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Research Paper

Inhibition of cell proliferation by siRNA targeting hPRLR in breast cancer MCF-7 cell line

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Abstract

Objective: To study the inhibition of proliferation of breast cancer by small interfering RNA(siRNA) targeting human prolactin (hPRLR) and the underlying mechanisms. **Methods:**The siRNA targeting hPRLR was chemically synthesized and transfected into MCF-7 cells, the expression of hPRLR was analyzed by real-time quantitive PCR, cell growth inhibition was measured with MTT assay, cell cycle of the transfected cells was examined by flow cytometry, meanwhile, expression of cyclin D1 was tested by semi-quantitative RT-PCR. **Results:**24 h after transfection with 100 nmol/L siRNA-PRLR, the expression of hPRLR mRNA was suppressed by 65%, cells in G1 phase increased, but cells in S phase decreased. Down regulated hPRLR expression exhibited significant inhibition in cell proliferation. And the expression of cyclin D1 was down regulated. **Conclusion:**The results indicate that siRNA-hPRLR is a useful tool for silencing hPRLR expression and inhibiting cell proliferation in breast cancer MCF-7 cell line, and it may be a possible new approach for breast cancer gene therapy.

Key words: human prolactin; breast cancer; siRNA; MCF-7

INTRODUCTION

Breast cancer is the leading cause of cancer-related death for women in the world. In recent years in our China the incidence of breast cancer has been increasing. However, even now the etiopathogenisis of breast cancer is not very clear. Perhaps the interaction of heredity, hormones, immunity and environmental factors play important roles in genesis of breast cancer. Meanwhile, being different from other tumors, the mammary gland responding to major hormones and many cytokines, which indicates that breast cancer is hormone dependent [1-2]. The hormone/cytokines human prolactin(PRL) is critical for development and differentiation of the mammary gland. The receptor of hPRL(hPRLR) is up regulated in mammary tissues during development and studies have shown that the majority of human breast tumors have higher hPRLR levels than surrounding normal mammary tissues[3-4]. It has also been demonstrated that both hPRLR degradation and hPRLR phosphorylation on Ser349 were impaired in breast tumor cells and tissues, and directly correlated with enhanced expression of hPRLR in malignant breast epithelium[5]. Therefore, inhibition or blocking of hPRLR expression may be a new method for breast cancer biotherapy. RNA interference(RNAi) is a mechanism by which double stranded RNA acts as a signal to promote degradation of mRNA with sequence identity[6]. In this study, we chemically synthesized siRNA targeting hPRLR to down regulate or block the expression of hPRLR mRNA in breast cancer MCF-7 cell line specifically, then detected the inhibition of cell proliferation and analyzed change in expression of cyclin D1(CCND1). This study aims to investigate the role of hPRLR in breast cancer

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development, and to get a new target for diagnosis and gene therapy of breast cancer.

MATERIALS AND METHODS

Materials and reagents

Bovine serum (Gibco,USA), Lipofectamin™ 2000 Reagent(Invitrogen,USA), MTT and DMSO were purchased from Sigma(USA), Taq DNA Polymerase, SYBR Green I and reverse PCR reagent(TAKARA, Japan), PCR primers were made by Shanghai Invitrogen Biotechnology Co,LTD. Three siRNA-hPRLRs, Non-scilencing siRNA and siRNA-GAPDH were synthesized by Shanghai Jikai Biotechnology Co.LTD. Human breast cancer MCF-7 cell came from American Type Culture Collection(ATCC).

Design and synthesis of siRNA

According to human *hPRLR* gene sequence(GenBank accession NM_000949.2), we designed and chemically synthysized three different siRNAs(siRNA-hPRLR$_1$, ssiRNA-hPRLR$_2$, siRNA-hPRLR$_3$) containing 21 base paires. All siRNA sequences were sourced in the National Center for Biotechnology Information's(NCBI)-we search for short nearly exact matched modes against all human sequences deposited in the GenBank and RefSeq databases and no significant homology(>17 contiguous nucleotides of identity) to genes other than the targets was found. Meanwhile, siRNA targeting GAPDH was used as a positive control and non-silencing siRNA was used as a negative control. All nucleotide sequences of siRNA were shown in ***Tab 1.***

