RESEARCH ARTICLE

shRNA Mediated RHOXF1 Silencing Influences Expression of BCL2 but not CASP8 in MCF-7 and MDA-MB-231 Cell Lines

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Abstract

   RHOXF1 has been shown to be expressed in embryonic stem cells, adult germline stem cells and some cancer lines. It has been proposed as a candidate gene to encode transcription factors regulating downstream genes in the human testis with antiapoptotic effects. Its expression in cancer cell lines has implied a similar role in the process of tumorigenesis. The human breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in DMEM medium and transfected with a pGFP-V-RS plasmid bearing an RHOXF1 specific shRNA. Quantitative real-time RT-PCR was performed for RHOXF1, CASP8, BCL2 and HPRT genes. Decreased RHOXF1 expression was confirmed in cells after transfection. shRNA knock down of RHOXF1 resulted in significantly decreased BCL2 expression in both cell lines but no change in CASP8 expression. shRNA targeting RHOXF1 was shown to specifically mediate RHOXF1 gene silencing, so RHOXF1 can mediate transcriptional activation of the BCL2 in cancers and may render tumor cells resistant to apoptotic cell death induced by anticancer therapy. shRNA mediated knock down of RHOXF1 can be effective in induction of apoptotic pathway in cancer cells via BCL2 downregulation, so it can have potential therapeutic utility for human breast cancer.

Keywords: RHOXF1 - hPEPP1 - apoptosis - cancer-testis antigen - BCL2 - CASP8

Introduction

   Homeobox (HOX) genes are a group of conserved genes coding for transcription factors. All subfamilies of homeobox transcription factors contain a conserved 60 amino acid DNA binding motif which recognizes DNA enhancers. The similarity between homeodomain of Hox proteins in different species is more than proteins encoded by neighboring genes within each Hox cluster. Accordingly, tandem duplication and subsequent divergence have been suggested as the mechanism of Hox gene clusters evolution (McGinnis and Krumlauf, 1992). These genes control many different processes including embryonic morphogenesis, cell proliferation, differentiation, adhesion and migration (Zhang et al., 2010). Recently, a cluster of HOX genes named Rhox (reproductive homeobox on the X chromosome) gene family has been discovered on the X chromosome. At least 33 genes have been found in mouse Rhox cluster, making it the largest homeobox gene cluster identified till now (MacLean and Wilkinson, 2010). Similar to Hox genes, Rhox genes are expressed in embryonic tissues. However, the distinctive feature of Rhox genes is that unlike most Hox genes, they continue to be expressed highly after birth. So they have been nominated as

essential factors for reproductive system. They have been shown to be expressed in both germ cells and somatic cells of reproductive tract (MacLean and Wilkinson, 2010). As most of Rhox genes are expressed on Sertoli cells which are known as "nurse" sells of the testis and have close interaction with developing germ cells, it has been deduced that Rhox genes products can regulate the expression of somatic cell gene products vital for germ cell development (Maclean et al., 2005). In addition, targeted Rhox5 knock out has resulted in increased male germ cell apoptosis and consequently reduced sperm count and fertility (Maclean et al., 2005).

   Two members of this gene family named RHOXF1 (hPEPP1) and RHOXF2A (hPEPP2) have been characterized on human X chromosome (Geserick et al., 2002; Wayne et al., 2002). RT-PCR experiments have shown RHOXF1 (initially named OTEX and hPEPP1) expression in the ovary, testis, epididymis, prostate and mammary gland (Geserick et al., 2002). Its protein has been shown to be exclusively localized in the nucleus. As, its expression has been shown to be androgen dependent and it is selectively expressed in the testes, it is likely that it has role in the spermatogenesis (MacLean and Wilkinson, 2010). However, in another experiment, northern blot analysis of RNA extracted from 10 human

tissues including ovary and prostate tissues failed to show RHOXF1 mRNA expression in tissues other than testis (Wayne et al., 2002). RT-PCR and ribonuclease protection assay have also demonstrated RHOXF1 mRNA expression in several tumor cell lines, such as HPB-ALL (acute lymphocytic leukemia), LNCaP (prostate), Hec1A (endometrial adenocarcinoma), and to a lesser extent in MDA-MB-231 (breast carcinoma) (Wayne et al., 2002). In addition, ESTs corresponding to this gene have been found in various human tumors (Wayne et al., 2002).

