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Increased expression of *SRp40* affecting *CD44* splicing is associated with the clinical outcome of lymph node metastasis in human breast cancer

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Abstract

Background: During the malignant transformation of breast tissues, the pre-mRNA precursor splicing of specific genes can be flexibly regulated, leading to the formation of different forms or amounts of mRNA in response to the cellular microenvironment, and is frequently associated with cell tumorigenesis and may even cause tumor metastasis. Regulation of pre-mRNA splicing by serine-arginine (SR)-rich phosphoprotein is hypothesized to be associated with tumor cell metastasis.
Methods: We enrolled 55 breast cancer patients (32 with lymph node metastasis; LNM) with paired tissue samples consisting of cancerous and tumor-adjacent normal portions and assayed these tissues for gene expression of the *SR* family using quantitative real-time RT-PCR (qRT-PCR), then evaluated an association with LNM of breast cancer. Furthermore, we examined whether increased expression of a specific *SR* gene was associated with the presence of specific *CD44* spliced variants using qualitative reverse-transcription PCR (RT-PCR).
Results: Support for our hypothesis came from the observations that breast tumor tissues displayed higher level of *SRp40* expression as compared with the paired non-cancerous tissues, which manifested the significant association between increased *SRp40* expression and LNM (OR=4.48, 95% CI, 1.08–19.50, $P=0.018$). In addition, the primary tumors of breast with increased expression of *SRp40* gene were associated with the presence of the large *CD44* inclusion variants, *CD44v2*, *CD44v3*, *CD44v5*, and *CD44v6* ($P<0.05$).
Conclusion: Increased expression of *SRp40* can be detected in breast tumor tissues with a high degree of sensitivity, and that higher expression of *SRp40* closely correlates with alternative pre-mRNA splicing of *CD44*, which may serve as an earlier marker in predicting the risk to breast cancer patients of developing LNM.

Keywords: Breast cancer; Lymph node metastasis, *SR*; *CD44*; Pre-mRNA splicing

1. Introduction

Tumorigenesis is a multistep process resulting from a series of genomic alterations that lead to the progressive disordering of the normal mechanisms controlling growth, death, and differentiation of the cell [1]. To account for the genomic alterations required for tumor progression, the genomes of cancer cells are unstable and this genomic instability is caused by mutators (the *mutator phenotype* theory) [2]. Instead of *irreversible* genetic mutations in tumor formation, clonal evolution during tumorigenesis, in particular, tumor metastasis, is characterized by unstable, phenotypic heterogeneity, which fluctuates frequently to be mediated by the reversible changes and their upstream mutators [3,4]. Thus, the reactivation of certain cancer-associated genes that have been lost during tumor initiation becomes essential for metastatic tumor cells to survive. SR proteins are encoded by a gene family of splicing factor of an arginine/serine-rich c-terminus (SFRS) and can

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collaborate with exonic splicing enhancers for the recognition of specific exonic sequences in pre-mRNA splicing [5,6]. SR factors of the SR family are characterized by the presence of an RNA recognition motif and are required at early stages of spliceosome assembly [7]. These proteins have distinct but overlapping specificities for different pre-mRNAs and can alter the splice site choice of many gene products. Recently, it has been reported that individual SR proteins have distinct tissue distributions [8–10], and that splicing of the pre-mRNA precursor of a specific gene that was flexibly regulated by SRs results in the formation of different forms of mRNA in response to the cellular microenvironment change [11,12]. Therefore, it is speculated that the differential expression of SR protein may correlate to different alternatively spliced gene products during tumor cell progression [13–15].

In breast cancer, the suitability of numerous genes encoding mRNA splice variants as prognostic markers had been investigated, including those for the estrogen receptor, osteopontin, RHAMM, and CD44 [16,17]. CD44, a lymphocyte homing receptor, was originally found to mediate the interaction between lymphocytes and high endothelial venules [18]. The *CD44* gene consists of 20 exons, at least 10 of which can be alternatively spliced, functioning in response to tumor cell development activities such as adhesion, migration and growth regulation [19]. Proteins encoded by specific alternatively spliced variants of *CD44* have been commonly shown in human cancers, including gastric, pancreatic, prostate, colon, and breast tumor tissues [20–23], suggesting that CD44 spliced variants can serve as the critical cellular receptor for the adhesive ligands of tumor cell progression, including tumor lymph node metastasis (LNM). Studies *in vivo* also indicated that increased expression of the *SR* gene during mammary cell tumorigenesis, and the affected splicing phenotype of CD44 was shown to be associated with neoplasm formation of cancer development among various tissues [24,25]. In addition, there has been investigated whether decreased binding capacity to hyaluronan in *CD44* knockdown breast cancer cells can potentiate the adherence of metastatic breast cancer cells to bone marrow endothelial cells [20]. However, the clinical evidence for the alternative splicing variants of the *CD44* gene that are affected by the differential expression of splicing factors in tumor tissues of breast and its association with the phenotype of lymph node metastasis (LNM) of mammary tumors is undetermined.

