Effects of Mfn2 Gene Overexpression on EGFR Expression and Angiogenesis of a Heterologous Graft Model for Human Breast Infiltrating Duct Carcinoma in Nude Mice

Hu Ji-wei[1], Yan Jin-yin[1], Wang Ya-qi[1,2], Zhang Jing-hua[1,2,*], Wang Lei[5], Li Yu-feng[3], Liu Yan[3,4], Ma Jie[1], Li Ning[1], Zhang Shun-li[1], Gu Zheng[1], Wang Hong[1]

[1]Department of Surgical Oncology, Tangshan People's Hospital, Tangshan, China
[2]Department of Oncology, North China University of Science and Technology, Tangshan, China
[3]Cancer Institute, Tangshan People's Hospital, Tangshan, China
[4]College of Life Science, North China University of Science and Technology, Tangshan, China
[5]Department of Pathology, Tangshan People's Hospital, Tangshan, China
*Corresponding author: jinghuazhang2014@163.com

Abstract **Objective:** To investigate the antitumor angiogenesis activity of *Mfn2* gene on a heterologous graft model for human breast infiltrating duct carcinoma in nude mice. To investigate the relationship between *Mfn2* gene and Epidermal Growth Factor Receptor (EGFR). **Methods:** Human breast cancer MCF-7 cells steady infected with *Mfn2* gene had been constructed. Western blotting was used to detect the overexpression of Mfn2 protein. Then the MCF-7 cells were injected subcutaneously into the breast pad of nude mice. Tumorigenicity and the growth of transplanted tumor in nude mice were observed. Growth curves were plotted based on mean tumor volume in each experimental group at the indicated time points. Meanwhile, the negative control group (NC group) which MCF-7 cells steady infected with PEGFP-N1 was set up. The expression of CD34 (reflect the microvessel density of tumor) and EGFR were detected by immunocytochemistry. **Results:** The tumor volume of the experimental group was smaller than the negative control group.The expression of CD34 of the experimental group was lower than the negative control group. The experimental group expressed low level of EGFR. **Conclusion:** Mfn2 significantly inhibits the growth of human breast cancer xenograft in nude mice. Downregulation of EGFR expression and decrease the potential of angiogenesis may be the potential mechanism.

Keywords: *breast cancer, EGFR, Mfn2, nude mice*

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1. Introduction

Breast cancer is one of the leading causes of cancer-related mortality worldwide. It has been estimated that a total of 989,600 new breast cancer cases and 738,000 deaths occurred in 2008 [1]. Countries in the Western Pacific Region have the highest reported incidence of breast cancer; in a recent survey, 47% of new breast cancer cases across the world occurred in China, and more than 90% of those cases were found to be advanced [1,2]. Surgery alone cannot guarantee good long-term survival for patients. Therefore, it is critical that we develop new therapeutic strategies, including molecule-targeted therapy.

Mitofusin-2 (Mfn2), a potential target molecule, has been extensively studied in Charcot-Marie-Tooth (CMT) disease (a heterogeneous group of inherited peripheral neuropathies). Point mutations in Mfn2 have been found in several types of CMT disease and are likely to be the fourth most common cause of CMT [3]. Mfn2 is a mitochondrial outer membrane protein that participates primarily in mitochondrial fusion [4]. It can also tether the ER to mitochondria and is required for efficient mitochondrial Ca^{2+} uptake [5]. Previously, it was known as hyperplasia suppressor gene (HSG). Studies of vascular proliferative disorders have demonstrated that the overexpression of HSG markedly suppressed serum-evoked vascular smooth muscle cell (VSMC) proliferation in culture and blocked balloon injury-induced neointimal VSMC proliferation and restenosis in rat carotid arteries [6]. Recently, Mfn2 has attracted interest in tumor research. Several studies have investigated the function of Mfn2 in different types of malignancies, including lung, liver and urinary bladder cancers; Mfn2 is thought to promote pro-apoptotic and anti-proliferative functions [7,8,9].

However, the mechanism underlying the anti-tumor effect of Mfn2 is unclear, and Mfn2 has never been studied in breast cancer. In the current study, we examined the expression of Mfn2 in tissues from 90 breast cancer cases and evaluated its prognostic significance in breast cancer. Furthermore, we explored the cellular function and mechanism of Mfn2 in vitro using breast cancer cell lines.

