Differences in Stemness Properties Associated With the Heterogeneity of Luminal-Type Breast Cancer

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Abstract

Luminal-type breast cancers are heterogeneous because of the fact that approximately 30% show poor response to endocrine therapy. We investigated the stemness properties of mammospheres prepared from clinical samples by analyzing surface cancer stem cell (CSC) antigens, stemness-related genes, and estrogen response element (ERE) activity. Assessment of mammosphere stemness properties could be a useful and novel approach to the subclassification of luminal-type breast cancer.

Background: Luminal-type breast cancers are the most abundant subtype. Endocrine therapies targeting estrogen receptor (ER) or estradiol (E2) synthesis have achieved marked improvement in disease-free and overall survival of ER-positive cancers. However, approximately one-third of these cancers are poorly responsive to endocrine therapies, suggesting nonuniform tumor cell characteristics of this subtype. Recently, the tumorigenesis theory which states that CSCs are capable of self-renewal, tumorigenicity, and therapeutic resistance, became widely accepted. We investigated the relationship between the heterogeneity of luminal-type breast cancer and stemness properties. **Materials and Methods:** CSC surface markers and expression of stemness-related genes, including *Octamer-binding transcription factor 4 (OCT4)*, Nanog homeobox (*NANOG*), and Kruppel-like factor 4 (*KLF4*), were analyzed in clinical samples. ER activities were analyzed using the adenovirus vector carrying the ERE-green fluorescent protein (GFP). We separated the luminal-type breast cancers into 2 groups according to stemness-related gene expression patterns in mammospheres. **Results:** The group that predominantly expressed *NANOG* mRNA showed a high percentage of the cells that were positive for CD44 and negative for CD24 and Hoechst (possessing high-stemness properties), younger patient age, higher p53 expression, and tended to show higher histological grade and higher topoisomerase IIα expression. The ERE-GFP assay revealed that the luminal-type breast cancer mammospheres were heterogeneous. Mammospheres from several specimens lacked ER activity and responsiveness to E2 but some retained ER activities. **Conclusion:** ERE activity differences might be associated with endocrine therapy effectiveness. Mammosphere stemness properties could be a useful and novel criterion for subclassification of luminal-type breast cancers.

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Introduction

Intrinsic subtypes of breast cancer have been defined using genome-wide gene expression microarray analysis to classify breast cancers. Several distinct subclasses are used to classify breast cancer: luminal/estrogen receptor (ER)-positive (ER^+; luminal type A and luminal type B), basal-like, human epidermal growth factor receptor 2 (HER2)-positive, and normal breast-like subtypes.[1,2] This classification was based on differences in disease-free survival and overall

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survival among the intrinsic subtypes. Clinically, discrimination of subtypes among breast cancer is performed by immunohistochemically determining ER, HER2, and Antigen KI-67 (Ki-67) expression.[3] In addition to the routine markers, other markers have been studied regarding their possibility to be a predictive marker of prognosis. Topoisomerase (topo) IIα protein according to immunohistochemistry and *topo IIα* mRNA expression levels in microarray analysis were strongly associated with cell proliferation, ER negativity, and a worse prognosis.[4,5] It has been also reported that tumors with a *tumor suppressor protein p53* mutation were related to ER negativity, and high histological grade compared with those without a *tumor suppressor protein p53* mutation.[6,7] However, the boundary line separating subtypes of breast cancer is a matter of debate especially in luminal type breast cancer because only a few parameters have been used. In fact, although endocrine therapies that target ER or estradiol (E2) synthesis have led to marked improvement in disease-free survival and overall survival in luminal-type breast cancer, approximately 30% of luminal-type breast cancers show poor response to endocrine therapy.[8] This finding suggests that luminal-type breast cancers are heterogeneous.

