Study of Molecular Mechanisms of PDLIM4/RIL in Promotion of the Development of Breast Cancer

Dmitry Sergeyevich Kravchenko1,2, Yuriy Nikolaevich Lezhnin1,2, Julia Evgenyevna Kravchenko1,2, Stepan Petrovich Chumakov1, Elena Ivanovna Frolova1,4

1Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 16/10, Miklukho-Maklaya St., GSP-7, Moscow 117997, Russia
2Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 119991, Russia

Abstract

PDLIM4 (RIL) gene is often subjected to epigenetic suppression in malignant tumors of various organs. At the same time, reasons of this suppression are not fully understood, and the only effect of the proposed mechanism of PDLIM4 impact on malignant transformation of epithelial cells is partially refuted by new experimental data. In order to determine a range of targets, which can be affected by changes in the level of PDLIM4 expression, a model system based on the breast cancer cell line MDA-MB-231 was created and characteristics were given to the model. The model had artificially suppressed PDLIM4 expression by introduced shRNA expression promoter. Comparative transcriptome sequencing before and after the suppression of PDLIM4 revealed a number of genes whose expression levels correlated with a change in PDLIM4 expression. Most of these genes related to the MAPK signal pathway. Based on the data obtained, the most likely candidate genes were offered, which may play the role of the end effectors that stimulate malignant transformation after PDLIM4 suppression.

Keywords

PDLIM4; Differential expression; Epithelial-mesenchymal transition

Introduction

RIL (reversion-induced LIM-domain containing) gene that subsequently received its present name PDLIM4 was firstly described in 1995 as a potential oncosuppressor [1]. In humans, RIL gene is localized on the 5th chromosome in 5q31.1 locus. This locus is often deleted in various human malignancies [2]. In addition to deletions, PDLIM4/RIL gene can undergo epigenetic suppression, which is often found in malignant cell transformation [3-6]. RIL may play a special role in the development of breast cancer (BC): a series of new data indirectly indicate the correlation of individual clinical parameters of the disease, such as size, ploidy, and degree of differentiation of cells, with impaired RIL expression [6]. However, despite the evidence of violations of the relationship of the gene expression with this and other types of cancer, the mechanisms of tumor development with RIL participation remain to be unknown. Up to date, there has been only one hypothesis postulating possible mechanism of RIL-mediated inactivation of c-Src kinase. However, an integral role of RIL in the malignant transformation of cells remains unexplored [7]. The purpose of the study is to find molecular mechanisms and signaling pathways involved in the development of breast cancer, which also can involve RIL gene.

Methods

Cell lines used in the study and their cultivation

Brief description of the cell cultures used in the study is shown in Table 1. The cells were cultured in the Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 2 mM l-glutamine, 4.5 g/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin supplemented with 10% fetal bovine serum (FBS) (BioWhittaker). Culturing was performed at 37°C, 5% CO₂.

Transfection

Cells were placed onto culture vessels for 24 h prior to transfection so that the density at the time of transfection did not exceed 70% of the monolayer. Transfection was performed using a liposomal method involving LIPOFECT Amine and Plus reagent (Invitrogen) according to the recommendations from the manufacturer. The transfected DNA and Plus reagent were diluted in a serum-free medium at the recommended ratios and incubated for 15 min at the room temperature. Then the diluted LIPOFECT Amine was added to the mixture, and the solution was incubated for another 15 min in order to form liposomes. Meanwhile, the cellular medium was changed to serum-free. The resulting liposome-DNA complexes were added to cells followed by incubation from 3 to 16 h depending on the cell line, and then the medium was replaced with a full one. For exogenous expression of proteins, cells were incubated for at least 20 h after transfection. However, shRNK was expressed for not less than 48 h for full RNA-interference and the maximum inhibition of target mRNA.

Packaging of lentiviral particles

In order to obtain lentiviral particles, 293T cell line was used, providing exceptionally high expression of exogenous protein structures. Cells were cultivated at the ratio of 1:4 for 16 h prior to transfection so that at the time of transfection their density consisted approximately 70% of monolayer. Transfection was performed by the method as described above. Cotransfection of 3 or 4 plasmids was conducted: actual lentiviral construction encoding a viral RNA genome; one (pCMVΔR8.2) or two (pGag and pRev) helper plasmids allowing expression of all structural proteins, reverse transcriptase, and protease; membranaceous vesicular stomatitis virus protein (pVSV-G).