2. Materials and Methods

2.1. Materials

The eukaryotic expression vector containing the *Mfn2* gene was constructed by Sangon Biotech. Human MCF-7 cells were obtained from the Cell Culture Center of Peking Union Medical College. G418 was purchased from Merck (Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone Company (USA). Lipofectamine^{TM} 2000 was purchased from Invitrogen Biotechnology (USA). The antibodies of anti-Mfn2 (bs-2988R), anti-EGFR (bs-0405R), anti- CD34 (bs-2038R), anti-β-actin (β-actin) were obtained from Beijing Biosynthesis Biotechnology Co., Ltd (China). Female BALB/C nude mice, 4-6 weeks old, were purchased from Beijing HFK Bioscience Co., Ltd (China) (SCXK (Jing) 2009-0004, SPF). All animal studies were conducted with a North China University of Science and Technology Animal Care and Use Committee protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals.

2.2. Stable Transfection

MCF7 cells were cultured in DMEM medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2. G418 dose used for selection of positive clones was determined by challenging MCF7 cells with gradient concentration of G418. The optimal dose was determined by titration to be 600 ug/ml.

The recombinant plasmid pEGFP-N1-*Mfn2* and the empty vector plasmid pEGFP-N1 were transfected into the MCF7 cells by mixing with Lipofectamine^{TM} 2000 reagent according to the instructions of manufacturer Forty-eight hours after transfection, cells were passaged and were cultured in DMEM medium containing 10% fetal bovine serum and 600 ug/ml G418. Ten days later the concentration of G418 changed to 300 ug/ml in order to maintain screening pressure. G418-resistant clones was visible approximately 2 weeks after the selection. Then the G418-resistant clones were picked up and cultured, after 4 weeks of cloning finally we got 2 cell lines with stable expression of pEGFP-N1-Mfn2 and pEGFP-N1 respectively. Cells were divided into three groups: Mock group (MCF7 cell), pEGFP-N1-*Mfn2* group (*Mfn2* group) and pEGFP-N1 group (N1 group).

Total cell lysates were obtained from MCF-7 cells RIPA buffer (Beyotime Institute of Biotechnology P0013B, Haimen, Jiangsu, China). The total protein concentration of the resulting supernatant was measured using the Bio-Rad Protein Assay kit (500-0201). Relative equal total amounts of proteins from tissue whole-cell lysate were separated by 10% SDS-PAGE. Mfn2 and β-actin proteins were detected by immunoblotting using Mfn2 (1:200) and β-actin (1:200) poly-clonal antibodies. Whole-cell lysate from cultured cells was mixed with 4×SDS loading buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 30% glycerol, 0.02% Bromophenol Blue containing 10% BME), and boiled for 5 min at 95°C. Denatured proteins were then separated by 10% SDS-PAGE, and specific proteins were detected by western blotting using indicated antibodies.

2.3. Construction and Evaluation of Xenograft Tumor in Nude Mice

Fifteen female BALB/C nude mice in a SPF grade aged 6 weeks (body weight 18-20 g,) were randomly divided into three groups (Mock group, N1 group and Mfn2 group). Subconfluent monolayer cells were used, they were detached from the bottom of the flask with trypsin and suspended in sterile PBS with density of $(1-2) \times 10^8$ /ml. The mice in three groups received injection of MCF7 cell, and MCF7 cell stably expressing pEGFP-N1, and pEGFP-N1-Mfn2 stable expressing MCF7 cells respectively. After injection, the mice were marked with picric acid on different location of the body, and then continued to be fed at SPF level. General conditions of nude mice were recorded every day.

Subcutaneous tumor: 0.2 mL of each suspension was subcutaneously injected into the right breastpad of the nude mice. The subcutaneous tumor formation, dynamic observation tumor size and weight of nude mice were observed. Long and perpendicular dimension (a, b) of the tumor were measured by using a caliper, the volume of the tumor was calculated according to the formula: $v = a \times b^2/2$. After 5 weeks, mice were killed by cervical dislocation.

Pathology examination: The thin sections of the tumors were prepared with HE staining and photographs were taken under optical microscope.

2.4. Immunohistochemistry

Immunohistochemical studies were done using paraffin-embedded material, heat-induced antigen retrieval, and antibodies specific for Mfn2 (1:200), EGFR (1:200), CD34 (1:200). The remaining procedures were adapted from the PV-6001 Two-Step IHC Detection Reagent illustration (ZSGB-BIO Corporation, Beijing, China); the brown color was developed using DAB (ZSGB-BIO Corporation, Beijing, China) and the sections were counterstained with hematoxylin (Baso Corporation, Zhuhai, China). All sections were semi-quantitatively analyzed using Image Pro Plus (IPP; Media Cybernetics Inc., Rockville, MD, USA) version 6.0 software, and the integrated optical density (IOD) was measured from the images at 400× (Mfn2, EGFR) or at 100× (CD34) magnification.

