**DOWN-REGULATION OF ABCC11 PROTEIN (MRP8) IN HUMAN BREAST CANCER**

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**Aim:** To investigate the expression of ABCC11 (MRP8) protein in normal breast tissue, and examine the difference in ABCC11 mRNA and protein expression between normal breast and breast cancer tissues taking into account ABCC11 genotype (a functional SNP, rs17822931) and estrogen receptor (ER) status. **Methods:** Sections of paraffin-embedded normal and malignant tissues from 10 patients with invasive ductal carcinoma were used for immunohistochemical analysis. DNA and RNA were extracted from the same sections and used for genotyping and ABCC11 transcript expression measurement by quantitative RT-PCR. **Results:** A strong expression of ABCC11 was found in epithelial and myoepithelial cells of normal breast lobules and ducts in individuals with different ABCC11 genotypes. A predominant decrease of ABCC11 expression was observed in malignant tissue compared to normal breast specimen (8 of 10 cases), despite four out of ten tumors showed the elevated ABCC11 mRNA level as compared to the normal counterpart. Neither ABCC11 mRNA nor protein expression in normal or cancerous tissue correlated with ER status. **Conclusion:** The expression of ABCC11 protein appears to be decreased in most BC. The effect of ABCC11 protein on breast cancer chemosensitivity is likely to be more complex than that which can be directly inferred from ABCC11 genotype and mRNA expression level in the tumor. **Key Words:** ABCC11 mRNA expression, MRP8 expression, normal breast, breast cancer.
mens were stained with hematoxylin and eosin and reanalyzed by an experienced pathologist to confirm that each BC specimen contained cancerous tissue, and each normal breast sample was free of malignant tissue.

**DNA extraction and genotyping.** DNA was extracted from paraffin-embedded sections with DEXPAT reagent (TaKaRa Bio Inc., Otsu, Japan) according to the manufacturer’s protocol. DNA was further precipitated with ethanol, reconstituted in TE buffer and 2 μl was used as a template in genotyping reactions. The samples were genotyped by TaqMan™ assay using the reagents, primers and probes (Applied Biosystems by Life Technologies, Foster City, CA, USA) and thermal cycling conditions described in our recent work [15]. The assays were run in a Rotor-Gene Q (QIAGEN, Tokyo, Japan). Four replicates of each sample were analyzed. Genotypes were assessed by automated allelic discrimination analysis and by comparison with external controls with known genotypes.

**Quantitative real-time (qRT)-PCR.** RNA was extracted from FFPE sections mounted on microscope slides with RNasy FFPE kit (QIAGEN, Tokyo, Japan) according to the manufacturer’s protocol with additional 3 min incubations at 50 °C after adding of 1 ml of xylene, and before the first centrifugation step. cDNA was then synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Three independent reverse transcription reactions were done for each sample, and the content of each of the three tubes was used as an individual template in triplicate qRT-PCR. Commercially available TaqMan® Gene Expression Assays (Applied Biosystems by Life Technologies, Foster City, CA, USA) were used to analyze the target (ABCC11 and ESR1) and reference cDNAs (MRLP19, TBP, TFRC). The respective assay IDs are listed in Table.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Assay ID</th>
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<tbody>
<tr>
<td>ABCC11</td>
<td>HS01090769_m1</td>
</tr>
<tr>
<td>ESR1</td>
<td>HS00174560_m1</td>
</tr>
<tr>
<td>MRLP19</td>
<td>HS000860822_m1</td>
</tr>
<tr>
<td>TBP</td>
<td>HS00497261_m1</td>
</tr>
<tr>
<td>TFRC</td>
<td>HS00951083_m1</td>
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Note: Assays were purchased from Applied Biosystems by Life Technologies (Foster City, CA, USA).

**MRLP19, TBP and TFRC** were selected as reference genes for normalization according to Drury et al. [16], who found these to be particularly suitable for gene expression analysis in FFPE material. To meet another important condition for qRT-PCR of FFPE samples [17], expression assays for all genes were selected to amplify the target as close to the 3’ end as possible. To comply with the MIQE Guidelines [18], each set of primers was tested for efficacy using serial dilutions of a control cDNA sample. Reaction was performed in TaqMan® Universal PCR Master Mix (Applied Biosystems by Life Technologies, Foster City, CA, USA) under the following thermal profile: after the initial incubation at 50 °C for 2 min followed by 95 °C for 10 min, reaction was cycled 55 times at 95 °C for 15 sec and at 60 °C for 1 min in a Rotor-Gene Q machine. Geometrical mean of the relative concentrations of ABCC11 and ESR1 against each of MRLP19, TBP, and TFRC was used as the expression index in further analysis.

