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Evaluating *RHOXF1* Gene Expression in Different Cell Lines

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ABSTRACT

Background: *RHOXF1* transcription factor is highly expressed in reproductive tissues and probably plays a role in spermatogenesis. *RHOXF1* is expressed in some cancer-derived cell lines, too. The anti-apoptotic role of *RHOXF1*, together with its hormone-dependent function, may increase its probable role in tumorigenesis as well as spermatogenesis. *RHOXF1* represents the properties of cancer-testis genes—the promising targets for immunotherapy. Therefore, its study in cancer cell lines can considerably contribute to future research on therapeutic interventions. This study used both experimental and in silico investigation of *RHOXF1* gene expression in different cancer cell lines.

Materials and Methods: In the present study, we investigated *RHOXF1* in silico gene expression levels in breast cancer and prostate cancer cell lines as well as in the Human Protein Atlas, GEMiCCL, and Expression Atlas databases. We also evaluated HEK 293 cells using RT-PCR. Finally, the results were compared and analyzed.

Results: The obtained data showed an increase in *RHOXF1* in cilico expression levels in the MCF7 cell line, which has a clear contradiction with the Human Protein Atlas and GEMiCCL databases. In this study, *RHOXF1* gene expression was not observed in HEK293, MDA-MB-231, PC3, and DU145. The expression of the *RHOXF1* gene in the database was also absent in some other cell lines, including HEK 293.

Conclusion: We discovered the discrepancy of some databases with the experimental findings, also we found higher expression levels of the *RHOXF1* gene as a cancer-testis gene in the MCF7 breast cancer cell line, which can be investigated as a possible candidate for immunotherapy.

Introduction

Homeobox genes are scattered throughout the genome encoding the transcription factors that direct embryonic development [1]. *HOX* genes family is a large subset of these regulatory genes being studied extensively, and its members are present in all animal species [2]. As their roles in setting cellular functions such as apoptosis, receptor signaling, differentiation, movement, and angiogenesis are crucial, their dysregulation

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can be influential in tumorigenesis or suppression of cancer depending on certain conditions [3].

The *Rhox* genes (reproductive homeobox X-linked) are a new gene cluster expressed selectively in the male and female reproductive tissues. The defects of these genes reduce fertility. *Rhox* genes are also highly expressed postnatally, so they are known as necessary factors for the reproductive system [4].

Two human *RHOX* genes, *RHOXF1* and *RHOXF2/2B*, are predominantly expressed in the testis and at lower levels in the ovarian tissue [5, 6]. Although *RHOX* genes commonly exhibit regulated and specific expression patterns, some members of the family, including *Rhox*5, *Rhox6*, and *Rhox9* in mice, as well as human *RHOXF1*, are expressed in tumors [6-9]. The anti-apoptotic function of *Rhox*5 in mice as an *RHOXF1* orthologue, besides their function of *RHOX*F genes as transcription factors and regulation of their expression by the hormone and their expression in cancer cells, may contribute to increasing the possible role of *RHOXF1* in carcinogenesis. Thus, the assessment of its role in the tumor cells can explain the carcinogenesis process. Moreover, *RHOXF1* expression has been reported in several cell lines, including HPB-ALL, Hec1A, A375, and HT29 [6].

Cancer cell lines are the necessary parts of biomedical investigation. However, the selection of appropriate cell lines for experimental objects is frequently challenging as the genotype information is either missing or scattered among various sources. In this study, to evaluate the gene expression levels of *RHOXF1* in cancer cell lines, we first carried out in silico study of the gene expression. Then we evaluated the gene expression levels in MCF7 and MDA-MB-231 cell lines, PC3 and DU145 cell lines, as well as HEK 293 in vitro system. Finally, we compared the results with the Expression Atlas database.