Sequence analysis of RHOXF1 has revealed that in its homeodomain it is most similar to a mouse Rhox gene, Esx1. However, RHOXF1 location on X chromosome is not syntenic to the region containing Esx1. Although the least similarity exists between RHOXF1 and Rhox5 (Pem), it is probable that RHOXF1 is the orthologue of Rhox5. Two distinctive characteristics of RHOXF1 that suggest it is the orthologue of Rhox5 are a helix-breaking proline at residue 29 and a lysine at residue 50 of the homeodomain (Wayne et al., 2002). As mouse and rat Rhox5 are identical at only about half of amino acids in the homeodomain region, Rhox5 divergence seems to be extremely rapid between species and low sequence similarity between RHOXF1 and Rhox5 does not rule out the possibility of being orthologues (Wayne et al., 2002). Besides, similar to RHOXF1, mouse and rat Rhox5 have been shown to be expressed in many malignant and immortalized cells (Wilkinson et al., 1990). Interestingly, unlike mouse Rhox genes, RHOXF1 and RHOXF2A are not expressed in placenta (Wayne et al., 2002).

RHOXF2A, the other member of this family, has been attributed to "cancer-testis" gene family, an expanding gene family with restricted expression in normal adult tissues other than testis and expression in a wide variety of tumors (Hofmann et al., 2008).

The antiapoptotic function of Rhox5 in mouse, the role of RHOXF genes as transcription factors and their expression regulation by hormones, in addition to their expression in cancer cell lines, raise the possibility that RHOXF1 expression has role in tumorigenesis process. So we analyzed the expression of RHOXF1 in two breast cancer cell lines and assessed the effect of its knock down on expression of two apoptosis related genes.

Materials and Methods

Cell lines and culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were kindly supplied by Dr MH Modarressi (Department of Medical Genetics, Tehran University of Medical Sciences, Iran). All cells were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated in an incubator containing 5% CO_2/95% humidity at 37°C.

shRNA constructs and transfection

Four unique 29mer RHOXF1 shRNAs constructs in retroviral GFP vectors were purchased from OriGene Technologies (Rockville, MD). The sequences in the shRNA expression cassettes were verified to correspond to the target gene with 100% identity. The sequences of shRNAs are listed in Table 1. The same plasmid without any insert as well as a plasmid with scrambled sequence cassette were used as negative controls. Cell viability was assessed by trypan blue staining before transfection. All shRNA transfections were performed with 1 μg DNA using Turbofectin 8.0 as a transfection reagent (OriGene Technologies) according to the manufacturer's instructions.

Cells were treated with 0.5 μg/mL of puromycin for 48 h. Transfection efficiency was monitored by the expression of green fluorescent protein (GFP) 24 hours post transfection. RNA was extracted from cells 48 hours after transfection and cDNA was synthesized.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells using AccuZol™ Total RNA Extraction Reagent (Bioneer, Korea) according to manufacturer's instruction. Then, 1 μg of RNA was applied to cDNA synthesis by accupower Rocketscript RT premix (Bioneer, Korea). After checking the quality of cDNA with primers designed to amplify HPRT gene, conventional RT-PCR experiment was done to assess RHOXF1 expression in MCF-7 and MDA-MB-231 cell lines. Amplifications was performed in 33 cycles of 30s at 95°C, 30 s at 61°C and 30 s at 72°C using an ABI thermal cycler 2720 (Applied Biosystems, Foster City, CA, U.S.A.). The reaction contained 1 μg cDNA, PCR set and Taq enzyme (Fermentas, Burlington, Canada).

Quantitative PCR reaction was performed on a rotor gene 6000 corbette detection system using LightCycler® FastStart DNA Master SYBR Green I (Roche, Germany) and following thermal cycling conditions: an initial activation step for 5 min at 95°C followed by 40 cycles including a denaturation step for 10 s at 95°C, 10 s at 60°C for annealing and 15 s at 72°C for extension step. Primer sequences are listed in Table 2. Melting curve analysis was done to confirm specificity of PCR products. Real time RT-PCR products were also electrophoresed on 2% agarose gel to evaluate product sizes and specificity.