**Antibodies.** The ERα was detected in human breast tissues with a mouse monoclonal antibody NCL-ER-6F11 (Novocastra Laboratories, Newcastle, UK) diluted 1:80. ABCC11 was detected with rabbit polyclonal antibody provided by Dr. K.Yoshiura at the dilution of 1:100. For the immunofluorescent detection of the proteins, we used secondary anti-mouse –Alexa Fluor 594 and anti-rabbit –Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) conjugates at 1:200 dilution. All antibodies were diluted in 1% BSA (Sigma, St Louis, MO, USA) in 0.01M PBS.

**Immunohistochemical double labeling for ER and ABCC11.** Sections of paraffin-embedded tissues were mounted on aminosilane-coated slides, deparaffinized, and rehydrated. The sections were sequentially incubated in four changes of boiling 0.01 M citrate buffer, pH 6.0, 5 min each, 2% hydrogen peroxide at room temperature for 15 min, three changes of PBS, 5 min each, and in 5% BSA blocking solution at room temperature for 20 min. Then the slides were washed in PBS for 10 min and incubated overnight at 4 °C in the mixture of primary anti-ER and anti-ABCC11 antibodies diluted as described above. After incubation the sections were washed in three changes of PBS, 10 min each, followed by 30 min incubation at room temperature with the mixture of the secondary antibodies. The slides were then rinsed in four changes of PBS, covered with Vectashield H-1200/DAPI mounting media (Vector Laboratories, Burlingame, CA, USA) and analyzed under a Biorevo BZ-9000 (Keyence Corp., Woodcliff Lake, NJ, USA) fluorescent microscope. The three-color images were acquired, merged and processed to remove haze and adjust the background using the built-in software. Green fluorescence intensity (ABCC11) was measured in the images and normalized to blue fluorescence intensity (nuclei) using Image-Pro software (v.4.5, Media Cybernetics, Bethesda, MD, USA).

**RESULTS**

**Localization of ABCC11 in normal breast tissue.** The localization of the ABCC11 protein product in normal breast lobules and terminal ducts was determined by immunochistochemistry. The high level of ABCC11 expression was seen in all 10 specimens (Fig. 1, 1N-10N). As shown in Fig. 1-3N, the ABCC11 protein was detected within the layer of both luminal epithelial (Fig.1-3N, solid arrow) and basal myoepithelial cells (Fig. 1-3N, hollow arrow). Of note, normal mammary cells appear to express ABCC11 regardless of the rs178829931 genotype or ER status.

**Expression of ABCC11 mRNA.** We compared ABCC11 transcript levels in normal breast tissues and in tumors. As shown in Fig. 2, the increased ABCC11 expression in cancerous tissue was seen in 4 of 10 patients (Patients 1, 6, 7, and 10). In six patients, the decreased expression as compared to normal breast was observed. The changes in ABCC11 expression
were irrelevant to the tumor ER status (the obtained ER staining results perfectly corresponded to those in patients’ medical records in all cases) or 538G > A (rs178829931) polymorphism. Moreover, no correlation was found between ABCC11 and ESR1 expression in tumors (Pearson’s correlation coefficient, $r = 0.175$) or in normal breast tissue ($r = -0.182$).

**Expression of ABCC11 in BC.** IHC analysis revealed an evident decrease in ABCC11 expression in tumor tissues in 8 patients as compared to the normal counterpart (Fig. 1, 1C–5C, 7C, 9C, and 10C). The quantification of green fluorescence intensity revealed 1.8- to 6.7-fold decrease of the signal (Fig. 3). ABCC11 levels in the remaining two BC samples were comparable to those in normal tissue (Fig. 1, 6C, 8C), 1.17- to 1.39-fold signal fading (see Fig. 3). Thus, none of examined tumor samples showed ABCC11 over-expression as compared to normal breast. Interestingly, in three BC samples a very low protein expression was detected despite the high mRNA levels (Fig. 1, 1C, 9C, and 10C).

**DISCUSSION**

In the present study we found a predominant decrease of the ABCC11 product, ABCC11 protein, expression in BC as compared to the normal breast tissue of the same patient, and such decrease did not correlate with ABCC11 mRNA level. Only two of ten BC specimens displayed ABCC11 expression similar to that in the normal tissue.