Tab 1 **Sequences of siRNA**

Gene and oligonucloitide	Sequence(5′-3′)
siRNA-PRLR1	
Sense:	GUUUCUCGGAUGAACUUUAtt
Antisense:	UAAAGUUCAUCCGAGAAACtt
siRNA-PRLR2	
Sense:	CACUACAGAGUACGUGAAAtt
Antisense:	UUUCACGUACUCUGUAGUGtt
siRNA-PRLR3	
Sense:	GCACACUGCUUAGUAUUCUtt
Antisense:	AGAAUACUAAGCAGUGUGCtt
Non-silencing	
Sense:	UUCUCCGAACGUGUCACGUtt
Antisense:	ACGUGACACGUUCGGAGAAtt
siRNA-GAPDH	
Sense:	GUGGAUAUUGUUGCCAUCAtt
Antisense:	UGAUGGCAACAAUAUCCACtt

Cell culture and siRNA transfection

MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum.Then, 24 h before transfection, the cells were seeded in six-well plate at 2×10^5 cells per well in 2 ml of antibiotics-free DMEM medium. Experiments were designed as blank group, negative group and positive group and three siRNA-PRLR group. Transfection of MCF-7 with siRNA used the Lipofectamine™ 2 000 reagent, following the protocol set by the manufacturer, and the final concentration of siRNA was 100 nmol/L. 24 h after transfection, cells were harvested for assays.

Quantitation of PRLR mRNA by real-time PCR assay

24 h after transfection, cells were collected and total RNA was extracted by Trizol. The concentration, purity and amount of total RNA were determined using ultraviolet(UV) spectrophotometry. Only samples exhibiting intact 28 S and 18 S ribosomal RNA were subjected to RT-PCR. 1 μg total RNA was reverse transcribed by Rever Tra Ace- α-™ in order to get the cDNA as template of real-time PCR. The appropriate forward primer(*hPRLR*):5′-GCGACCTTCATTCAGATAC C-3′,and reverse primer:5′-CCAGCAAGTCCTCA TAGTCA-3′were used. β-actin was used as the internal control with forward primer:5′-AAAGACCTG TACGCCAACAC-3′, and reverse primer:5′-GTC ATACTCCTGCTTGCTGAT-3′. After reaction, we can get the number of Ct, which was used to calculate level of gene expression. The formulae are as follows: $\triangle Ct=Ct_{objective\ gene}-Ct_{\beta\text{-}actin}$, $\triangle\triangle Ct=\triangle Ct_{test\ group}-\triangle Ct_{control\ group}$, test group/control group=$2^{-\triangle\triangle Ct}$.

Measurement of cell growth by methylthiazolyltetrazolium assay

The effect of siRNA-hPRLR on cell proliferation was measured by MTT colorimetric assay. The day before transfection, MCF-7 cells were seeded at a density of 8×10^3 cells/well into 96-well plates. Experiments were designed as blank group(DMEM as the substitute of transfection complex), negative group(100 nmol/L non-scilencing siRNA) and siRNA-hPRLR group(100 nmol/L siRNA-hPRLR). After transfection, cells were incubated at 37℃, 5%CO_2 incubator for 24 h. MTT(5 g/L) was added to the wells(10 ml/well) at the end of experimental period. After 4 h incubation at 37℃, the medium was removed from the wells, and dimethylsulfoxide(DMSO) was added to each well(150 ml/well). The plates were vibrated at room temperature for 5 min. Absorbance of each well at 570 nm was read on an enzymelinked immunosorbent assay reader.

Flow cytometric analysis of the cell cycle

The day before transfection, MCF-7 cells were seeded at a density of 2×10^5 cells/well into 6-well plates. Experiments were designed as blank group(DMEM as substitute of transfection complex), negative group (100 nmol/L non-scilencing siRNA) and siRNA-hPRLR

group(100 nmol/L siRNA-hPRLR). 24 h after transfection, cell modality was observed by the inverted microscopy. Then cells were harvested, washed twice with 1×PBS, fixed with ice-cold 70% ethanol at –20℃ for 24 h, and stained with 20 g/L Propidium iodideI(PI: 300 μl) for 20 min. Cell cycle analysis was done with FAC station equipped with Cell Quest.