Statistical analysis

Fold changes in gene expression were calculated by LinRegPCR(2) (Software for analysis of quantitative

Table 1. shRNA Sequences

ID	Sequences
GI330053	CAGCATCAAGCGCAGAAGGCCATGTTGGC
GI330054	ACGAAGTTCACGCTGTTGCAGGTGGAGGA
GI330056	ACTTAGGTGTGACTGAAGACAAAGTGCGG
GI330055	GCCAGATGTAGGCGACATCAGAGAGAATT

Table 2. Sequences of Primers

HPRT forward	GGCTCCGTTATGGCGACCCG
HPRT reverse	CGAGCAAGACGTTCAGTCCTGTCC
RHOXF1 forward	TGCCCACAAGAAGGGAACTTGCC
RHOXF1 reverse	ACAGTCGTCTGGGTCAGCACG
BCL2 forward	GATGTCGCCCCTGGTGGACAAC
BCL2 reverse	CACCAGGGCCAAACTGAGCAGAG
CASP8 forward	GGTGCGTCCACTTTCTGGGCA
CASP8 reverse	CCAGGCTCAGGAACTTGAGGGAGG

real-time PCR data) and Relative Expression Software Tool-RG©-version 3 (Calculation Software for the Relative Expression in real-time PCR using Pair Wise Fixed Reallocation Randomization Test©). HPRT was amplified as normalizer gene. The amounts of mentioned mRNAs in the tissue, standardized to the HPRT mRNA, were calculated as follows: $-\Delta CT=-[CT$ Gene of interest $- CTHPRT]$.

Results

Expression of RHOXF1 in MCF-7 and MDA-MB-231 cell lines

Normal testis cDNA was used as a positive control for RHOXF1 expression. RT-PCR showed that both cell lines expressed RHOXF1 mRNA. The size of product was similar in testis and cancer cell lines (146 bps).

Observation of transfection results

After transfection with the recombinant plasmids, the breast cancer cell lines MCF-7 and MDA-MB-231 showed green luminescence (green fluorescent protein, GFP), suggesting the correct expression of shRNAs (Figure 1).

Real-time RT-PCR results

Real-time RT-PCR products of HPRT, RHOXF1 and BCL2 genes in both cell lines are shown in Figure 2. We could not detect CASP8 expression in either of cell lines before and after transfection.

The relative RHOXF1 expression ratio in MCF-7 and MDA-MB-231 cell lines

RHOXF1 expression was evaluated in MCF-7 and MDA-MB-231 cell lines before transfection by quantitative real time PCR. Real time RT-PCR results showed that RHOXF1 expression when normalized with
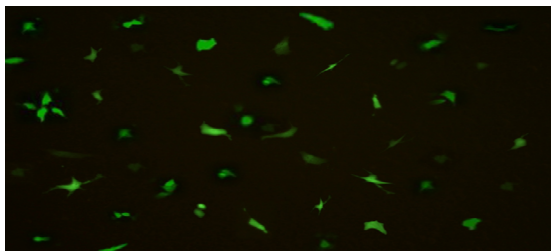

Figure 1. 70% Confluent MCF-7 Cells in a 96-well Microtiter Plate were Transfected with shRNA Containing Vectors using Turbofectin 8.0. Fluorescence Images were Taken 48 hrs Post Transfection.
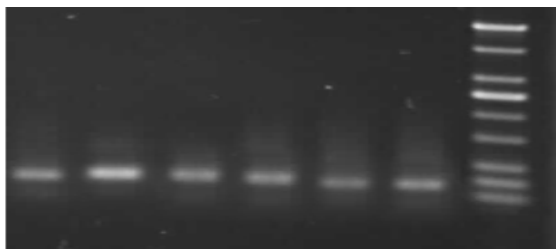