Recently, the cancer stem cell (CSC) theory regarding tumorigenesis has become widely accepted. The theory is that only rare populations, termed CSCs, have high tumorigenicity, self-renewal ability, and can generate tumors. It is thought that CSCs have a pivotal role in recurrence and metastasis because of their characteristic of resistance to chemotherapy[9] or radiotherapy.[10] CSCs are enriched in sphere cultures and are identified by several markers, such as Hoechst 33342 exclusion (side population: SP)[11] and cell surface antigen or aldehyde dehydrogenase 1 activity.[12] CSCs have been detected in a wide variety of human solid tumors, such as of the breast,[13,14] brain,[15] prostate,[16,17] colon,[18] and pancreas.[19] In breast cancer, a subpopulation with the phenotype showing CD44-positive ($CD44^+$)/CD24-negative or low ($CD24^-$/low) cells[13] or high aldehyde dehydrogenase 1 activity[12] has been proposed as a CSC type. These cells have been shown to overexpress transcription factors, such as *octamer-binding transcription factor 4* (*OCT4*),[14,20,21] *Nanog homeobox* (*NANOG*),[21] and *Krueppel-like factor 4* (*KLF4*).[22] Stemness-related genes, such as *OCT4, NANOG*, and *KLF4*, are known to be key regulators that maintain the self-renewal potential and pluripotency of embryonic stem cells.[21,23] OCT4 and KLF4 are included in the Yamanaka factors that can induce pluripotent stem cells.[24] High expression of activation targets of NANOG, OCT4, sex determining region Y-box 2, and proto-oncogene c-Myc was observed in high-grade ER^- breast cancers with poor clinical outcome.[25] Moreover, OCT4 expression was observed in $CD44^+/CD24^-$ cells that have been reported to be CSCs of breast cancer.[14] The role of KLF4 in tumorigenesis is still controversial because tumorigenesis and tumor suppressor functionalities have been reported.[22,26,27] Nagata et al studied the expression of pluripotency-inducing factors in breast cancer specimens using immunohistochemistry and reported that KLF4 was a favorable prognostic indicator.[28] Akaogi et al reported that KLF4 inhibited ER target gene transcription in an estrogen-dependent manner and abrogated breast cancer cell growth.[29]

A high proportion of the $CD44^+/CD24^-$ phenotype or high expression of stem cell-like genes has been reported in basal-like breast cancer.[30] It was proposed that the stemness property is greater in basal-like breast cancer than in other subtypes. However, in luminal-like breast cancer, the $CD44^+/CD24^-$ phenotype in situ was not sufficient to evaluate stemness properties.

In the present study, we investigated the relationship between the heterogeneity of luminal-type breast cancer and stemness properties. Practically, it is very difficult to obtain sufficient amounts of cells from all clinical samples to perform fluorescence-activated cell sorting (FACS) analysis (side population analysis) or xenograft experiments (to examine tumorigenesis). Therefore, in this study, we assessed the stemness properties of individual breast cancers by preparing mammosphere cultures from surgical specimens and measured the expression of surface CD44 and CD24 antigens using the Hoechst 33342 dye exclusion method. mRNA from these mammospheres was prepared, and expression of stemness-related genes, including *OCT4*, *NANOG*, and *KLF4*, was measured. Moreover, immunohistochemical analysis of ER expression was performed, and the estrogen response element (ERE) activity of the mammospheres, activity which is thought to be an important factor in endocrine therapy, was examined by the adenovirus ERE-green fluorescent protein (GFP) assay.

Materials and Methods

Tumor Samples and Clinicopathological Factors

Primary breast cancer tissues were obtained from women who underwent surgery in the Department of Surgery at Tohoku Kosai Hospital (Miyagi, Japan) between 2009 and 2012. Informed consent was obtained from all patients before surgery. This study was approved by the Tohoku Kosai Hospital Ethics Committee and Tohoku University Ethics Committee (Tohoku Kosai Hospital, Tohoku University Ethics Committee 2009-306). ER, progesterone receptor, HER2, and Ki-67 status was obtained from the original pathological reports. Topoisomerase IIα (topo IIα) and p53 was reviewed by one of the authors (TM) without any knowledge of the clinicopathological data. Immunohistochemical assessment of topo IIα and p53 used antibodies for topo IIα, anti-topo IIα (Dako, Copenhagen, Denmark) diluted at 1:300, and for p53, anti-human p53 monoclonal antibody (DO7, Immunotech, Marseille, France), diluted at 1:200. Immunoreactivity for p53 and topo IIα was detected in the nuclei, and cases that had more than 10% of positive (with any staining intensity) carcinoma cells were considered positive for p53. Scoring of topo IIα in invasive carcinoma cells was counted and the percentage of immunoreactivity in 500 carcinoma cells at the hot spot in any staining intensity was considered as labeling index.

Isolation of Primary Breast Cancer Cells

Tumor tissues were shredded finely and washed with Hank's Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA) twice and then digested with collagenase solution (2.5 mg/mL collagenase, 40 mg/mL bovine serum albumin [BSA], 2 mg/mL glucose, 1 × antibiotic-antimycotic, and 50 μg/mL gentamicin in HBSS) for 30 minutes at 37°C. Then, the cell suspensions were sieved through a 40-μm strainer (Becton-Dickinson, Franklin Lakes, NJ) and centrifuged. The cells were washed with HBSS twice.

Mammosphere Culture

Primary breast tumor cells were suspended in mammary epithelial basal medium (MEBM) (Lonza, Basel, Switzerland)