1.5, 211 bp). The procedures and conditions of amplification were as follows: initial denaturation at 95°C for 3 min to activate the HotStar Taq, with subsequent three-step cycling of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Triplicate reactions on each sample cDNA were carried out simultaneously for Nav1.5 and β-actin. Blank reactions without added cDNA were performed in

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order to control cross-contamination from other sources. The values of amplification cycle (Ct) were analyzed by the $2^{-\Delta\Delta Ct}$ method to determine target expression levels[6].

1.4 Immunofluorescence

MDA-MB-231 and MCF-7 cells were cultured on glass cover-slips for 24 h and were fixed for 10 min in 4% paraformaldehyde in PBS at room temperature. Unspecific sites were saturated by incubating for 30 min with 3% normal goat serum in PBS. Nav1.5 was detected by incubating the cells for 60 min with a rabbit polyclonal anti-Nav1.5 antibody (Alomone labs, Isr) diluted to 1:100 in PBS. Cells were then washed and incubated for 60 min with a goat anti-rabbit conjugated to FITC secondary antibody (Pierce, USA). For each sample four microscopic fields (×200) in three different sections were counted.

1.5 Western Blot

Total proteins were extracted from the two cell lines studied. Total protein concentrations were determined by the bicinchoninic acid (BCA) method. The proteins samples were boiled for 10 min in a 5×SDS sample buffer before being loaded at a total protein concentration of 60 μg/well and run on a 6% acrylamide gel. Protein samples were then transferred to PVDF membranes. After saturating for 3 h in 5% nonfat milk TBST solution (containing 0.5% Tween 20), the membrane was incubated overnight at 4°C with the Nav1.5 rabbit primary antibody (1:500) or a polyclonal rabbit anti-β-actin antibody. The membrane was then incubated for 1 h at room temperature, with a goat anti-rabbit secondary antibody (1:5000). Immunoblots were visualized with the ECL immuno detection system (Pierce, USA).

1.6 Cell Proliferation Assays

Cells were seeded at a density of 1×10^4 cells/per well in a 96-well plate, and were incubated with TTX (0–30 μmol/L) for 48 h. Proliferation and viability of cells were measured by MTT assay**.** Data were obtained from 3 separate experiments and each treatment versus control was performed in triplicate.

1.7 Migration and *in vitro* Invasion Assay

Migration was analyzed in 24-well plates receiving 8 μm pore size polyethylene terephtalate membrane cell culture inserts (Corning, USA). The upper compartment was seeded with 4×10^4 cells in DMEM. The lower compartment was filled with DMEM supplemented with 20% FBS, instead of 10%, as a chemoattractant. After 7 h at 37°C, remaining cells were removed from the upper side of the membrane, and cells that had migrated and were attached to the lower side were stained with HE and counted in the whole insert, using a light microscope at ×100 magnification. *In vitro* invasion was assessed using the same inserts and the same protocol as above but with the membrane covered with a film of Matrigel (BD, USA), an extracellular mimicking matrix. Migration and invasion assays were performed in triplicate in 2 separate experiments (control group and 30 μmol/L TTX groups). For easier comparison between groups, results obtained for migration and invasion were normalized to the control condition.

1.8 The mRNA Expression of MMP-2 and MMP-9

After MDA-MB-231 and MCF-7 cells were treated by TTX for 24 h, total cellular RNA was extracted. RT-PCR was used to analyze the changes in the MMP-2 and MMP-9 mRNA expression. Primers of targeted genes were as follows: MMP-2, GTG CTG AAG GAC ACA CTA AAG AAG A/TTG CCA TCC TTC TCA AAG TTG TAG G, 605 bp; MMP-9, GAC TCG GTC TTT GAG GAG CC/GAA CTC ACG CGC CAG TAG AA, 350 bp; β-actin, TCA CCC ACA TGT GCC CAT CTA CGA/CAG CGG AAC CGC TCA TTG CCA ATG G, 295 bp. The reaction cycles for all genes begun with: 95°C for 5 min, 94°C for 30 s, 57°C for 30 s, 72°C for 30 s and 72°C for 7 min. Forty amplification cycles were necessary to achieve exponential amplification. PCR products were run on 1.5% agarose gels with DNA markers and were visualized under the ultraviolet light.

1.9 Statistical Analysis

All quantitative data were determined to be normally distributed and were presented as $\bar{x}\pm s$. Statistical significance was determined with Student's t test using SPSS11.5 statistical software. Results were considered significant at $P<0.05$.