2.5. Statistical Analysis

All data were analyzed using SPSS 17.0 software (IBM Corporation, Armonk, NY, USA) and results are expressed as means ± standard error of the mean (SEM). The Shapiro-Wilk test for normality and Bartlett test for homogeneity of variance were performed. Comparisons between groups were tested using a one way analysis of variance (ANOVA) and Fisher's least significant

difference (LSD) t-test. A p-value <0.05 was considered statistically significant.

3. Results

3.1. The Expression of Mfn2 in Three Groups MCF7 Cells

To study the expression pattern of the *Mfn2* gene in MCF7 cells, the levels of the Mfn2 protein were quantified by Western blot in three groups. Expression levels of protein extracted from each sample were assessed in the three groups with calibration of β-actin amounts. A significant difference was observed between Mfn2 group and the Mock group. But there was no statistical difference between the Mock group, N1 group (Figure 1).
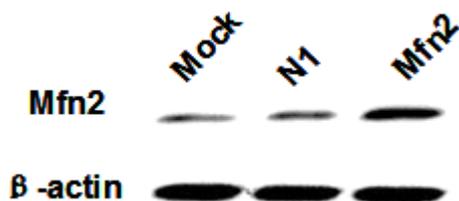
Figure 1. Levels of the Mfn2 protein quantified by Western blot in three groups

3.2. General Condition of Nude Mouse

The life state of nude mouse in the Mock group, N1 group and Mfn2 group carrying xenograft were normal all the time, voracious and active.

3.3. Mfn2 Inhibited Tumor Growth

The tumor suppressor efficacy of Mfn2 was firstly evaluated by its inhibition on the tumor volume of the xenograft. As shown in Figure 2, Mfn2 group significantly reduced the tumor size compared to control ones. Mfn2 also did not affect body weight. These data collectively suggest the potency of Mfn2 in suppressing in vivo growth of breast cancer, without significant adverse effects.

Figure 2. Representative tumor bearing nude mice and tumor

3.4. Mfn2 Inhibited Tumor Angiogenesis

The levels of the Mfn2 protein of the xenograft were quantified by immunohistochemistry in three groups (Figure 3). Because angiogenesis plays an important role in the progression and metastasis of cancers, we examined the effect of Mfn2 on intratumoral microvessel density (MVD), using the endothelial cell-specific marker CD34. As shown in Figure 4, the percentage of CD34-positive cells in Mfn2 group mice was significantly reduced compared to control animals. The expression of EGFR was also detected. As expected the expression of EGFR suppressed by Mfn2 (Figure 5). These results illustrate the inhibition of angiogenesis of breast cancer by Mfn2.
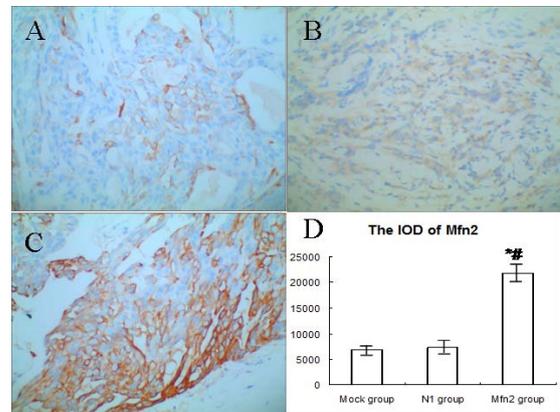
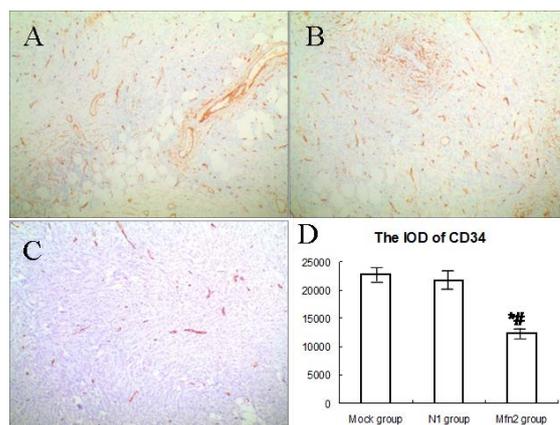**Figure 3.** Immunohistochemistry assay for Mfn2 in each group. (**A**) Mock group; (**B**) N1 group; (**C**) Mfn2 group

Figure 4. Immunohistochemistry assay for CD34 in each group. (**A**) Mock group; (**B**) N1 group; (**C**) Mfn2 group
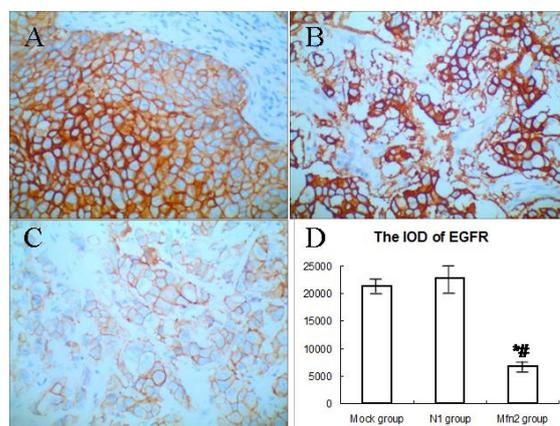
Figure 5. Immunohistochemistry assay for EGFR in each group. (**A**) Mock group; (**B**) N1 group; (**C**) Mfn2 group