Materials and Methods

In silico investigation of *RHOXF1* gene expression in different cell lines

The expression of the *RHOXF1* gene was investigated in The Human Protein Atlas, GEMiCCL (Gene Expression and Mutations in Cancer Cell Lines), and Expression Atlas databases. The Human Protein Atlas (www.proteinatlas.org) database consists of three separate sections, each focusing on a particular aspect of the human protein genome analysis. One of these sections shows the local distribution of the protein inside the cells, which contains mRNA expression profiles from different panels of human cell lines (n=64), representing the various tissues and germinal layers.

The GEMiCCL is a database consisting of the human cancer cells which provide information about genotype and gene expression. Mutation data, gene expression, and Copy Number Variations (CNVs) are collected from the following three cell lines: CCLE (Cancer Cell Line Encyclopedia), COSMIC (The Catalog of Somatic Mutations in Cancer), and NCI60.

Finally, we compared the data obtained from this study to Expression Atlas (www.ebi.ac.uk), which is a comprehensive database capable of covering almost all cell lines and displaying the gene expression or loss of expression in each of the selected cell lines.

In vitro investigation of *RHOXF1* gene expression in different cell lines

Cell Culture

The investigations were carried out using MCF7 and MDA-MB-231 cell lines, PC3 and DU145 cell lines, and HEK293. All study procedures followed the ethical principles and the national norms and standards of the institutional Research Ethics Committee of Tehran University of Medical Sciences (Ethical approval ID: IR.TUMS.MEDICINE.REC.1398.190). The cells were obtained from the cell bank of the Department of Medical Genetics, Tehran University of Medical Sciences, Iran. Then, they were grown in RPMI1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin at incubator under the following conditions: 37° C, 5% CO^2, and 95% humidity.

RNA Extraction, cDNA Synthesis, and RT-PCR:

Total RNA was extracted using the RiboExTM total RNA solution (GeneAll®) by following the manufacturer's instructions. Then the quality and quantity of RNA were measured by using the ND-2000 NanoDrop instrument (Thermo Fisher, USA). Afterward, 1 µg of the extracted RNA was used for the cDNA synthesis using the Yekta Tajhiz Azma kit. Amplification was performed in 25 cycles of the 30 s at 94° C, 30 s at 59° C, and 30 s at 72° C using an ABI thermal cycler 2720 (Applied Biosystems, Foster City, CA, USA).

Primer design

NCBI Primer-Blast designed the primers, and GeneRunner software tested their efficacy and specificity. Forward and Reverse primers were designed for the PGM1 (Phosphoglucomutase 1) as a housekeeping gene, as well as *RHOXF1*. Primers are annealed with various exons of each gene to prevent false-positive results as

Table 1. Sequences of primers used for RT-PCR

Primer	Sequence (5'-3')
RHOXF1-F	ATGGTTTGATAGTGGGCTGGG
RHOXF1-R	TGTAAGGCAGGCAGGTAGAC
PGM1-F	ACTTGACTGCTTACCACCTCC
PGM1-R	GGGTAATTTCTTCCTGTGCCTG

there is a possibility of DNA contamination during RNA extraction. Table 1 lists the sequence of the primer pairs.

Results

In silico investigations

Data obtained from the Human Protein Atlas database showed that the *RHOXF1* gene was expressed in the HEK293, HDLM-2, NB-4, and SH-SY5Y cell lines. This gene had no or low expression in other cell lines (Figure 1). The data collected from the GEMiCCL database showed *RHOXF1* gene expression in several cell lines, including DND-41, CCK-81, and CW-2 (Table 2).

