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Original contribution

Frequent epigenetic inactivation of the receptor tyrosine kinase *EphA5* by promoter methylation in human breast cancer☆

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Summary *EphA5* is a member of the Eph receptor tyrosine kinase family, which plays a critical role in the regulation of carcinogenesis. Our previous DNA methylation microarray results suggested that the CpG islands in the *EphA5* promoter exhibited higher methylation levels in breast cancer tissues. In this study, we further analyzed *EphA5* gene expression profiles, methylation status, and clinical implications in breast cancer. We found that the level of *EphA5* mRNA was dramatically decreased in 5 different breast cancer cell lines. After treating the cell lines with 5-aza-2′-deoxycytidine (5-aza-dC, a demethylation agent), the levels of *EphA5* mRNA and protein were significantly increased. Bisulfite sequencing and methylation-specific polymerase chain reaction detection showed that decreased expression of *EphA5* was associated with its methylation status. We also found a significant correlation ($P = .017$) between the reduction of *EphA5* mRNA levels and aberrant methylation of *EphA5* in 31 paired tissue samples. In clinical samples, *EphA5* methylation was detected in 64.1% (75/117) of breast tumors and 28.2% (33/117) of paired normal tissues ($P < .001$), which was associated with higher tumor grade ($P = .024$), lymph node metastasis ($P = .004$), and progesterone receptor–negative status ($P = .008$). Our data indicate that *EphA5* might be a potential target for epigenetic silencing in primary breast cancer and a valuable molecular marker for breast cancer carcinogenesis and progression.

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1. Introduction

Aberrant methylation of CpG islands, which are located in the promoter region of tumor-related genes, has been firmly established as the most frequent mechanism for gene inactivation in cancers [1,2]. During the development and progression of breast cancer, a number of genes involved in DNA repair, cell cycle regulation, cell adhesion, as well as cell signal transduction could be silenced by aberrant methylation [3]. Clinically, identification of specific hypermethylated genes in breast cancer could improve our understanding of carcinogenesis and progression; furthermore, these genes may also serve as valuable biomarkers for early detection and prognosis prediction of breast cancer. Recently, in an effort to identify new genes that are functionally affected by altered methylation status in breast cancer, we performed a DNA methylation microarray-based analysis on 16 paired breast cancer and normal breast tissue samples. *EphA5* displayed the most significant change among the hypermethylated genes.

EphA5 is a member of the Eph receptors, the largest subgroup of the receptor tyrosine kinase family. Recent studies demonstrated that Eph and their ephrin ligands are involved in a variety of biological activities and developmental processes [4,5] and play an important role in oncogenesis and progression of many types of cancer [6] including colon cancer [7], lung cancer [8], prostate cancer [9], and breast cancer [10-12]. They were found to be valuable candidates in cancer diagnosis and prognosis prediction [7-10]. Furthermore, it was shown that targeting this receptor tyrosine kinase receptor and/or its ligand may lead to development of novel inhibition strategies and improve cancer treatment [13].

In breast cancer cell lines, a detailed study of Eph family gene expression profiles had indicated a subset that may be important in the progression of breast cancer from a noninvasive to an invasive phenotype [14]. A recent study revealed that *EphA2* activity appeared to promote mammary tumor progression and invasion [15], which could be used as an attractive target for antioncogenic therapy [13,16]. Meanwhile, several reports suggested that some *Eph* receptors might be modulated by methylation, such as *EphA3* in hematopoietic tumors [17]; *EphA7* in colorectal cancer, gastric cancer, and B-cell lymphomas [18-20]; and *EphB6* in breast cancer [21].

The function of the *EphA5* receptor is well characterized as an axon guidance molecule during neural development [22]. However, the potential role of *EphA5* in human carcinogenesis has never been addressed. Here, we provide evidence that *EphA5* is frequently downregulated in breast cancer cell lines and tumor tissues via aberrant hypermethylation of its promoter. We also found that *EphA5* methylation in breast cancer was significantly associated with some clinical characteristics. These findings suggested that *EphA5* might be a valuable biomarker for diagnosis and prognosis prediction in breast cancer.

2. Materials and methods

2.1. Cell lines and 5-aza-2′-deoxycytidine treatment

Seven breast cancer cell lines (T47D, MCF-7, Bacp37, ZR-75-30, SKBR-3, MDA-MB-231, and MDA-MB-435s) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The nontumor galactophore cell line HBL-100 was obtained from the Cell Bank of Chinese Academy of Sciences. Cell lines ZR-75-30, Bacp-37, MCF-7, T47D, and SKBR-3, and cell lines MDA-MB-231 and MDA-MB-435s were maintained in Dulbecco's Modified Eagle Medium and Leibovitz's L-15 medium, respectively. Both media were supplemented with 10% fetal bovine serum (GIBCO; Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 5% CO_2 humidified atmosphere.

Cells were seeded at a density of 5×10^4 cells per square centimeter in a 6-well plate. After 24-h incubation, fresh culture medium with or without the demethylating agent 5-aza-2′-deoxycytidine (Sigma-Aldrich Co Ltd) was added to a final concentration of 5 μmol/L and incubated for 24 and 48 hours.

2.2. Human breast tumor and paired normal tissue

Between April and June 2006, 117 primary breast carcinomas and paired normal tissue specimens, from which 31 pairs of RNA samples were available, were obtained from the Cancer Hospital of Fudan University, Shanghai, China. Informed consent was obtained from all patients before tissue acquisition. Immediately after resection, the specimens were flash frozen in liquid nitrogen and stored at −80°C until further treatment. The pathologic information, including histologic tumor type, tumor size, axillary nodal status, tumor grade (according to the Scharf-Bloom-Richardson grading system [23]), estrogen (ER) and progesterone receptor (PR) status, and HER2/neu expression status, was determined by a breast histopathological specialist in our hospital. ER and PR status were assayed by means of immunohistochemistry as previously described in detail [24], with the standard that tumors were considered as ER or PR positive if more than 10% of the cells were stained. HER-2/neu status was determined by immunohistochemistry using the Dako HercepTest and scored with the Dako scoring system [25]. Her-2/neu was defined as negative for scores of 0, +, and ++ in the Dako scoring system and as positive for strong membranous staining with a Dako score of +++. The mean age was 48 years (range, 26-81 years). Detailed clinicopathologic parameters are listed in Table 1.

2.3. Gene expression analysis by RT-PCR and real-time RT-PCR

Total RNA was isolated with RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and treated with DNase,