INHIBITION OF PROLIFERATION OF HUMAN BREAST CANCER MCF-7 CELLS BY SMALL INTERFERENCE RNA AGAINST LRP16 GENE

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ABSTRACT

Objective: Our previous studies have firstly demonstrated that 17β-E2 up-regulates LRP16 gene expression in human breast cancer MCF-7 cells, and ectopic expression of the LRP16 gene promotes MCF-7 cells proliferation. Here, the effects of the LRP16 gene expression on growth of MCF-7 human breast cancer cells and the mechanism were further studied by establishing two stably LRP16-inhibitory MCR-7 cell lines. Methods: Hairpin small interference RNA (siRNA) strategy, by which hairpin siRNA was released by U6 promoter and was mediated by pLPC-based retroviral vector, was adopted to knockdown endogenous LRP16 level in MCF-7 cells. And the hairpin siRNA against green fluorescence protein (GFP) was used as the negative control. The suppressant efficiency of the LRP16 gene expression was confirmed by Northern blot. Cell proliferation assay and soft agar colony formation assay were used to determine the status of the cells proliferation. Cell cycle checkpoints including cyclin E and cyclin D1 were examined by Western blot. Results: The results from cell proliferation assays suggested that down-regulation of LRP16 gene expression is capable of inhibiting MCF-7 breast cancer cell growth and down-regulation of the LRP16 gene expression is able to inhibit anchorage-independent growth of breast cancer cells in soft agar. We also demonstrated that cyclin E and cyclin D1 proteins were much lower in the LRP16-inhibitory cells than in the control cells. Conclusion: These data suggest that LRP16 gene play an important role in MCF-7 cells proliferation by regulating the pathway of the G1/S transition and may function as an important modulator in regulating the process of tumorigenesis in human breast.

Key words: Estradiol; LRP16; Small interference RNA; MCF-7 Proliferation; Soft agar assay; G1/S control

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Estrogen and its receptor (ER) play important roles in several physiological processes, including development of the female and male reproductive systems as well as bone, vascular and neuronal functions[14]. They are also important pathological factors for genesis and malignant progression of breast cancer[5-6]. ERα binds to estrogen response elements or interacts with other transcription factors upstream of the target genes[7-9], thus regulates the transcription of various genes as a transcription factor. There are many reports concerning target genes that are transcriptionally activated by ERα, but the entire mechanism of the pathway from ERα leading to the proliferation and progression of mammary tumors is far from being clarified[10,11]. Breast cancer cell lines have been extensively used as models for understanding the mechanisms associated with 17β-estrodial (E2) induced cell growth and for development of antiestrogenic and anticarcinogenic agents for treatment of this disease[12-14]. MCF-7 cells were among the first ER-positive human breast cancer
cell lines characterized as responsive to the mitogenic effects of estrogens in cell culture and in athymic nude mice bearing MCF-7 cell xenografts\textsuperscript{[14-17]}. LRP16 is a human gene which was originally recognized from peripheral lymphocyte cells by our group in 1999 using restriction length genomic scanning (RLGS), and then the cDNA was isolated using the rapid amplification of cDNA end (RACE) technique (GenBank Accession No. AF202922)\textsuperscript{[18]}. LRP16 contains an open reading frame for a protein of 325 amino acids and the encoding product mainly distributes in the nucleus\textsuperscript{[19]}. Based on mRNA analysis showing that LRP16 is higher in primary acute leukemias and cell lines than in normal bone marrows, LRP16 has been implicated as a candidate oncogene\textsuperscript{[20]}. Previous study has demonstrated that E2 activates the LRP16 mRNA levels in MCF-7 human breast cancer cells and also reporter gene activities in cells transfected with pGL3-S0, which contains the growth-responsive--2600 to --24 region of the LRP16 gene promoter\textsuperscript{[21]}. Further study identified an estrogen response element half site (ERE1/2)/GC-rich Sp1 binding site within the --251 to --225 upstream region of the LRP16 gene which confers the E2-action via ERA/Sp1 binding to DNA. Ectopic expression of the LRP16 gene in MCF-7 significantly stimulated cells proliferation and markedly increased the cyclin E protein level\textsuperscript{[21]}. These findings imply that LRP16 may play an important role in carcinogenesis and/or progression of hormone-dependent breast cancer.

The aim of the present study was to further examine the effect of different LRP16 expression level on the growth of human breast cancer cells and to investigate the possible mechanism. MCF-7 breast cancer cells were used as a model and we stably transfected hairpin small interference RNAs targeting LRP16 mRNA, which were released by retrovirus, into human MCF-7 breast carcinoma cells. LRP16-inhibitory MCF-7 cells normally show the characteristics with slow-growing and lower colony efficiency on soft agar.

**MATERIALS AND METHODS**

**Chemicals, Biochemicals, and Oligonucleotides**

Puromycin was purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from GibicoBRL (Grand Island, NY, USA). Transfection reagent SuperFect, PCR product extraction kit and plasmid purification kit were purchased from Qiagen (Hilden, Germany). \([\alpha^{32}P]dCTP\) was purchased from YaHui Chemical Co. (Beijing, China). Various restriction enzymes were purchased from TaKaRa (Ostu, Shiga, Japan). RNA extraction reagent TRIZOL were purchased from Gibco BRL (Grand Island, NY, USA). Random primer labeling kit was purchased from Promega Corp. (USA). Antibodies, including anti-human cyclin E, cyclin D1 and β-actin used for the Western blot analysis and the chemiluminescence luminal reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Structures of the synthetic oligonucleotides are summarized in Figure 1.

### L374 Sequence:

| 5'-ttt gcagcggaggaacattac ttcaagaga | glaagttctccgctgcgtcgg tttg-3' |
| 3'-tctccttagtttaag aagttcact | cattacaagggggecgacgaa aaactttaa-5' |

### L668 Sequence:

| 5'ttt gactggcaagggcagata ttcaagaga | gatacgctcgcgtgctgctgg tttg-3' |
| 3'-tctccttagtttaag aagttcact | ctgaaccggaacagctcggaa aaactttaa-3' |

### GFPi Sequence:

| 5'ttt gaagagcttcgctctccttttttt tttg-3' |
| 3'-tctccttagtttaag aagttcact | ctgaaccggaacagctcggaa aaactttaa-5' |

**Fig. 1.** Synthesized sequence of nucleotide acids.

The restriction sites for \textit{BbsI} and \textit{EcoRI} were added at 5'- and 3'-terminals, respectively. The sequence for forming loop structure was underlined. The inverted repeat sequences flanking the loop were specific for LRP16 or GFP gene mRNA.

**Plasmid Constructs**

The RNA polymerase III mU6-RNA gene promoter was used in our study. mU6pro vector containing the mouse U6 snRNA promoter was kindly provided by Dr. Turner from University of Michigan in the USA. To generate the mU6-driven hairpin siRNA plasmids mU6-L374, mU6-L668 and mU6-GFPi, the mU6pro vector was digested with \textit{BbsI} and \textit{EcoRI} and the annealed oligos (L374, L668, and GFPi) were ligated into the vector. Plasmids were confirmed by DNA sequencing.