*Corresponding author: Frolova EI, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, 16/10 Ullitsa Miklukho-Maklaya, GSP-7, Moscow 117997, Russian Federation; E-mail: eifr@ibch.ru

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pseudotyping virions, which does not require cell receptor to initiate membrane fusion, thus ensuring a high transduction efficiency and a wide range of susceptible cells. The medium with the transfected cells containing recombinant virions was collected at 24, 36, 48, and 72 h, filtered through a 0.44 micron sterile filter (Millipore), and used for contamination of target cells. Viral effluents were stored frozen at −70°C. If necessary, a medium with reduced serum content (5% or 2.5% FBS) was used in order to reduce the concentration of toxic cellular metabolites in viral supernatants.

**Purification of virions and transduction**

For infection of target cells, they were stored at 16-24 h before handling in such a way that their density was 50-70% of the monolayer. Viral supernatants were diluted with a complete medium. Cationic agent polybrene at a concentration of 4-8 μg/mL was added to the cells in order to enhance the efficiency of contamination. Contamination was carried out in a minimal volume of medium for 4-8 h. Incubation was carried out on a rotary shaker (20-30 rpm) in order to increase the likelihood of transduction of cells with difficulties of contamination.

In order to reduce the toxicity of viral supernatants and enhance the titer of viral particles, they were concentrated with PEG-8000 where appropriate. Freshly harvested virus-containing supernatants were added with one-third volume of sterile, 40% PBS-derived PEG-8000 solution and mixed and incubated on ice for at least 8 h (in this state virus particles are stable for at least 7 days). Bounded virions were harvested by centrifugation at +4°C. 8,000 for 15 min, the supernatant was carefully removed and the pellet resuspended in phosphate-buffered saline or a complete growth medium, and the aliquots were stored frozen at −70°C.

**The genetic constructs used in the study**

pLSLpw-siRIL lentivector was used for RNA interference. This vector contains H1 promoter, which controls an expression of RNA hairpins composed of 3′ LTR. This position of H1+shRNK expression cassette plays a dual role. In the first place, during reverse transcription (in case of lentivirus infection of target cells), the promoter in 3′ LTR position (inactivated by H1+shRNK insertion) replaces the functional one in 5′ LTR position, thereby preventing transcription and generation of virus particles of infected cells. In the second place, during reverse transcription there is duplication of H1+shRNK of the expression cassette, resulting in obtaining two copies by each infected cell, amplifying RNA interference. Also, this vector has a selective marker (puromycin-resistance gene) under the control of H4 promoter.

The corresponding vector contained preselected oligonucleotide sequences homologous with cDNA RIL (homology with the cDNA RIL (GenBank AF153882) is underlined):

siRIL-1 direct 5′-/P/gattccagacaagcgtttcacttctcttcgcaagtagaa
ccacgcgtctggtttt/3′

siRIL-1 reverse 5′-/P/aactcaaaaaagcagcgtttcacttctcttgcaagagaa
gtagccaacgcgtcggg/3′

**RT-PCR**

Reverse transcription in order to obtain cDNA was performed using SuperScript II RT (Invitrogen) reverse transcriptase involving oligo (dT)$_{12-18}$ primer per 2 μg of total RNA. The method of reverse transcription is described in IFU for SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) reagent kit. Aliquots of 2 μL of cDNA were added to the standard mixture for PCR amplification (25 μL). The following primer sequences were used for RIL amplification: RIL direct: 5′-CTCGGTTCCTACGGCTTACAGAT; RIL reverse: 5′-CTCAGATGCCTGCAAGATCG. Each primer weighing 12.5 pmol was used. Annealing temperature was calculated using OligoAnalyser available on the web page of IDT DNA (www.idtdna.com/scitools) company. Regions of mRNA “household” genes, RP-2 and GAPDH, were amplified in order to monitor the efficiency of reverse transcription and leveling.

