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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 beast 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.**

Human ATP-binding cassette (ABC) transport proteins have an essential function of extruding toxins from cells [1]. Namely this function brings ABC transporters into the focus of the studies of multidrug resistance of tumor cells. Starting with the *ABCB1* gene product, MDR1, several other transporters have been shown to cause anti-cancer drug resistance in cell culture through an increased efflux and decreased intracellular accumulation of chemotherapeutic agent [2]. Most ABC transporters associated with tumor resistance belong to the ABCC subfamily.

The *ABCC11* gene product (also known as MRP8) is one of nine multidrug resistances (MDR)-associated proteins of the ABCC subfamily. ABCC11 substrates include cyclic nucleotides, monoanionic bile acids, steroid sulfates, estradiol 17-β-D-glucuronide [3–4]. ABCC11 has been proved to confer resistance to chemotherapeutic drugs 5-fluorouracil (5-FU) [5] and pemetrexed (MTA, Alimta) [6].

Profiling of MDR proteins expression in cancer cells is an important direction in exploring of drug resistance mechanisms and discovering biomarkers of a particular tumor type. Breast cancer (BC), as the most common type of non-skin cancer in women and the fifth most common cause of cancer death, involves an intense research effort in this regard. Apart from MDR1 [7], no evidence has been reported yet on the relationship of ABC transporters with drug resistance of BC cells. At the same time, MDR genes transcripts, including *ABCC11* mRNA, have been shown to be overexpressed in BC [8–9]. Elevated expression levels of *ABCC11* in estrogen receptor (ER)α-positive, as compared to ERα-negative BC, were reported by Honorat *et al.* [10]. The authors also observed the regulation of the *ABCC11* expression by estrogen in MCF7 breast cancer cell line [10]. However, no studies addressing differential *ABCC11* expression in normal and cancerous breast tissues have been done so far. Similarly, nothing currently is known about the ABCC11 protein expression in normal breast tissue in comparison to the tumor.

This work was set out to examine the *ABCC11* transcript and ABCC11 protein expression in BC and matched normal breast specimens in 10 women in relation with ER status. We also analyzed *ABCC11* expression levels with regard to a functional SNP (rs17822931) in the *ABCC11* gene that apparently affects the transport activity of the protein [11–14].

MATERIALS AND METHODS

Samples. The study protocol was approved by the Committee for the Ethical Issues of Human Genome and Gene Analysis of Health Sciences University of Hokkaido. A total of 10 BC and normal mammary gland specimens which were located away from the tumor of the same patient were selected from pathological archives of the Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki University, Japan. Clinicopathological information on BC samples including ER status (positive/negative, as a part of routine pathological diagnosis of BC) was obtained from patients' records. Serial 5 μm sections of normal tissue and BC surgical specimen mounted on microscope slides were available for the study. Sections of all speci-

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Abbreviations used: BC – breast cancer; ER – estrogen receptor; FFPE – formalin-fixed paraffin-embedded; MPR – multidrug resistance protein; SNP – single nucleotide polymorphism.

mens were stained with hematoxylin and eosin and re-analyzed 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 RNeasy FFPE kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol with additional 3 min incubations at 50 ºC 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. Gene Expression Assays used as primers for quantitative RT-PCR

Gene symbol	Assay ID
ABCC11	Hs01090769_m1
ESR1	Hs00174860_m1
MRLP19	Hs00608522_m1
TBP	Hs00427621_m1
TFRC	Hs00951083_m1

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 ºC for 2 min followed by 95 ºC for 10 min, reaction was cycled 55 times at 95 ºC for 15 sec and at 60 ºC 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 aminoalkylsilane-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, hollow arrow) and basal myoepithelial cells (Fig. 1-3N, solid 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.

