High Expression of the RECK Gene in Breast Cancer Cells is Related to Low Invasive Capacity

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OBJECTIVE To investigate the expression of the RECK gene in human breast (cancer) cell lines, and to determine the relationship between RECK gene expression and the invasive capacity of the breast cancer cell lines.

METHODS The invasive capacity of breast (cancer) cell lines including HBL-100, MCF-7 and MDA-MB-435S were determined by the Transwell method. The protein expression levels of RECK, MMP-2 and MMP-9 genes in these three cell lines were measured by immunocytochemical methods. The expressions of the RECK gene and protein level were measured by RT-PCR and Western blots in the cell lines respectively.

RESULTS The order of the invasive capacity of the breast (cancer) cell lines was MDA-MB-435S, being the highest, and HBL-100, being the lowest. The invasive capacity difference between any two groups among the three groups was significant (P<0.01). The protein expression level of the RECK gene in the HBL-100 cell line was highest, and no expression was detected in MDA-MB-435S cells. Moreover, the expression of the RECK gene was negatively correlated with the expression of the MMP-2 and MMP-9 genes. The mRNA level of the RECK gene in HBL-100 cells was the highest, but no expression was found in the MDA-MB-435S cells (P<0.001).

CONCLUSION There was a significant negative correlation between the expression level of the RECK gene and invasive capacity in vitro, and the RECK gene expression showed an inverse proportion to that of the MMP-2, MMP-9 genes.

KEYWORDS: breast neoplasms, RECK gene, invasive capacity.

The degradation of the extra-cellular matrix plays a key role in the process of cancer growth, parenchymal invasion, metastasis and angiogenesis. Cancer cells degrade the extra-cellular matrix with the help of MMP (matrix metalloproteinase) to expedite their infiltration and metastasis. Research shows there is a relationship between breast cancer progression and the increase of MMP expression.[1,2] Inhibitors of MMP are regarded as promising agents to inhibit cancer growth and metastasis as a result of their inhibitory activity on angiogenesis.[3] One of the recently found inhibitors is RECK (reversion-inducing-cysteine-rich protein with Kazal motifs), thought to be a newly discovered cancer prognosis gene. In cases of pancreatic carcinoma, those with a high expression of RECK have a better prognosis than those with lower expression.[4] However in cases of breast cancer, this relationship between the RECK and MMP genes expression, relative to invasive capacity was not found. Therefore, we attempted to examine the correlation between the invasive capacity and the expressions of the RECK gene in three breast (cancer) cell lines with different inva-
MATERIALS AND METHODS

Cell lines and cell culture
HBL-100 cells are human breast non-tumor cells; MCF-7 cells are human breast adenocarcinoma cells and MDA-MB-435S cells are human breast ductal carcinoma cells. All cells were maintained in 25-cm² flasks in a humidified 5% CO₂/95% air incubator at 37°C in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Hyclone laboratories, Inc., Logan, UT). Cell counts were performed with the aid of a hemocytometer in the presence of 0.02% trypan blue to assess cell viability.

Cell invasion assay
Transwells (Corning Costar) with 12-mm polycarbonate filters, 12-µm pore size, were used. Twenty-five microliters of 100 µg/ml fibronectin (Sigma) was applied on the lower side of the filters, which were left for 1 h in a laminar hood to dry. The upper side of the filters was coated with 100 µl of 1 mg/ml Matrigel (Becton Dickinson) and 100 µl of RPMI 1640 medium was added. The Matrigel was dried overnight at room temperature in a laminar hood and reconstituted with 200 µl of medium for 1 h at 37°C. The upper compartments were filled with 200 µl of cell suspension, final concentration 1 x 10⁵/ml, and the lower compartments were filled with 800 µl medium. The plates were incubated for 24 h at 37°C in 5% CO₂. The cells on the upper side of the filters were scraped and the wells put upside down. The filters were fixed using methanol and stained with Giemsa. The number of cells invaded into the filters was counted. All tests were performed in triplicate.

Immunocytochemical procedures
The cells were seeded on glass coverslips and allowed to grow until half confluence. They were washed with PBS three times and then fixed by immersion in cold acetone for 10 min. After several washes in PBS, the cells were treated with 0.3% hydrogen peroxide for 10 min. After rinsing, the cells were blocked with non-immune animal serum, 50 µl for 15 min and subsequently incubated overnight at 4°C in a moist chamber with primary antibodies (anti-RECK, from BD Biosciences Inc., USA; anti-MMP-2 and anti-MMP-9, from Zhongshan Bio-technique Inc., Beijing, China) diluted in PBS. After three washes with PBS, the slides were incubated for 1 h at 37°C with secondary antibodies (rabbit anti-mouse IgG, Zhongshan Bio-technique Inc.), diluted and washed in PBS and incubated with the avidin-biotin-peroxide reagents (Zhongshan Bio-technique Inc.) for 30 min at 37°C. Then the slides were washed in PBS, and the antigen-antibody complex visualized using diaminobenzidine (Stable DAB, Zhongshan Bio-technique Inc.). Negative controls were treated identically with omission of the primary antibody. The immunocytochemical reactions were visualized using Olympus AX70 equipped with a 400× magnification. Cells were scored as positive for membrane or cytosol immunoperoxidase stain by two independent observers. The RECK, MMP-2 and MMP-9 protein expression levels in these three cell lines were calculated using an image analysis program, Metamorph 4.5 (Universal Imaging Corp., West Chester, PA) by taking two slides from each cell line, and measuring the OD values of the 5 same areas on each slide. The protein level was proportional to the OD value.

Western blot analysis
Initially, the cells were washed twice in ice-cold PBS. After this step, total cell extracts were prepared from cells which were lysed in 50 µl of RIPA buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid and 1 mM EDTA). Following 10 min of incubation on ice, insoluble material was removed by centrifugation at 14,000 rpm for 10 min. The supernatants were transferred to fresh test tubes and the total protein concentration was determined (Bradford assay Bio-Rad). The protein (50 µg) of whole cell lysates was resolved by 8% SDS-PAGE, and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) in a transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, and 15% methanol). These membranes were blocked in PBS containing 5% non-fat milk for 1 h. They were then washed in PBS containing 0.1% Tween 20 (Bio-Rad) three times. Mouse anti-RECK antibody (1:250) and goat anti-actin(1:500) were used as primary antibodies diluted in 0.1% PBST (phosphate buffered saline containing 0.1% Tween 20) overnight at 4°C. After that step, they were washed in 0.1% PBST several times. Rabbit anti-mouse and rabbit anti-goat IgG antibodies were used as secondary antibodies at a 1:5000 dilution for 30 min. Chemiluminescence (Amersham Biosciences) was then used to visualize immunoreactive protein complexes. The exposure time for the detection of RECK and actin was 1