**RNA isolation, quality assessment, and hybridization on microchips**

Isolation of the total RNA was carried out using Trizol reagent following the protocol recommended by the manufacturer (Invitrogen). During the operation the samples containing 1 mL of lysate were complemented with 200 μL of chloroform followed by gentle stirring. The resulting suspension was centrifuged at 4°C, 12,000 g for 15 min. The upper portion of the solution was transferred to a clean tube and complemented with 500 μL of isopropanol. Samples were incubated on ice for 1 h and then centrifuged (10 min, 12,000 g, 4°C), after which the isopropanol was removed. The RNA pellet had been washed with 75% ethanol, which was removed during the following centrifugation (10 min, 12,000 g, 4°C). Thereafter, the RNA pellet was dried up for 5-10 min at the room temperature and resuspended in 50 μL of purified water free from RNase and treated with DNase I (Promega). Concentration of the resulting RNA was measured by using a spectrophotometer, ND-1000 NanoDrop (Thermo Scientific, USA). Its integrity was determined in denaturing agarose gel electrophoresis, and then verified during capillary electrophoresis (Agilent Bioanalyzer, Palo Alto, CA). In the first stage of sample preparation, ribosomal RNA was removed using RNAminus Transcriptome Isolation Kit (Invitrogen). Purified total RNA samples were fragmented into small parcels in the presence of bivalent cations at a high temperature. Thereafter, RNA fragments were used as a matrix for cDNA synthesis using oligo-dT 18 primer and reverse transcriptase. The complementary DNA chain was further synthesized using DNA polymerase I and RNase II. Then double-stranded cDNA fragments were ligated with adapters and amplified during PCR in order to obtain full cDNA library. Sequencing of the cDNA was performed using Illumina HiSeq 2000 System (Moscow State University of Lomonosov). Differential analysis of the data obtained was carried out using CLC Genomic Workbench 6.0.5.
The study of RIL-mediated intracellular processes that control the development of tumors began with an analysis of RIL expression in different cell lines of breast cancer. Based on RT-PCR, the analysis of RIL expression obtained in the previous work stages the fact that studied breast cancer cells were divided into two groups: lines included in the first group were characterized by normal or elevated levels of RIL expression (MDA-MB-231, MDA-MB-436, SUM-159PT, etc.). The second group consisted of cell lines with suppressed expression of the protein (BT-20, T47D, MDA-MB-468, etc.); the data are presented in Figure 1. These results are consistent with those presented in the reference on the variability of RIL expression in the cells of mammary tumors of different histogenesis [8].

MDA-MB-231 cell line characterized by RIL overexpression was selected for the further study of the first group. The next step was suppression of the RIL level in the selected cells using the mechanism of RNA interference. A special vector was used for this purpose. Being under the control of H1 promoter, this vector contained a region encoding a double-stranded RNA with a hairpin structure, which subsequently, in the process of RNA interference, was converted into the small interfering RNA specific for RIL, which were able to suppress the expression level of the gene.

The described genetic construction was used in the lentiviral transfection for a stable transfection of cell line MDA-MB-231. As a result, MDA-MB-231 siRIL transgenic cell lines were obtained with suppressed RIL expression. The effectiveness of changes in the RIL expression has been confirmed during PCR, and the results are presented in Figure 2.

The objective of the further work was to identify epigenetic changes in the cells after the suppression of RIL expression. For this purpose, iRNA was isolated from cells of base and transgenic lines (MDA-MB-231 and MDA-MB-231 siRIL). iRNA then was used to analyze the transcriptome during deep sequencing. As a result of this study, we obtained quantitative expression data of a number of genes for both cell lines of breast cancer. The next logical stage of the study was the comparison of the expression profiles of the original and transgenic cell lines by using iPathwayGuide (Advanta Corporation 2015) service in order to identify changes that correlate with RIL expression. Due to the fact that the difference in expression profiles between the studied cell lines was directly related to RIL, the analysis revealed genes whose products directly interact with RIL or participate in related intracellular processes.

The main focus in the analysis of the results was aimed at the search of genes associated with the processes of cancerous degeneration, control of proliferation, migration activity, and cell apoptosis. During the processing of the data on genes correlating with changes in RIL expression, we identified components of MAPK (mitogen-activated protein kinase) signaling pathways being key regulators of carcinogenesis. The further analysis was focused on the study of these genes (Figure 3).