RHOXF1 gene expression increase in breast cancer cell line

RT-PCR data analysis showed the expression of the *RHOXF1* gene only at the MCF7 cell line. However, it

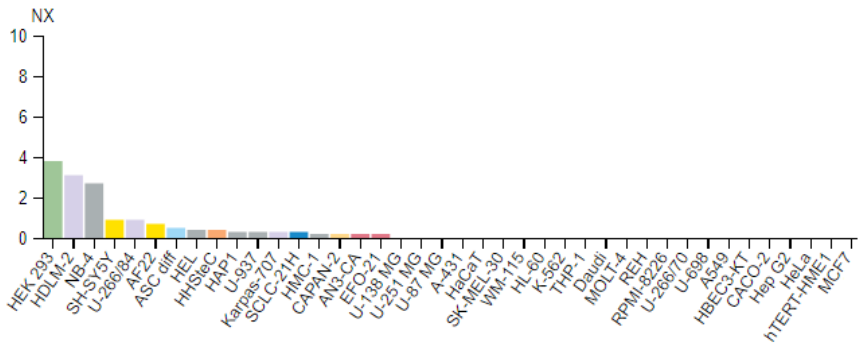

Figure 1. *RHOXF1* gene expression levels in cell lines obtained from the Protein Atlas database indicated the expression of this gene in only five cell lines of HEK293, HDLM-2, NB-4, SH-SY5Y, and U-266/84

Table 2. *RHOXF1* gene expression levels in various cell lines obtained from the GEMiCCL database indicated the expression of this gene in several cell lines, including DND-41, CCK-81, CW-2, SNU-1040, HT115, NCI-H2342, and NCI-H2347

Gene	Cell Line	Entrez	Source(%)	
			CCLE	COSMIC
RHOXF1	DND-41	158800	29.81	22.28
RHOXF1	CCK-81	158800	27.22	19.02
RHOXF1	CW-2	158800	28.29	17.45
RHOXF1	SNU-1040	158800	27.22	19.76
RHOXF1	HT115	158800	26.16	18.82
RHOXF1	NCI-H2342	158800	29.24	19.42
RHOXF1	NCI-H2347	158800	26.37	19.34

CCLE: Cancer Cell Line Encyclopedia; COSMIC: The Catalog Of Somatic Mutations In Cancer

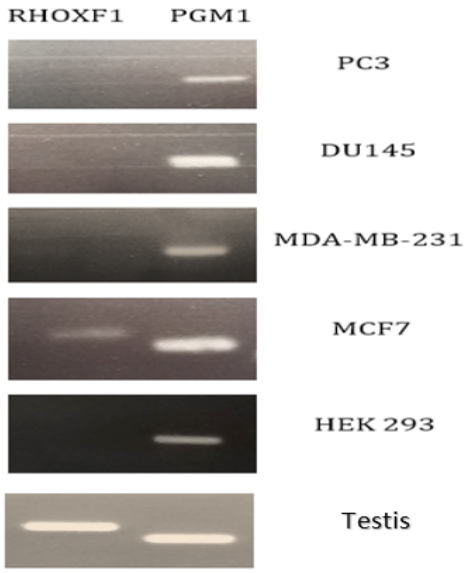

Figure 2. RT-PCR data analysis for PGM1 and *RHOXF1* genes in cancer cell lines together with normal testis tissue showed the expression of the *RHOXF1* gene at the MCF7 cell line as well as testis

is less expressed compared with the healthy tissue of the testis (Figure 2).

Finally, the obtained data from the Expression Atlas database were investigated, which confirmed *RHOXF1* gene expression in the MCF7 cell line (Figure 3). The expression of this gene was not observed in HEK, PC3, DU145, MDA-MB-231 cell lines.

Discussion

Besides their fundamental role in embryogenesis, homeobox genes are involved in tumorigenesis, and a large number of these genes are disturbed in several cancers [2]. *RHOXF1* is a human gene of this family specifically expressed in reproductive tissues and different cancer cell lines. Also, the anti-apoptotic effects of its orthologue, as well as its hormone-dependent manner, increase its possible role not only in spermatogenesis but also in tumorigenesis [10]. RT-PCR experiments have revealed *RHOXF1* expression in the ovary, testis, epididymis, and prostate [5]. Nevertheless, in other studies, the northern blot analysis did not reveal *RHOXF1* expression in tissues other than testis [6]. RT-PCR and RNase protection assay have illustrated *RHOXF1* mRNA expression in different tumor cell lines, such as HPB-ALL, Hec1A, and to a lesser extent, in MDA-MB-231 [6].