The function of ABCC-subfamily transporters and their role in tumor resistance are intensively investigated. ABCC11 mRNA expression data are also available from rather numerous BC analyses. Several studies reported ABCC11 mRNA over-expression in BC tissue and BC cell lines [8–9, 19–20]. Park et al. [19] observed the increased expression of ABCC11 in BC patients with residual disease compared to those who achieved a complete response, although the authors did not include ABCC11 in their optimal molecular prognosticator of BC response to neoadjuvant chemotherapy. Honorat et al. [10] pointed at the possibility of estrogen involvement in the regulation of ABCC11 expression. On the other hand, estrogen-responsive genes have been implicated in acquired resistance to tamoxifen or aromatase inhibitors [21]. Taken together, the existing knowledge on ABCC11 expression in BC indicates that this protein may play a role in the regulation of chemotherapy response.

Most studies of tumor resistance are performed using cell cultures. However, the origin of cells used to establish cell lines does not represent all tumor types and the conditions of cell culturing appear to limit translational application of the results obtained in cell lines. Our results show that in a real tissue, ABCC11 mRNA level poorly correlates with protein expression. This finding emphasizes the importance of parallel examination of ABCC11 mRNA and protein product in normal and malignant breast tissue. As we demonstrated, surgical samples stored as FFPE tissues could be successfully used for such an analysis.

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**Fig. 1.** Expression of ABCC11 and ERα in normal mammary (N, left column) and breast cancer (C, right column) tissues of 10 patients. The ABCC11 protein (green) and the ESR1 protein (red) were detected on 5 μm FFPE sections and merged as described in Materials and Methods. The DAPI counterstaining of the nuclei appears in blue. The genotype at rs17822931 of each patient is indicated on the right of each normal-cancer image pair.
Relative ABCC1 level  

ER -  

ER +  

Fig. 2. Expression of ABCC11 and ESR1 transcripts in normal mammary tissue and breast cancer tissue of 10 patients. Expression was measured by qRT-PCR, and the geometrical mean of ABCC11 and ESR1 relative concentrations against three reference genes was used as an estimate of gene expression level. Black diamonds represent the ratio of ABCC11 mRNA expression level in BC to that in normal tissue. Circles represent the changes in ESR1 mRNA expression.

Fig. 3. Down-regulation of ABCC11 protein in BC tissues. The relative intensity of green signal was determined in normal and malignant tissue images as described in Materials and Methods. The decrease of ABCC11 fluorescence intensity from normal (N) to cancer (C) tissue was observed in each patient irrespectively of ER status or genotype.

To better understand the role of ABCC11 in BC, the knowledge of protein localization in normal mammary gland is essential. Our experiments employing immunohistochemical staining demonstrated that ABCC11 is expressed in epithelial and myoepithelial cells of breast lobules and ducts. The presence of ABCC11 in epithelial cells of normal terminal duct lobular unit (TDLU), the structural and functional unit of the breast, implies the involvement of this transporter in secretion function of the mammary gland, and is consistent with the finding that the volume of colostrum secretion depends on ABCC11 genotype at rs17822931 [12]. Of interest is the observation that ABCC11 is expressed also in myoepithelial cells which do not express ERα [22]. Our examination of ABCC11 localization may suggest that the protein participates not only in apocrine secretion, but also in metabolite transport into the stroma embedding ducts and lobules.

Transport activity of ABCC11 is strongly affected by a SNP at nucleotide 538 (538G > A, rs17822931) of ABCC11 [14]. This SNP determines human earwax [11]. This SNP determines human earwax [11]. Although ABCC11 expression in normal breast or BC is independent of rs17822931, functional studies of the ABCC11 SNP are potentially useful. This could be illustrated by the study of the ABCC11 role in lung cancer cell resistance to MTA, in which ABCC11 expression level did not correlate with IC_{50} for MTA; yet ABCC11 genotype affected chemosensitivity [6].

In conclusion, the expression of ABCC11 protein which localizes in epithelial and myoepithelial cells of normal breast lobules and ducts is likely to be decreased in the majority of BC or it may be comparable to that in normal tissue in some cases. It remains to be elucidated whether ABCC11 loss or retention in BC is functionally relevant to tumor development or may affect clinical course and prognosis. Therefore, further studies of ABCC11 expression in BC are warranted to determine its usefulness for decision making on BC therapy protocol.

ACKNOWLEDGEMENTS

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