Expression of CCND1 mRNA by semi-quantitative RT-PCR

CCND1 gene and β-*actin* gene were amplified by PCR, with the template of cDNA as forward. The appropriate forward primer(CCND1):5′-GCCTCTAAG ATGAAGGAGAC-3′, and reverse primer:5′-CGGTAGTAGGACAGGAAGTT-3′ were used and the amplification fragment length was 356 bp. β-actin was used as the internal control with primers as forward. CCND1 and β-actin were amplified in separate tubes. The PCR procedure contained 28 cycles of 94℃ for 5 min, 58℃ for 45 s, and 72℃ for 1.5 min. The PCR products were analyzed on 1.5% agarose gels. Densitometric analysis were performed by using Sensiansys Image software and the inhibitory rates of CCND1 mRNA expression were calculated as follows:[CCND1 group(gradation$_{CCND1}$:gradation $_{β\text{-actin}}$) / Negative group (gradation$_{CCND1}$: gradation $_{β\text{-actin}}$)] × 100%.

Statistical analysis

SPSS 10.0 software was used to perform statistical analysis. *T* test was used for comparison between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Down-regulating effects of siRNA on hPRLR mRNA expression

Compared with the blank group and the negative group, among three siRNA-hPRLRs, siRNA-hPRLR1 can specifically inhibit the expression of PRLR mRNA ($P < 0.05$). According to the formulae, the inhibitory rate was 65%(***Tab 2***). However, the other two siRNA-hPRLRs had no significant inhition on hPRLR expression. Besides, there was no significant difference between the blank group and the negative group($P > 0.05$).

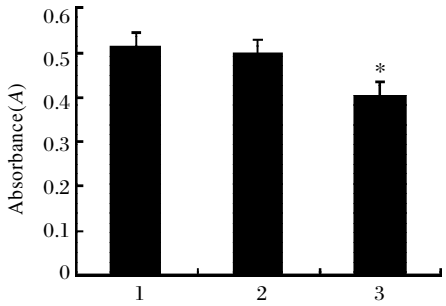
Tab 2 **Quantification of hPRLR mRNA expression by real-time RT-PCR** ($\bar{x} \pm s$)

Groups	Ct_{PRLR}	$Ct_{β\text{-actin}}$	$2^{-\triangle Ct}$	$2^{-\triangle Ct}$
Blank group	23.63 ± 0.37	14.03 ± 0.27	1.40E-03 ± 1.11E-05	–
Negative group	24.33 ± 0.21	14.55 ± 0.07	1.13E-03 ± 7.83E-06	–
siRNA-PRLR	25.40 ± 0.36	14.16 ± 0.28	4.19E-04 ± 5.81E-06*	0.37

Compared with the blank group and the negative group, *$P < 0.05$.

Inhibition of cell growth by siRNA-hPRLR

Because PRLR overexpression is known to stimulate cell growth, we examined growth rate of cells in which the hPRLR had been silenced by chemically synthesized siRNA. As shown in ***Fig 1***, siRNA PRLR could inhibit the proliferation of MCF-7 cells($P < 0.05$). In contrast, there was little effect for non-scilencing siRNA on cell proliferation.



1: Blank group; 2:Negative group; 3:siRNA-hPRLR(100 nmol/L)
Compared with the blank group and the negative group, *$P < 0.05$.

Fig 1 Effect of transfection with siRNA on cell growth

Changes in cell cycle by siRNA-hPRLR

24 h after transfection of siRNA-hPRLR, under the inverted microscopy, we found that cells of two control groups grew well , while PRLR scilenced cells exhibited a worse growth state. Cell cycle distribution was examined in cells with inhibited expression of hPRLR by chemically synthesized siRNA. The proportion of cells in G1 stage was significantly higher in siRNA-hPRLR group than in two control groups, and this increase was accompanied by concurrent decrease in the number of cells in S stage. These findings suggested that hPRLR affected cell cycle regulatory mechanisms that control the G1-to-S transition(***Fig 2***).

