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Organic anion transporting polypeptide 2B1 expression correlates with uptake of estrone-3-sulfate and cell proliferation in estrogen receptor-positive breast cancer cells☆

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ABSTRACT

Estrone-3-sulfate (E1S) is thought to be a major estrogen precursor in estrogen receptor (ER)-positive breast cancer. Since E1S is a hydrophilic compound, the uptake of E1S into cancer cells is probably mediated by transporters, such as organic anion-transporting polypeptide (OATP, *SLCO*) family. In this study, we investigated the relationship between expression of OATP2B1 and cell proliferation in ER-positive breast cancer. Cell-based assays were carried out in MCF-7 cells both with and without over-expression of OATP2B1. Normal breast and tumor tissues were collected and used in this study. Cell proliferation, ER-mediated transcriptional activities and estradiol secretion were stimulated by addition of E1S to the culture medium of MCF-7 cells. These stimulatory effects were significantly greater in MCF-7 cells overexpressing OATP2B1 than in control cells. The expression level of SLCO2B1 mRNA was significantly correlated with histological grade, Ki-67 labelling index and mRNA expression of steroid sulfatase. The expression level of SLCO2B1 mRNA in luminal B-like cancers was higher than that in luminal A-like cancers. Uptake of E1S resulted in down-regulation of ERα protein and induction of Ki-67 in MCF-7 cells. The present study suggests that OATP2B1 is involved in cell proliferation by increasing the amount of estrogen in ER-positive breast cancer cells.

1. Introduction

About two thirds of invasive breast cancers express an estrogen receptor (ER), and estrogens play an important role in the proliferation of ER-positive breast cancer cells [1]. Two major pathways, aromatase and sulfatase pathways, are involved in the production of biologically active estrogens (mainly estradiol, E2) in breast cancer cells, and sulfatase activity has been reported to be higher than aromatase activity [2]. Recent studies have revealed that steroid sulfatase (STS), which converts steroid sulfates into the unconjugated forms in the sulfatase pathway, may have a key role in the growth of cancer cells. Thus, inhibition of STS activity has been proposed as a strategy to treat ER-positive breast cancer [3,4]. In the sulfatase pathway, estrone-3-sulfate (E1S) is a substrate of STS and precursor/reservoir of estrogens. The half-life of E1S in plasma is much longer than that of its unconjugated form, and the concentration of E1S in malignant breast tissues is greater than that in non-malignant tissues [2]. Taken together, E1S may play an

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important role in the proliferation of ER-positive breast cancer cells. Since E1S is a highly hydrophilic compound, it likely requires a solute carrier to cross plasma membranes. Current evidence suggests several classes of transporter specialized for entry of E1S into ER-positive breast cancer cells.

Human organic anion-transporting polypeptides (OATPs, *SLCO/SLC21*) belong to the family of solute carrier transporters. OATPs have been characterized in detail and shown to be transporters responsible for the uptake of organic anions. So far, 11 human OATP family members have been identified, and several members of the OATP family have been shown to be capable of transporting steroid sulfates, including E1S [5,6]. OATP2B1 recognizes various types of organic anions as substrates and has unique substrate specificity with high affinity to E1S. OATP2B1 is highly expressed in the liver, small intestine, placenta, and platelets and has been reported to be ubiquitously distributed in many tissues [7]. Importantly, the abundant expression of OATP2B1 has been detected in the human mammary gland, and it has been reported that OATP2B1 might be the most important transporter accounting for delivery of E1S in both normal breast and malignant tissues [8]. However, information on the association between expression level of OATP2B1 and proliferation of breast cancer cells is limited.

ER-positive breast cancers are classified into at least two subtypes, luminal A-like and luminal B-like cancers, by gene expression profiling [9]. Luminal B-like cancers show worse outcomes than those of luminal A-like cancers [10]. Although luminal B-like cancers are defined as a subtype of ER-positive breast cancers, they show high expression of proliferation markers and low or lack expression of estrogen-regulated genes compared with those in luminal A-like cancers. Moreover, luminal B-like cancers are thought to rely on another pathway of growth in addition to the estrogen pathway [11,12]. Therefore, chemotherapy is required in addition to endocrine therapy in luminal B-like cancers. Conversely, luminal A-like cancers respond well to endocrine therapy. Thus endocrine therapy alone should be applied to luminal A-like subtype from the viewpoint that chemotherapy causes more serious adverse events. Consequently, it is of great importance to clarify luminal subtypes to predict a benefit of an endocrine treatment and to identify patients who will receive potential benefit from a particular chemotherapy agent in a clinical setting.