Herein, the present study reports the frequency of increased expression of a subset of *SR* members, *SRp20* (*SFRS3*), *SRp30b* (*SFRS2*/*SC35*), *SRp40* (*SFRS5*), and *SRp55* (*SFRS6*), in breast tumor tissues. We examined whether expression of *SRs* may be involved in unique splicing patterns through alternative splicing of certain target genes that contribute to LNM of breast tumors, i.e. the correlations between the presence of altered variants of *CD44* and LNM which was modified by the increased expression of individual *SR* genes.

2. Materials and methods

2.1. Patients and tumor tissue

The present study is part of an ongoing cooperative study aimed at discovering markers for the prediction of breast cancer with LNM in Taiwan, in which breast cancer is characterized by a low incidence, early tumor onset [26], reproductive hormone dependency [27], and novel genomic alterations [26,28]. Patients enrolled with breast disease were a subset of women randomly selected from this ongoing hospital-based breast cancer cohort collected at the clinical surgery department. Their ages ranged from 30 to 83 y and none had a family history of breast cancer (mother or sisters). The tumors were diagnosed as incident, histologically confirmed, invasive ductal carcinoma of the breast. The clinical pathological characteristics of breast cancers categorized according to the TNM staging system are listed in Table 1. None of the patients were receiving neoadjuvant treatment at the time of primary surgery, thus avoiding up- or down-regulation of gene expression. Informed consent was obtained from each patient prior to tissue acquisition.

Table 1
Clinico-pathological characteristics of the 55 breast cancer patients

Characteristic*	Number	%
Tumor size		
<2 cm	10	18.18
2–5 cm	27	49.09
>5 cm	17	30.91
N.D.	1	1.82
Histologic grade		
I	15	27.27
II	24	43.64
III	14	25.45
N.D.	2	3.64
Lymph node metastasis		
Negative	23	41.82
Positive	32	58.18
ER		
Negative	16	32.65
Positive	33	67.35
PR		
Negative	9	18.37
Positive	40	81.63

*ER, estrogen receptor; PR, progesterone receptor. Minor difference of the sample size in individual comparison was due to missing data in the analysis. N.D., non-detected.

During surgical intervention, breast tissues taken separately from cancerous and surrounding non-cancerous parts of the resected specimens were immediately frozen in liquid nitrogen until analysis. Tissue samples collected from all breast cancers were approved histopathologically by experienced clinico-pathologic physicians. The LNM status of each patient was determined by the presence of tumor cells in lymph nodes using immunohistochemistry staining of at least 10 lymph nodes collected from each tumor lesion of the patient.

2.2. RNA extraction and reverse transcription of cDNA synthesis

Total RNA was extracted from the tumor and normal breast tissue of individual cases using an RNeasy RNA extraction kit (QIAGEN, Valencia, CA, USA). Total RNA (1 μg in a volume of 5 μl) was reverse-transcribed for 70 min at 42 °C using 5 units of Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA), and 10 mM oligo dT primers (Promega, Madison, WI) in a reaction volume of 20 μl, and the cDNA concentration was determined by spectrophotometry.

2.3. PCR amplification

The primers used for the qualitative amplification of the cDNAs for individual *SR* genes are shown in Table 2. In addition, the exons of interests in altered variants of the *CD44* gene, including *CD44v2*, *v3*, *v5* and *v6* were examined (Fig. 1), and the amplification primers to detect the presence of those spliced variant exons in PCR are listed in Table 2. All PCR experiments were performed under an initial step of 94 °C for 5 min, 32 thermal cycles consisting of 94 °C for 40 s and 72 °C for 40 s with a different annealing temperature for 40 s, and a final extension step of 72 °C for 10 min. Stepwise, 10 μl of the PCR product was electrophoresed on a 1.8% agarose gel and the expression of individual genes determined by the presence of a cDNA band of the appropriate molecular weight. In addition, each cDNA band was purified from the agarose