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Interference of Frizzled 1 (FZD1) reverses multidrug resistance in breast cancer cells through the Wnt/β-catenin pathway

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ABSTRACT

Multidrug resistance (MDR) represents a major obstacle in the successful treatment of breast cancer. The MDR1 gene is a direct target of the Wnt/β-catenin signaling pathway, which controls tumor development. Overexpression of P-glycoprotein, encoded by the MDR1 gene, is one of the most common causes of MDR. We found that the Frizzled 1 (FZD1) protein, which is an essential component of the Wnt/β-catenin pathway, is overexpressed in the multidrug resistant breast cancer cell subline MCF-7/ADM, coincident with MDR1/P-gp. FZD1 silencing induced down-regulation of MDR1/P-gp, restored sensitivity to four chemotherapy drugs, and significantly decreased cytoplasmic and nuclear β-catenin levels. FZD1 appears to mediate multidrug resistance by regulating the Wnt/β-catenin pathway.

1. Introduction

The development of multidrug resistance (MDR), in which tumor cells become resistant to a wide spectrum of anti-cancer agents with different structures or different target sites [1], severely limits the success of chemotherapy in breast cancer. The mechanisms of MDR acquired in the course of chemotherapy are numerous and complex. The human multidrug resistance 1 gene (MDR1) encodes the membrane-located efflux pump P-glycoprotein (P-gp). Overexpression of MDR1/P-gp results in an active efflux of anticancer agents from cells, thus lowering intracellular drug concentrations and inducing cancer cells to resist chemotherapeutic drugs, especially substrate anti-cancer drugs, such as doxorubicin and paclitaxel [2–5]. Several strategies have been developed to restore chemotherapeutic sensitivity in MDR cells with limited success [6,7]. MDR1 has been demonstrated to be a direct target gene of the β-catenin/Tcf4 transcriptional complex [8]; thus, MDR1 expression may be down-regulated by inhibiting the Wnt/β-catenin pathway.

The Wnt/β-catenin signaling pathway plays a key role in controlling multiple aspects of tumor development, including breast cancer [9–13]. In this pathway, interactions of Wnt proteins with the cell surface of Frizzled receptors and their associated membrane proteins lead to the inactivation of glycogen synthase kinase-3 (GSK-3), resulting in cytosolic β-catenin stabilization. Free β-catenin accumulates and is translocated to the nucleus, where it binds to the transcription factor TCF/LEF to regulate target gene expression [14]. It has been reported that inhibitors of Wnt/β-catenin signaling have been developed as potential anti-cancer drugs, with gene therapy using Wnt/β-catenin-restricted expression of a therapeutic gene drawing the attention of the scientists [15].

The Wnt receptors Frizzled 1 and 2 (FZD1/2), which are known to be essential components of Wnt/β-catenin, have been found to be overexpressed in advanced infiltrating ductal breast cancer [16]. Flahaut et al. [17] confirmed that FZD1 silencing induced a strong parallel decrease in MDR1/P-gp expression in two doxorubicin-resistant neuroblastoma cell lines; yet, it restored different extents of sensitivity to doxorubicin, etoposide, paclitaxel, and cisplatin. However, FZD1 has not been reported to mediate chemoresistance in breast cancer.

We previously detected the expression of FZD1 in the breast cancer cell lines MCF-7 and MDA-MB-231, as well as in the multidrug resistant subline MCF-7/ADM [18]. FZD1 was found to have higher mRNA and protein levels in relation to MDR1/P-gp expression in ADM cells, compared to the sensitive cell lines MCF-7 and MDA-MB-231 (Fig. 1). These results suggest the potential roles of FZD1 and the Wnt/β-catenin pathway in the development of multidrug resistance in breast cancer cells. We propose that the silenc-

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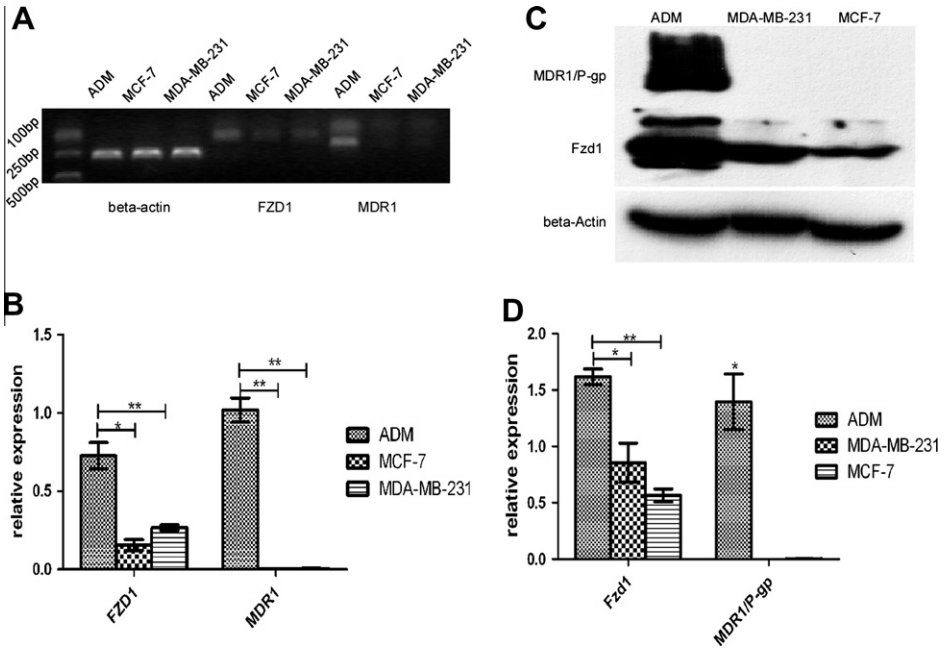

Fig. 1. The expression of FZD1 and MDR1/P-gp in ADM, MCF-7, and MDA-MB-231 cells. (A) RT-PCR results showed a stronger expression of FZD1 and MDR1 mRNA in ADM compared to MCF-7 and MDA-MB-231 cell lines. (C) Western blot results showed a higher level of Fzd1 and MDR1/P-gp proteins expressed in ADM cells. (B and D) Histograms showed a significant difference, error bars indicate standard error. ($^{*}P < 0.05$, $^{**}P < 0.01$, respectively).

ing of FZD1 by plasmid-mediated expression of small interference RNA (siRNA) might result in the down-regulation of MDR1/P-gp, and thus reverse multidrug resistance in MDR cells through the Wnt/β-catenin pathway.