Discussion

MAPK signaling pathway is one of the main pathways associated with cancer development. It plays an important role in regulating the expression of genes involved in the control of cell growth and survival. MAP-kinase cascade is activated by extracellular signal mediators, such as hormones, growth factors, chemokines, and neurotransmitters, that are recognized by the corresponding receptor tyrosine kinases or receptors associated with G-protein [9]. In mammals there are 4 major MAPK signaling pathways: ERK (extracellular signal-regulated kinase), ERK5 (extracellular signal-regulated kinase 5), JNK (c-Jun N-terminal kinase), and p38. Typically, ERK signaling pathways respond to growth factors, while JNK and p38 respond to extracellular stress signals [10].
In most cases, activation of ERK family of protein kinases is associated with stimulation of cell survival and proliferation, while activation of JNK and p38 protein kinases families is associated with induction of apoptosis [11].

Violation of the MAPK signaling pathways can lead to an uncontrolled cell growth and division, cell resistance to apoptosis, and development and progression of various forms of cancer [10,12]. A large amount of data point to an important role of MAPK signaling pathway in the formation and development of breast cancer [13-16]. Activation of different MAPK signaling pathways differently impacts the processes occurring in the cells. Thus, according to the recent studies, activation of p38 cascade regulating cell cycle and cell proliferation under conditions of stress inhibits metastasis of breast tumors, reducing the intensity of cell migration [17]. At the same time, a number of other data suggest that triple negative and basal-like subtypes of breast tumors are characterized by a widespread transcriptional activation of Ras/MAPK [18]. An increased activity of the components of MAPK pathways are also observed in the loci of metastatic lesions of breast cancer [9].

Activation of the individual components of MAPK pathway identified during the suppression of RIL expression may indicate that the studied protein is involved in key processes that control cell growth and division. Taking into account the heterogeneity of the MAPK signaling pathway, it is necessary to analyze the changes in the major genes that correlate with RIL expression discreetly.

c-Jun

c-Jun gene is one of the most important genes activated by suppressing of RIL expression. c-Jun proto-oncogene is a component of AP-1 transcription factor. Its increased activity is observed in various types of cancer [19]. An expression of c-Jun is mostly typical for invasive cancers, and it is associated with angiogenesis and increased proliferative activity of cells. According to some data, endogenous c-Jun reduces cell adhesion and stimulates the migration activity of breast epithelial cells [20]. c-Jun activation is not a predictor by itself. Nevertheless, it can indirectly stimulate angiogenesis, tumor progression, and their increased invasiveness [21,22].

Despite the large amount of information postulating an expressed oncogenic effect of c-Jun, recent data may indicate a more diverse role of this gene. According to recent studies, c-Jun promotes the preservation of differentiation status of somatic cells by inhibiting the genes responsible for pluripotency. Suppression of c-Jun provides acquiring of the pluripotent status by the cells, while maintaining its expression interferes with this process [23]. An analysis of recent data in the light of cancerous transformation of cells may cause scientists to reconsider the role of c-Jun, as the decrease in cell differentiation is one of the most important stages of epithelial-mesenchymal transition and serves as an important predictor of a greater malignancy and negative prognosis of the disease.

c-Fos

It was c-Fos being another AP-1 component that demonstrated an inverse correlation with the level of RIL expression. c-Fos is an important modulator of cell proliferation, differentiation, and transformation. Depending on the cell type, it can serve both as an activator and as a suppressor of transcription. Switching between two patterns is done by posttranslational modifications of C-terminal region of the protein [24]. c-Fos plays an important role in the development of breast cancer. According to some studies, suppression of c-Fos expression increases the survival rate and inhibits proliferative activity and the invasive capacity of this type of tumors. Although there is a number of alternative evidences that this gene is involved in the normal development and control of cell growth and can exhibit antiproliferative properties, inducing apoptosis by p53 activation cascade in response to cellular injury, c-Fos has been identified as an independent marker associated with a negative prognosis and low survival rates in case of breast cancer [25,26].

An inverse correlation of c-Jun and c-Fos expression with RIL gene may indicate the oncosuppressive role of RIL. We can assume that the low activity of the studied genes associated with an increased expression of both proto- oncogenes is typical for breast tumors with increased malignancy and poor prognosis of the disease. Based on these data, we can also assume that the suppression of RIL will be associated with an increase in proliferative activity and the rate of cell migration. However, this assumption is not consistent with the results of our previous studies in which there were no significant changes in migration and proliferative activity of the transgenic cells. The discrepancy between these results may be caused by heterogeneous action of c-Jun and c-Fos as well as influence of the activation of alternative pathways.