Since *RHOXF1* is mainly expressed in the testis tissue and has no or lower expression in any other tissues, its non-expression in the human embryonic kidney, HEK293 cell line, is justified. This result is consistent with the data obtained from Expression Atlas and GEMiCCL database, while it is contrary to Human Protein Atlas data.

In our study, the lack of *RHOXF1* gene expression in PC3 and DU145 cell lines suggests that this gene is not expressed in prostate cancer, which is compatible with the

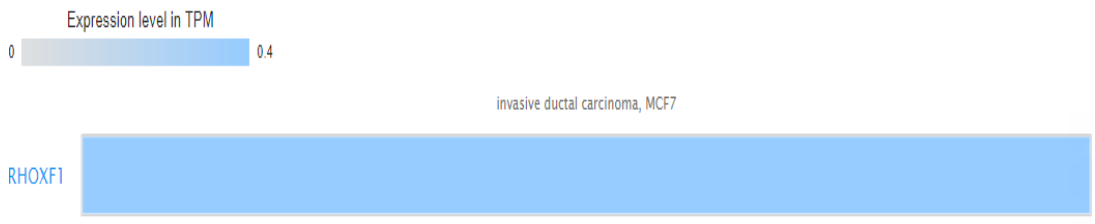

Figure 3. *RHOXF1* gene expression in MCF7 cell line obtained from Expression Atlas

results in all databases. Regarding breast cancer, the gene expression shows different results. We observed *RHOXF1* gene expression in MCF7 cell line, which is an $ER^{positive}$ breast cancer cell line, and the Expression Atlas database confirmed these results while other databases did not.

Because *RHOXF1* is a hormone-dependent gene, its expression in MCF7 is expected, but its expression was not observed in other breast cancer cell lines, MDA-MB-23, or its expression was less than the detection limit of our experiment. Because MDA-MB-231 is an $ER^{negative}$ cell line, *RHOXF1* is probably expressed only in the early stages of cancer, and in the later stage, when the cancer is metastatic and invasive, its gene expression level decreases. On the other hand, the *RHOXF1* gene was not expressed in the normal breast tissue but highly expressed in the MCF7 cell line. These findings can open a new window of future studies on the development of cancers.

Nowadays, because of the increasing prevalence of various cancers and their complications, scientists and researchers are paying so much attention to cancer all over the world. Considering the inefficiency and the aggressive mode of cancer treatments, numerous efforts were made to find decent therapies with more specificity and safety among them during recent years. The most appropriate candidate in this area is the genes explicitly expressed in the testis tissue while not expressed or little expressed in other healthy tissues of the body [11, 12]. Accordingly, testis cancer genes are promising targets for cancer vaccines and immunotherapy. The *Rhox* gene family shows the properties of the testis cancer genes. Therefore, they can be proper targets for cancer vaccine development. Given that the characteristics of these genes are not yet fully identified, the efforts to explain the expression, function, and interactions of these genes in cancer biology as well as reproduction are crucial.

Generally, the results of the present study revealed some differences in the experimental and in silico approach, meaning that in silico data are not entirely reliable. In this study, we demonstrated the elevation of *RHOXF1* gene expression in the breast cancer cell line. As breast cancer is the most common cancer among women and accounts for about one-third of all new cancer incidence [13], further study of this gene is likely to be the inspiring hope for the future treatment of this cancer. The present experiment provides information for examining breast cancer to evaluate the function of the *RHOXF1* gene, and the study of this gene as a possible candidate for immunotherapy is strongly recommended.

Ethical Considerations

Compliance with ethical guidelines

This research does not contain any studies with human participants or animals performed by any of the authors.

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Authors contribution's

All authors contributed in designing, running, and writing all parts of the research.

Conflict of interest

The authors declare no conflict of interest.

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