Figure 2. Real time RT-PCR Products for MCF-7 and MDA-MB-231 Cell Lines Electrophoresed on 2% Agarose Gel. lanes 1,2: BCL2; lanes 3,4: HPRT, lanes 5,6: RHOXF1, lane 7: DNA size marker.
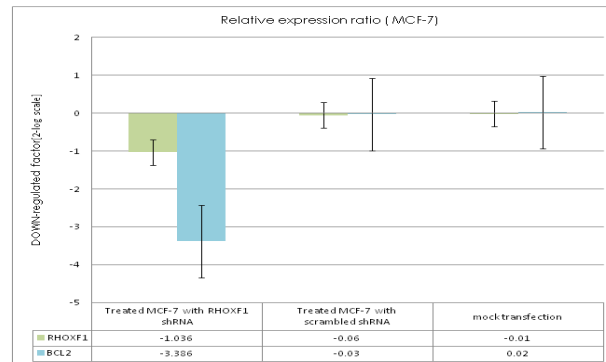

Figure 3. Relative Expression Ratios of RHOXF1 and BCL2 Before and After Transfection in 3 Groups for MCF-7 Cell Line.
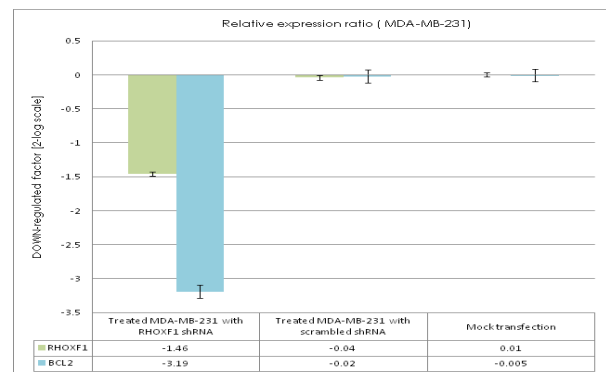

Figure 4. Relative Expression Ratios of RHOXF1 and BCL2 Before and After Transfection in 3 Groups for MDA-MB-231 Cell Line.

HPRT expression, is higher in MDA-MB-231 than MCF-7 by the factor 131 (data is not shown).

The mRNA expression intensity of RHOXF1 after transfection of cells with shRNAs

shRNA targeting RHOXF1 inhibited RHOXF1 mRNA expression in MCF-7 and MDA-MB-231 cells by the factors 0.48 and 0.36 respectively (Figures 3 and 4).

The mRNA expression intensities of target genes (BCL2 and CASP8) after transfection of cells with shRNAs

The statistical analysis showed that BCL2 mRNA of MDA-MB-231 and MCF-7 cells were down-regulated significantly after transfection with shRNA plasmids, compared with that in the control group (P<0.01).

The inhibition rates for BCL2 were 0.09 and 0.10 in the MCF-7 and MDA-MB-231, respectively.

We could not detect any alteration in expression of CASP8 after transfection in either cell lines (Figures 3 and 4).

Discussion

A large body of evidence indicates the involvement of HOX genes in tumorigenesis process. Many of them have been shown to be aberrantly expressed in different cancers (Wu et al., 2006). As they have critical roles in regulation of cellular functions such as apoptosis, receptor signaling, differentiation, motility and angiogenesis, alteration of their expression can be important for both tumorigenesis

and cancer suppression, depending on the situation (Shah and Sukumar, 2010).

The functions of RHOX genes in embryonic development have been studied deeply for over two decades and their role in postembryonic developmental processes have started to be examined. However, little is known about their function in tumorigenesis events.

The putative mouse orthologue of RHOXF1 has been shown to be expressed in pluripotent embryonic stem cells, primordial germ cells, teratocarcinoma cell lines, and some transformed cell lines, suggesting a possible role for it in cellular differentiation (Fan et al., 1999). It has been demonstrated that forced expression of Rhox5 in mouse embryonic stem cells inhibits the in vitro and in vivo differentiation of the cells (Fan et al., 1999).

A population of cells in tumors which are thought to be the sources of metastasis and tumor relapse demonstrates stem like features and expresses stem cell markers (Shipitsin and Polyak, 2008). The absence or very low expression of cancer-testis genes in normal differentiated tissue as well as their expression in normal stem cells have raised the possibility that their expression in tumor tissues is limited to cells presenting stem cell characteristics (Costa et al., 2007). As RHOXF1 and RHOXF2A genes express the features of both cancer-testis genes and stem cell markers, the evaluation of their function in tumor cells can help to elucidate the tumorigenesis mechanism.