In the present study, the relationship between the expression level of OATP2B1 and proliferation of ER-positive breast cancer cells was examined by using the MCF-7 cell line, which is widely utilized as a model of ER-positive breast cancer cells. We overexpressed OATP2B1 instead of knockdown to clarify potential role of the transporter on the cell proliferation, because the expression level of SLCO2B1 mRNA in MCF-7 cells was far lower than that in breast cancer cells. This is the first study to overexpress OATP2B1 in a breast cancer cell line and to directly elucidate the effects of overexpression of the transporter for an estrogen precursor on proliferation of ER-positive breast cancer cells.

2. Methods

2.1. Cell culture

The human breast cancer cell line MCF-7 was obtained from RIKEN Bioresouce Center (Tsukuba, Japan) and routinely cultured as described previously (estrogens-contained medium) [13]. In the experiments using E2 and E1S, phenol red-free DMEM (Wako, Osaka, Japan) containing charcoal dextran-treated FBS (Funakoshi, Tokyo, Japan) (estrogens-free medium) was used. To establish MCF-7 cells overexpressing OATP2B1, MCF-7 cells were transfected with OATP2B1 cDNA subcloned into pcDNA3 (Life Technologies Japan, Tokyo, Japan) by using TransFast Transfection Reagent (Promega, Tokyo, Japan) according to the manufacturer's instructions. The cells were cultured in a medium containing 1.0 μg/μL G418 (Wako). HEK293 cells overexpressing OATP2B1, which was established in a previous study [14], was used as a positive control. In this paper, MCF-7 cells transfected with a control vector and those overexpressing OATP2B1 are designated as MCF-pcDNA3 and MCF-OATP2B1, respectively.

2.2. Tissue samples

Malignant breast tumor tissues were donated from 49 female patients with primary invasive breast carcinoma at Chiba University Hospital from 2011 to 2012. Non-malignant breast tissues distant from the carcinoma region were obtained from 16 female patients who underwent total mastectomy. These tissue samples were immediately soaked in RNAlater® RNA Stabilization Solution (Life Technologies Japan) and stored at −20 °C until use. None of patients who participated in this study received irradiation or chemotherapy before undergoing the surgical operation. Data on clinicopathological variables were collected as described previously [15]. This study was approved by the Research Ethics Committee of the Graduate School of Medicine, Chiba University (Approval No.100) and all patients provided written informed consent.

2.3. RNA preparation, cDNA synthesis and real-time RT-PCR

Total RNA was extracted from breast tissues and cell lines by using TRIzol® (Invitrogen, Carlsbad, CA). Quantitative RT-PCR or detection of mRNA expression of each gene was carried out as described previously [13]. The specific primers used for amplification are listed in Table 1.

2.4. Protein preparation and deglycosylation

Total protein from MCF-7 cells was collected as described previously [13]. The ProteoExtract Transmembrane Protein Extraction Kit (Novagen, Darmstadt, Germany) was used to extract plasma membrane fractions from cells according to the manufacturer's instructions. Enzymatic deglycosylation of plasma membrane fractions was performed with Protein Deglycosylation Mix (New England Biolabs, Ipswich, MA) according to the manufacturer's recommendations. The deglycosylation was carried out to confirm whether OATP2B1 overexpressed in MCF-7 cells was identical with that overexpressed in HEK293 cells.

2.5. Immunoblot analysis

SDS-PAGE and Western blot analysis were performed as described previously [13]. Polyclonal rabbit anti-OATP2B1 antibody [16], monoclonal mouse anti-alpha 1 Sodium Potassium ATPase antibody (Abcam, Cambridge, MA), polyclonal rabbit anti-ERα antibody (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-Ki-67 antibody (abcam) and polyclonal rabbit anti-β-actin (Santa Cruz Biotechnology) were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Wako) and goat anti-mouse IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

2.6. Immunocytochemistry

Cells were plated on a cover glass. On the day after plating, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min. After washing with ice-cold PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated with 3% H_2O_2 for 10 min to block endogenous peroxidase