4. Discussion

Given recent advancements in medicine, especially the improved early diagnosis rate, the morbidity and mortality

of gastric cancer have declined in many advanced countries. However, these advances have not changed the treatment of breast cancer by much. Surgery remains the most efficient therapy, but a high survival rate cannot be attained with advanced stage patients, and chemical therapy and radiotherapy lack specificity. Recently, with the development of molecule-targeted therapy, scientists have focused increasingly on breast cancer treatment. Many relevant target molecules or pathways have been identified, including epidermal growth factor receptor, vascular endothelial growth factor receptor, vascular endothelial factor receptor, insulin-like growth factor receptor, and the PI3k/Akt/mTor pathway [10,11,12,13,14]; however, these target molecules are less than satisfactory. Therefore, it is critical that we find more specific and more useful genes or biomarkers. In our research, we investigated Mfn2, which has been studied widely in CMT disease. Researchers have reported that Mfn2 is correlated with anti-tumor activity in several malignancies [7,8]. The current study confirmed such an association in breast cancer cell lines, and we found a negative relationship with tumor size.

Mfn2, which controls mitochondrial fusion, is a highly conserved GTPase [15]. Similar proteins are found in yeasts, *Caenorhabditis elegans*, fruit flies and mammals [16]. Mfn2 possesses two transmembrane domains spanning the outer mitochondrial membrane, a possible PKA/PKG phosphorylation site and a p21 (Ras) signature motif (amino acids 77–92), which plays an important role in signaling [15,17].

Previous studies have demonstrated that the mutation of Mfn2 can cause CMT neuropathy type 2A [3,18,19]. Additionally, reduced expression of Mfn2 plays an important role in insulin resistance [20]. One recent study revealed that Mfn2 markedly reduced the proliferation of VSMCs [21]; further research confirmed this anti-proliferative effect in many tumor cell lines, including hepatocellular carcinoma cell line, HepG2, urinary bladder carcinoma cell lines, T24 and 5637, lung cancer cell line, A549, and colon cancer cell line, HT-29 [8,22]. Moreover, in BM-1 cells, the anti-proliferative effect of Mfn2 has been shown to be stronger than that of the anti-tumor gene, p53 [23,24].

In our preliminary study, we had demonstrated that Mfn2 suppressed the proliferation of MCF7 breast cancer cells. Moreover, we found that Mfn2 caused a cell cycle arrest in the G0/G1 phase. Taken together, we have reason to consider Mfn2 to be a potential anti-tumor gene. Meanwhile, we also observed a significant difference between the expression of Mfn2 and TNM stage or clinical prognosis, though the one-year, two-year and three-year overall survival of the Mfn2 low-expression group was lower than the moderate and high-expression group. However, the findings may have been affected by limited sample size or follow-up time.

Our previous study *in vitro* showed overexpress Mfn2 could downregulated the expression of EGFR. In this study, in mouse xenograft models, we observed that Mfn2 could not only downregulated the expression of EGFR but also inhabit angiogenesis. Petit *et al.* reported in 1997 that the oncogencity of the EGF receptor might partially be mediated through promotion of angiogenesis by upregulating VEGF [25]. Since then there has been growing evidence that stimulation or inhibition of EGFR

also has significant consequences for tumor-induced angiogenesis [25,26,27]. EGFR and its downstream signaling cascade is one of the most well-studied pathways in solid tumors. Although the normal function of EGFR signaling in adult physiology remains an active area of investigation, preclinical data suggest that EGFR ligands induce potent mitogenic signals via binding to the EGFR, and that this pathway is involved in cellular development, proliferation, and apoptosis [28]. However, in the pathologic state, dysregulation of EGFR-induced signaling cascades is involved in tumorigenesis and malignancy by facilitating cellular transition to a continually dividing and proliferating state [29,30]. Meanwhile, we did not observe the potential mechanism between Mfn2 and EGFR.

5. Conclusion

In a word, *Mfn2* significantly inhibits the growth of human breast cancer xenograft in nude mice. Downregulation of EGFR expression and decrease the potential of angiogenesis may be the potential mechanism.

Acknowledgments

Conflict of Interest

All authors declare no conflicts of interest in this study.

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