DUSP1 (MKP-1)

Among the genes being most important for the regulation of MAPK cascade and the level of expression that was activated during RIL suppression, DUSP1 was also identified. DUSP1 (also known as MKP-1) refers to the dual specificity phosphatases that catalyze the dephosphorylation of active MAPK. It was shown that MKP-1 inhibits a number of cellular processes regulated by ERK1/2, JNK, and p38 MAPK and provides anti-inflammatory and antiproliferative effect in various types of cancer, including breast cancer [27,28]. It is noteworthy that in case of the breast cancer, the level of DUSP1 expression is lower than that in the tumors of triple negative subtype. Thus, in the ER-positive cell lines (MCF-7, BT-474) there was an overexpression of DUSP1, while in ER-negative cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-468, MDA-MB-453, SK-BR-3), the level of this protein was suppressed [29]. Today there are studies of the possibility of using DUSP1 as a potential target for the treatment of triple negative subtype of breast cancer [29].

The data on the correlation of DUSP1 expression with a subtype of the breast cancer are of great interest to us because the cell line used to suppress the expression of RIL refers to ER-negative subgroup. An increased DUSP1 expression in response to RIL suppression indirectly proves the acquisition of MDA-MB-231 cell line of features typical for triple negative subtype of breast cancer, suggesting association in the changes of RIL expression with this subtype of cancer.

DUSP10

DUSP10 (also known as MKP-5) as DUSP1 refers to the family of phosphatases that regulate the activity of mitogen-activated protein kinases. DUSP10 is involved in the inactivation of stress-activated JNK and p38 kinases. Some data suggest that DUSP10 reduces the invasiveness of certain types of cancer due to inactivation of p38 [30]. According to the recent data, MKP-5 also interacts with ERK, suppressing its activation and reducing the level of ERK-dependent transcription. In a number of tumors, the expression of MKP-5 is increased, except for breast cancer. In breast cancer there were notable differences between cancer and normal cells [31].
Activation of DUSP1 and DUSP10 in response to the suppression of RIL expression can be assessed in different ways. On the one hand, we can assume an oncosuppressive role of the studied protein, because the suppression of its expression leads to the activation of intracellular protective mechanisms providing anti-inflammatory and antiproliferative effects. On the other hand, you can assume participation of RIL in signaling cascades that suppress activation of dual specificity phosphatases characterized by oncosuppressive action. These results require further experiments and in-depth analysis.

**GADD45B**

GADD45B is a member of the evolutionarily conserved family of genes that are activated in response to cellular stress and DNA damage and associated with the control of cell growth and apoptosis [32]. Genes of this group provoke activation of p38/INK signaling pathway by increasing the activity of MTK1/MEKK4 kinase with subsequent activation of both of these signaling pathways.

**DDIT3**

The expression of DDIT3 gene is increased in response to the suppression of RIL. DDIT3, also known as GADD153 or CHOP, encodes a transcription factor belonging to the C/EBP family. According to some data, the induction of DDIT3 may be caused by DNA damage or exposure of the cells to toxins or other types of stressful agents. DDIT3 activation leads to apoptosis of damaged cells, while inhibition of this gene increases the number of cells that carry genetic mutations [33,34]. According to more recent studies, GADD153 effect may differ depending on its cytoplasmic or nuclear localization in a cell. Cytoplasmic GADD153 affects more than 90 genes associated with cell migration, whereas its nuclear form affects genes that control the cell cycle. Cytoplasmic form inhibits cell migration, while a nuclear form inhibits cell division [35].

**Conclusion**

The data obtained regarding an increase in the intensity of gene expression associated with the cellular stress as response to the suppression of RIL expression are an issue of great interest. If the suppression of RIL expression exposes cells to stress, it can be concluded about the importance of the studied gene for normal cells functioning. In summary, we can conclude that suppression of RIL expression in breast cancer cells results in modulation of expression of important genes involved in key processes providing the control of the cell cycle, migration activity, inflammatory processes, and antimtress action. This proves a direct association of the studied gene with key signaling cascades associated with the development of cancer. The data are contradictory in many respects. This does not allow us to make an unambiguous conclusion about the role of RIL in the development of tumors. Nevertheless, the revealed correlation will serve as a basis for planning further studies in order to reveal the role of RIL in the development of cancer.

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