2 RESULTS

2.1 The mRNA Expression of Nav1.5 Channels in Breast Caner Cell Lines

Nav1.5 mRNA expression levels in MDA-MB-231 cells were increased by (88.2±1.24)% as compared with those in MCF-7 cells ($P<0.05$).

2.2 Expression of Nav1.5 Protein in Breast Caner Cell Lines

Western blots assay detected a typical band of approximately 220 kD corresponding to the normal Nav1.5 protein in MDA-MB-231 cells, which was increased by (83.1±1.46)% as compared with that in MCF-7 cells. There was another faint brand less 30 kD than the normal Nav1.5 (fig. 1A). Interestingly, these results were in accordance with the immunofluorescence assay that the addressing of Nav1.5 to the membrane was evaluated in MDA-MB-231 cells while there was only cytoplasm labeling in MCF-7 cells (fig. 1B).

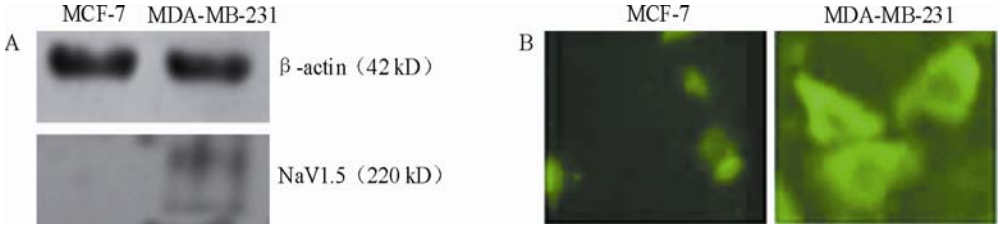

Fig. 1 Expression and location of NaV1.5 protein in MDA-MB-231 and MCF-7 cells

A: Western blot evaluated the expression of Nav1.5 protein; B: Immunofluorescent assay evaluated the cellular localization of Nav1.5 (×200).

2.3 Involvement of Nav1.5 in the Oncogenic Properties of Breast Cancer Cells

Cell proliferation and migration had no obvious changes in MDA-MB-231 and MCF-7 cells treated with TTX (fig. 2A and B). As shown in fig. 2C, the relative number of MDA-MB-231 cells through the Transwell membrane pro field in control group and TTX group was respectively 56.30±4.78 and 29.38±1.56. As compared with control group, the invasion ability in TTX group was decreased to (47.82±0.53)% (P<0.05).

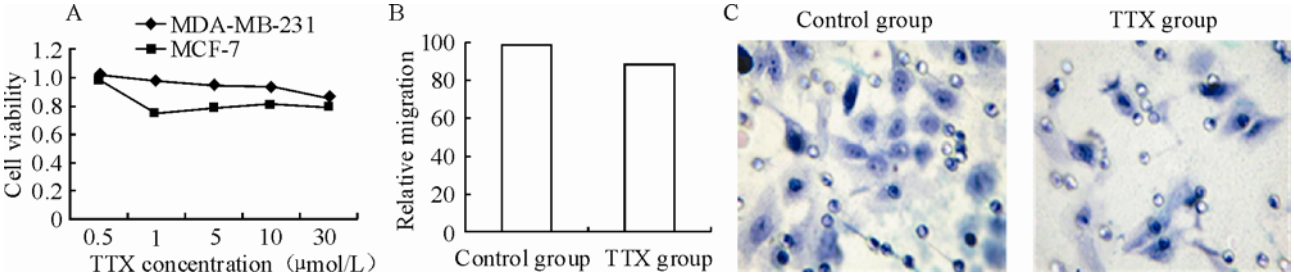

Fig. 2 Effects of TTX on the proliferation (A), relative migration (B) and invasion capability (C, ×100) of MDA-MB-231 cells

2.4 Changes in the Expression of MMP-9 mRNA in MDA-MB-231 Cells Treated with TTX

The expression of MMP-9 mRNA in MDA-MB-231 cells was up-regulated compared to MCF-7 cells. Before and after treatment with TTX for 24 h, the MMP-9/β-actin levels in MDA-MB-231 cells were respectively 0.93±0.02 and 0.52±0.02. TTX significantly reduced the MMP-9 mRNA by (43.97±0.64)% in MDA-MB-231 cells (P<0.05), but there were no obvious changes in MCF-7 cells. In both MDA-MB-231 and MCF-7 cell lines, no MMP-2 mRNA expression was detectable (fig. 3).