Microarray analysis of 15P-1 Sertoli cell clones expressing physiological levels of Rhox5 from a stably transfected expression vector, has demonstrated Rhox5 induced alteration in expression of many genes such as those encoding transcription factors, proteins controlling cell cycle regulation, signal transduction, apoptosis, metabolism, and cell-cell interactions (Hu et al., 2010). Among them was Unc5c with a proapoptotic function which was down regulated in Sertoli cells expressing Rhox5. Interestingly, transient transfection of above mentioned cells with expression vectors encoding either RHOXF1 or RHOXF2 caused the down-regulation of Unc5c (Hu et al., 2010). This study has demonstrated the ability of human RHOXF1 and RHOXF2A in regulation of Rhox5 gene targets in mouse Sertoli cells. However, to our knowledge, their human target genes have not been studied before. Previous studies demonstrated that the pro-survival role of Rhox5 (and its probable human orthologue) in the testis is mediated by Unc5c down regulation. Here we reported another mechanism for RHOXF1 pro-survival function in cancer cell lines which is down regulation of BCL2 expression.

We chose 2 breast cancer cell lines with distinct features regarding estrogen receptor expression (ER) as MCF-7 is classified as ER positive and MDA-MB-231 as ER negative (Weigel and deConinck, 1993). MCF-7 is believed to have a relatively benign phenotype, while MDA-MB-231 human breast cancer cells are a highly invasive metastatic cell line. Expression profile of these 2 cell lines have been compared with specific focus on genes related to metastasis, cell motility and apoptosis (Zheng et al., 2006). MDA-MB-231 cells have shown to have a much more malignant molecular profile than MCF-7 cells with regard to expression of genes having

functions in cell migration and invasion, the ability to suppress apoptosis and the ability to metastasize (Zheng et al., 2006). The significant higher expression of RHOXF1 in MDA-MB-231 than in MCF-7 implies a role for this gene in malignant phenotype, especially when considering the antiapoptotic role of RHOXF1 in germ cells. In addition, we have shown that moderate RHOXF1 knock down can significantly down regulate expression of BCL2. BCL2 is an oncogene which acts as a critical regulator of apoptosis and is shown to be overexpressed in various solid tumors and hematologic malignancies (Zhang et al., 2010). The exact mechanism of BCL2 action is inhibition of cytochrome C release from the mitochondria and subsequent prevention of caspases induced apoptosis (Yang et al., 1997). However, it has been shown that Bcl2 down-regulation by siRNA induces massive autophagy in a caspase-independent manner in human leukemic HL60 cells (Saeki et al., 2000). It has been shown that overexpression of Bcl2 protein in tumoral cells inhibits the cell reaction to apoptotic signals induced by conventional chemotherapy and radiotherapy, whereas inhibition of Bcl2 production by siRNA has a therapeutic effect in various malignancies (Zhang et al., 2010). So this study provides insight in developing novel cancer treatment strategies.

A previous study has shown higher levels of caspase-3 and caspase-8 activities in MCF-7 and MDA-MB-231 cell lines and breast carcinoma tissue specimens compared with normal human fibroblast and mammary epithelial cell line in the absence of apoptotic stimuli (Yang et al., 2003). In addition, in a study of mRNA and protein levels of 7 caspases in 18 breast cancer cell lines, researchers could detect CASP8 expression in MDA-MB-231 cell line in spite of frequent down regulation of it in other breast cancer cell lines (Yang et al., 2007). However, a more recent study indicates that the CpG sites in the promoter region of CASP8 are methylated in MCF-7 and MDA-MB-231 cancer cell lines but not in two non-tumorigenic breast cell lines, while treatment with 5-FU increases unmethylated CASP8 and CASP8 mRNA in mentioned cancer lines (Wu et al., 2010). Our data is compatible with the latter study, as we could not detect CASP8 mRNA either before or after transfection. If CASP8 expression is epigenetically regulated, it is logical that RHOXF1 knock out does not have any effect on it. However, future experiments are needed to illuminate the mechanism of its expression regulation.

Acknowledgements

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