Effects of CDK4 antisense RNA on Human breast cancer cell proliferation and expression of cyclinD1, cyclinE and CDK2

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Abstract The role of CDK4 in human breast cancer cell proliferation and expression of cyclinD1, cyclinE and CDK2 has been investigated using inhibition of CDK4 expression by antisense RNA. When CDK4 expression was inhibited, the rate of cell proliferation and tumorigenecity decreased apparently. This indicates that CDK4 plays an important role in formation and development of breast tumor. The results of Northern blot analysis showed that the levels of cyclinD1 and CDK2 mRNAs changed slightly whereas the level of cyclinE mRNA decreased obviously. It is suggested that the expression of CDK4 is necessary for induction of cyclinE expression. Thus, inhibition of CDK4 expression affects not only the role of CDK4 itself but also the role of other genes.

Keywords: CDK4, breast cancer cell, antisense RNA, cyclinE.

The loss of normal regulatory control of cell cycle, leading to unrestrained cell proliferation, is the main property of cancer cells. The mutations of cyclins and CDKs play a key role in resulting in uncontrolled proliferation since cyclins and CDKs are involved in the regulation of the cell cycle progression[1, 2]. CyclinD1 was identified originally as a putative proto-oncogene[3], then the expressions of other cyclin genes in human breast cancer were reported[4]. To investigate the role of CDK4 in tumorigenesis and the effect on expression of cyclin and other CDK genes, we analyzed the proliferation, tumorigeneity and expression of cyclinD1, cyclinE and CDK2 of human breast cancer cell line Bcap-37 in which the expression of CDK4 was inhibited by antisense RNA.

1 Materials and methods

(i) Materials. Human breast cancer cell line Bcap-37 was cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in 5% CO₂.

(ii) Construction of antisense RNA vector and transfection to cells. CDK4 cDNA was digested with BamH I and Kpn I, and the resulting fragment was then gel purified. The plasmid pXJ41-neo as a vector was digested with the same enzymes, then the fragment was ligased in the plasmid. Transfection of cells was carried out with calcium phosphate system.

(iii) Growth curve of cell and flow cytometry analysis. Cells were diluted to 1×10⁴/mL and cultured in plates, then were harvested at intervals of 24 h. And the cell density was measured. The growth curve was drawn according to the cell number at each time. For flow cytometry analysis, cells were harvested by centrifugation at 500 g for 5 min and washed in ice-cold PBS, fixed in 75% ethanol, and stored overnight at 4°C prior to analysis. The cells were washed with ice-cold PBS twice, suspended in PBS containing 100 µg/mL RNase A, incubated at 37°C for 30 min, washed once with PBS and stained with 50 µg/mL propidium iodide in the dark for 30 min. Cells were filtrated through a 55 mm-nylon mesh. DNA content was measured on a flow cytometer system.

(iv) Soft-agar and tumorigenecity assay. For the bottom layer of agar, 1 mL of 0.5% agar was placed in a 35-mm plate. Then 2 mL of 0.35% top agar containing 1×10⁴ cells was layered on the top of the solidified layer of bottom agar. Conon formation was monitored by microscopy for up to 18 d. Cells were trypsinized, collected and washed with PBS. Then 6×10⁶ cells were injected subcutaneously per site into 6 weeks old nude mice. Tumor development was monitored twice every week.

(v) Northern blot analysis. RNA prepared from cells was separated by agarose (1%) gel electrophoresis under denaturing conditions. The integrity of the 18s and 28s bands of the extracted RNA indicated that RNA molecules had not degraded during the extraction process. RNA was transferred onto a nylon membrane by vacuum automated transfer system. The RNA blots were hybridized with 32P-labeled gene antisence RNA.

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2 Results

(i) Effect of inhibition of CDK4 expression on cell proliferation. Comparing the cells in which the expression of CDK4 was inhibited by antisense RNA with the control cells, the reduction in proliferation rate was apparent. The ratio of growth inhibition was about 51% (fig. 1). Changes in cell phase distribution of cells with CDK4 antisense RNA were observed by flow cytometry analysis. The proportion of cells in G1 phase rose from 40% to 55%, while the proportion of cells in S phase fell from 56% to 41% (figure 2).

(ii) Effect of inhibition of CDK4 expression on cell tumorigenicity. Cells with CDK4 antisense RNA showed a decreased cloning efficiency (69%) and smaller colonies in soft agar (fig. 3), compared to the control cells. When these cells were tested for tumor formation in nude mice, none of tumor formed after nude mice was injected for ten weeks, whereas the control cells formed obvious tumors in about two weeks.

(iii) Effect of inhibition of CDK4 expression on the expression of cyclinD1, cyclinE and CDK2. Total RNA of cells with antisense RNA and control cells was extracted, then analyzed by Northern blot. Hybridization was conducted with a 32P-labeled RNA probes of cyclinD1, cyclinE and CDK2, which were transcripted in vitro (fig. 4). The level of cyclinD1 mRNA did not show a significant change, the levels