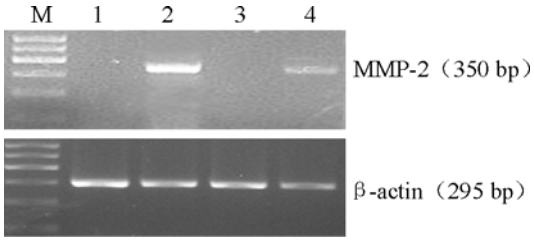

Fig. 3 Detection of the MMP-9 mRNA expression in MDA-MB-231 and MCF-7 cells by RT-PCR
1: MCF-7 cells; 2: MDA-MB-231 cells; 3: MCF-7 cells treated with TTX for 24 h; 4: MDA-MB-231 cells treated with TTX for 24 h

3 DISCUSSION

Many reports elucidated that VGSCs had strong correlation with tumor malignant behaviors, such as migration, invasion and secretory membrane activity [7]. In human and rat prostate cancer cell lines AT-2 and LNCaP with low metastasis no functional expression of VGSCs was detectable, while in PC3 and Mat-Ly-Lu cells with high metastasis, there was aberrant over-expression of VGSCs[8]. In addition, cultured normal cells (smooth muscle cells, retinal pigment epithelial cells) expressed VGSC, but there was no expression of VGSC in the freshly isolated cells, deducing that VGSCs might be one of the embryonic genes which were silent in the cells of the mature organ, and re-expressed in cancer cells[9, 10].

This study found the expression levels of Nav1.5 mRNA and protein were significantly increased in highly metastatic MDA-MB-231 cells as compared with those in lowly metastatic MCF-7 cells. Western blot analysis showed Nav1.5 mRNA was translated into proteins of two molecular weights: the large one was in accordance with the expected normal size of the glycosylated Nav1.5, and the small one might be non-functional truncated proteins produced through a "fail-safe" mechanism. Immunofluorescent technique revealed that Nav1.5 was mainly distributed on the membrane of MDA-MB-231 cells, and mainly locating in the cytoplasm of MCF-7 cells. Therefore, it was presumed that the aggressive phenotype might be due to the addressing of the Nav1.5 to the membrane.

Different aspects of cancer cell lines biology can refer to different aspect of solid tumor. For example, cell proliferation *in vitro* can be an index of the kinetics of tumor proliferation *in vivo*; the cells migrate through filters, covered or not with Matrigel (mimicking the extracellular matrix, ECM) are considered as indexes of invasion and migration, respectively[2]. Interestingly, Roger *et al* previously have reported that 30 μmol/L TTX could completely block the activity of VGSCs in non small lung cancer cell lines[11]. In this study, it was found that pre-treatment with 30μmol/L TTX could reduce the invasive capacity of MDA-MB-231 cells by almost 50% without affecting cell proliferation and migration, while it had no obvious effect on lowly metastatic MCF-7 cells. Importantly, incubation of highly metastatic prostate cancer cells Mat-Ly-Lu and PC-3 with TTX reduced their invasive capacity by about 33%[12].

Metastasis is a process whereby cells escaping from a primary tumor, migrate and lodge at tissue-specific or non-specific sites. To set in a distant site, tumor cells need to produce proteases (e.g. matrix metalloproteases, MMP) to degrade the ECM. At present, the cellular mechanisms of VGSCs participating in the modulation of metastatic cascade remain unknown. Previously researches reported that the invasive properties of non small cell lung cancer cells with functional VGSCs might be correlation with the secretion of MMPs[2,11]. MMPs are zinc proteinases that have been implicated in tumor invasion and metastasis through degrading many ECM proteins, especially MMP-2 and MMP-9. The expression of MMP-9 in beast cancer was an important prognostic marker of invasive potential of pancreatic

tumors[13]. In this study, RT-PCR assay revealed that the MMP-9 mRNA expression levels were significantly decreased by (43.97±0.64)% in MDA-MB-231 cells treated with TTX for 24 h, but there were no obvious changes in MCF-7 cells. It might be inferred that Nav1.5 present in MDA-MB-231 cells controlled the secretion of MMP-9. Interestingly, in metastatic prostatic cancer cell lines VGSCs were related to the cell endocytosis and secretion cycle activity; and numerous cell lines from small cell lung cancers with functional VGSCs expression had also been postulated that these VGSCs activity were involved in secretory activity[14, 15].

In conclusion, Nav1.5 might potentiate the invasive behavior of human breast cancer MDA-MB-231 cells by increasing the secretion of MMP-9 and might be a new anti-breast cancers therapeutic target. However, the overall situation is likely to be much more complex and potential clinical study needs further investigation.

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