Down-regulating expression of CCND1 gene

Compared with untransfected siRNA-hPRLR groups, the expression levels of CCND1 gene declined. Densitometric analysis was performed using Sensiansys Image software and the inhibitory rate of CCND1 mRNA expression was 33.3%(***Fig 3***).While there was no obvious difference between the blank group and the negative group in CCND1 mRNA expression. The expression level of β-actin mRNA was similar in all groups of cells($P > 0.05$).

DISCUSSION

As is reported, in most of breast cancer patients, the

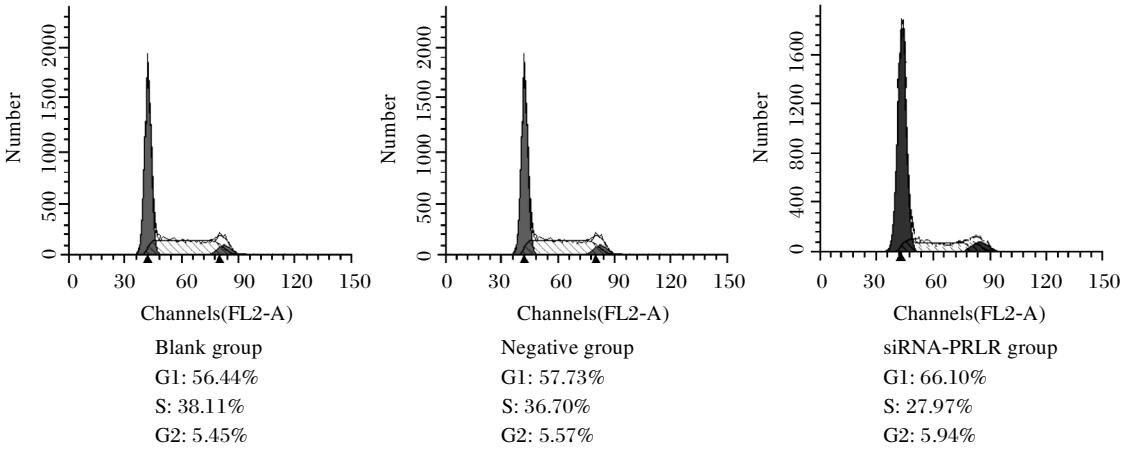
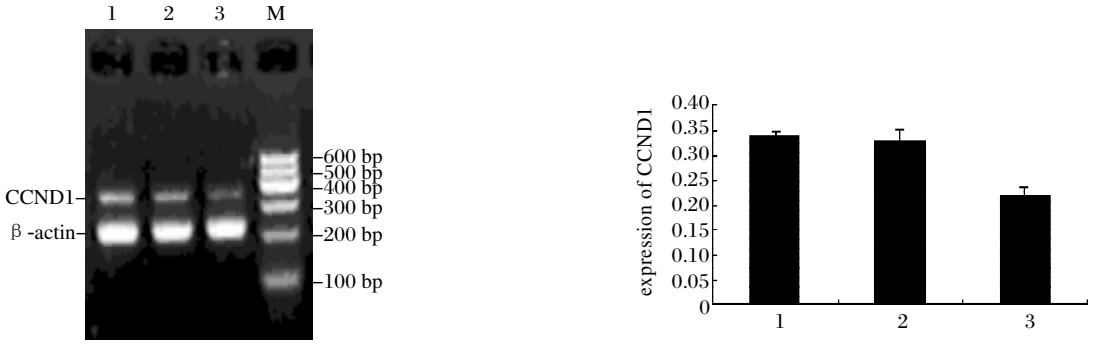


Fig 2 Change in cell cycle distribution analysis by flow cytometry



1: Blank group; 2: Negative group; 3: siRNA-hPRLR group; M: DNA Marker

Fig 3 mRNA expression of CCND1 detected by semi-quantitative RT-PCR

neoplastic tissues express higher level of hPRLR than normal adjacent tissues[7]. Human prolactin antagonist, hPRL-G129R, can inhibit breast cancer cell proliferation through induction of apoptosis[8-9]; in rPRL(rat prolactin, rPRL) transgene mouse, rPRLR(rat prolactin receptor, rPRLR) of breast tissue became more active, which lead to higher incidence of breast cancer[10]. All these experiments indicated that: up-regulation of hPRLR will cause wrong signaling caspades, and finally induce abnormal proliferation of breast epithelium.