2. Materials and methods

2.1. Cell lines and cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere. The MDR subline MCF-7/ADM was obtained from Tianjin Blood Institute, and maintained in RPMI-1640 culture medium (Hyclone, Logan, UT, USA) containing 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. All cells were free from mycoplasma infection.

ADM cells overexpressing MDR1/P-gp were generated by sequential exposure to increasing concentrations of Adriamycin (doxorubicin) [19], and were protected from paclitaxel, cisplatin, and 5-Fu toxicity. The MDR phenotype was stable in doxorubicin-free medium for at least 4 months. To obtain more MDR cell sublines, we transduced MDA-MB-231 and MCF-7 cell lines with the retroviral vector SF91m3PRE, containing the human multidrug resistance 1 gene (MDR1) [20].

2.2. Construction of siRNA vectors

siRNA oligonucleotide sequences targeted at FZD1 were selected according to Ulivieri et al. [21], as follows:

Forward: 5′-GATCCCCACTCCTGGAGGAAGTTCTATTCAAGAGATAGAACTTCCTCCAGGAGTTTTTTGGAAA-3′; and Reverse: 5′-AGCTTTTCCAAAAAACTCCTGGAGGAAGTTCTATCTCTTGAATAGAACTTCCTCCAGGAGTGGG-3′.

Underlined nucleotides show the restriction endonuclease sites for Bgl II and Hind III. Small hairpin siRNA sequences were synthesized as 64 oligonucleotides, annealed, and then cloned into the pSUPER. neo + GFP expression vector using T4 DNA ligase to generate pSUPER-siFZD1 plasmids. After amplification using standard methods [22], the recombinant plasmid was extracted.

Non-targeting (Notarget) siRNAs were designed as negative controls using the sequences:

Forward: 5′-GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTGGAAA-3′; and Reverse: 5′-AGCTTTTCCAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGGG-3′.

The control vector pSUPER-siN was constructed in a manner similar to that described for targeting siRNAs.

2.3. Transfection of breast cancer MDR cells

An approximate number of 1×10^6 cells were seeded in 4 mL of RPMI-1640 culture medium with 10% FBS in each well of 6-well plates for 24 h prior to transfection. For each well, 4 μg of pSUPER-siFZD1 plasmid DNA was diluted in 400 μL of serum-free RPMI-1640 medium. Then, 8 μL of TurboFect *in vitro* Transfection Reagent (Fermentas, Burlington, Canada) was added to the diluted DNA, and mixed by pipetting before incubation for 20 min at room temperature. To each well, 400 μL of the TurboFect/DNA mixture was added drop-wise, without removal of the medium from the cells. The plates were gently rocked to distribute the complexes evenly, and were then incubated at 37 °C in an atmosphere with 5% CO_2. Nontarget siRNA plasmid pSUPER-siN was used as a control. Three groups of MDR cells were transduced, namely, ADM siN, ADM siFZD1, MDA-MB-231/MDR1 siN, MDA-MB-231/MDR1 siFZD1, MCF-7/MDR1 siN, and MCF-7/MDR1 siFZD1. For stable transfection, cells were grown in medium containing 400 μg/mL of G418 for at least 15 d.

Stably transfected ADM siFZD1 cells were transduced with SF91m3PRE (ADM siFZD1/MDR1), to determine if the treatment could reverse increased cell sensitivity to anti-cancer drugs, which was caused by MDR1/P-gp.

2.4. Semi-quantitative RT-PCR analysis

An RNA Iso-Plus kit (Takara, Otsu, Japan) was used to extract total cellular RNA, and the first strand DNA was synthesized with Omniscript Reverse Transcriptase (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The primers were: FZD1 forward primer 5′- CGGTAAAATCTAAGCGCAGG-3′, FZD1 reverse primer 5′- AGCTTTGTGTGGGTTGGAAG-3′; MDR1 forward primer 5′- TCAGCCTCACCACAGATGAC-3′, MDR1 reverse primer 5′- TCACTTCAGGAAGCAACCAG-3′; β-actin forward primer 5′- CTCCATCCTGGCCTCGCTGT-3′, and β-actin reverse primer 5′-GCTGTCACCTTCACCGTTCC.

PCR amplification was performed using 1 μL cDNA template, with an initial step of 95 °C for 5 min, followed by 32 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and then a final extension cycle at 72 °C for 10 min. RT-PCR products were analyzed by electrophoresis on 2% agarose gel. Each band was quantified using a gel figure analysis system (Gel Doc 2000, Hercules, Germany). β-actin was used as the internal control.

2.5. Preparation of cell lysates

Total protein extracts were obtained from cells using RIPA lysis buffer, which was composed of 50 mM Tris with pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 0.05 mM EDTA. Prior to cell lysis, 0.2 mM phenylmethylsulfonyl fluoride, a protease inhibitor, was added to the extracts. After 20 min on ice, the complex was centrifuged at 12,000 × g for 10 min at 4 °C.