RNA interference(RNAi) is a genetic interference phenomenon directed by double-stranded RNA (dsRNA). It could specifically and efficiently degrade mRNA, resulting in post-transcriptional gene scilencing (PTGS), which has become a new technique in knocking gene down and probably plays an important role in gene func-tion and gene therapy[11-12].

In this study, we chemically synthesized siRNA targeting PRLR gene, then transfected MCF-7 cells with siRNA. Results revealed that,among siRNA-hPRLR$_1$, siRNA-hPRLR$_2$ and siRNA-hPRLR$_3$, only siRNA-hPRLR1 had distinct effect on hPRLR silencing(about 65%), compared with the control groups. This means different siRNAs, targeting different regions of hPRLR mRNA, possess diverse silencing effects. Because of the down regulation of PRLR, signaling triggered by PRL-PRLR complex was blocked, which subsequently influenced the combination of STAT(Signal Transducers and Activators of Transcription) and the promoter of target gene[13]. Therefore we observed the cell growth inhibition by MTT, detected a block in the G1 phase of cell cycle distribution by flow cytometry(FCM). We believe that the above data have laid a foundation for further tumor therapeutic investigations to follow.

To investigate the mechanism of proliferation inhibition by siRNA-hPRLR, we observed CCND1 expression after siRNA transfection by semi-quantitative RT-PCR, the result of which indicated that, down regulation of hPRLR mRNA induced reduction of CCND1. CCND1 is one of main target genes of PRL-PRLR signaling pathway[14]. PRL binding at the PRLR induces activation of Jak2(Janus kinase 2), phosphoration of PRLR. PRLR signaling can activate STAT 1,3, or 5, which then form homo-or heterodimers that translocate to the nucles where they bind to specific response elements(GAS sites) in the promoters of target gene (CCND1)[15-16]. So, when expression of PRLR is down regulated, binding sites of PRL is surely reduced, the Jak2/Stat pathway is blocked, which leads to inhibition of CCND1 expression. Therefore, the experiments not

only once again validated higher expression of CCND1 in breast cancer was associated with enhanced PRL-PRLR signaling, but also explained the reason why cells were arrested in G1 phase[17-18]. We can conclude that the reverse of cell malignant phenotype by siRNA-hPRLR may be associated with down regulation of CCND1 gene. In conclusion, siRNA inhibits the hPRLR gene expression and proliferation of breast cancer MCF-7 cells with specificity, and is a possible new approach for breast cancer gene therapy.

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·*Abstract*·

A novel genetic screen for leukemia stem cell immortalization genes

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Leukemia, like many other cancers, is thought to arise from a small population of stem cells that have the capacity to self-renewal extensively and to initiate, sustain or regenerate the disease. Elimination of the leukemia stem cells (LSCs) will likely be essential, and probably sufficient, for curing this disease.Recent studies have shown that LSCs can be derived from early hematopoietic progenitors as well as more differentiated derivatives; the key feature being these cells have acquired an increased proliferative capacity and the ability to self-renew extensively. Genes that make this possible are attractive drug targets for treating leukemia. In our laboratory we have developed a novel in vitro genetic screen that uses retroviral insertional mutagenesis as a tool for identifying genes that are able to convert both normal hematopoietic progenitors and committed myeloid progenitor cells into cells that resemble LSCs. Interestingly, the genes identified in this screen are either known human leukemia genes or genes thought to regulate them ? Recently, insertional mutations in these same genes were identified in the blood of X-linked granulomatous disease patients following retroviral gene therapy and likely reinforced the therapeutic efficacy in this trial. The insertional mutations were associated with a 3-4 expansion in the myeloid compartment of gene-corrected patients, indicating that mutations in these genes also have an effect on the self-renewal of human hematopoietic cells. Currently, we are determining whether this screen can be extended to other cells types(i.e., epithelial cells) and whether we can use this information to develop better methods for killing LSCs.

Key words: leukemia; stem cell; genetic screen