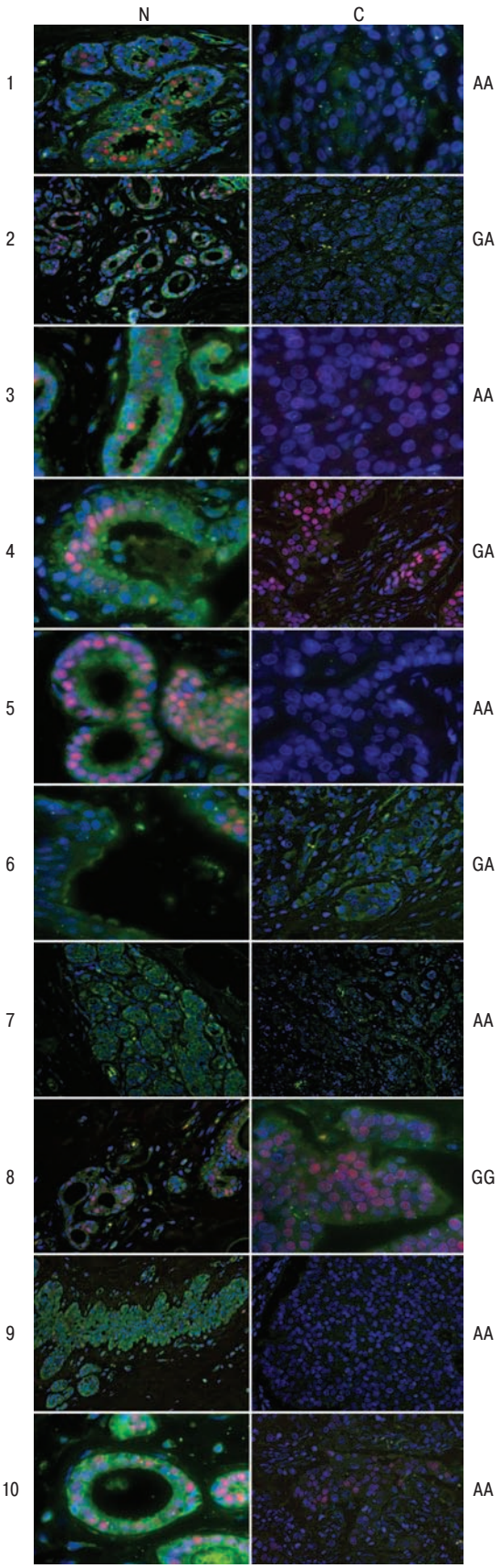

Fig. 1. Expression of ABCC11 and ERα in normal mammary (N, left column) and breast cancer (C, right column) tissues of 10 patients. The ABCC11 protein (green) and the ESR1 protein (red) were detected on 5 μm FFPE sections and merged as described in Materials and Methods. The DAPI counterstaining of the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

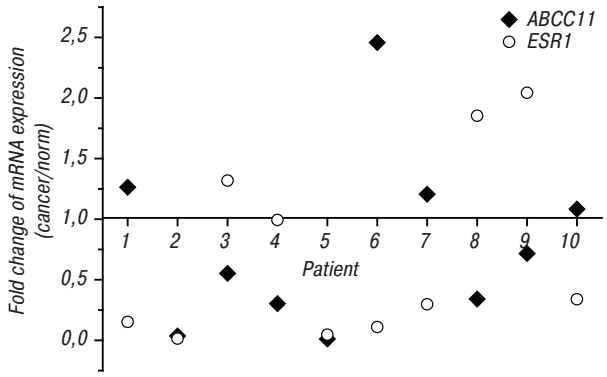

Fig. 2. Expression of *ABCC11* and *ESR1* transcripts in normal mammary tissue and breast cancer tissue of 10 patients. Expression was measured by qRT-PCR, and the geometrical mean of *ABCC11* and *ESR1* relative concentrations against three reference genes was used as an estimate of gene expression 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

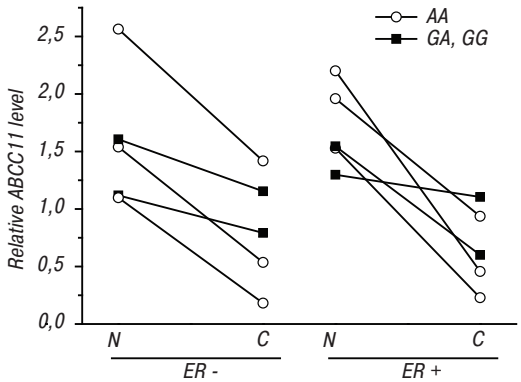

Fig. 3. Down-regulation of ABCC11 protein in BC tissues. The relative intensity of green signal was determined in normal and malignant tissue images as described in Materials and Methods. The decrease of ABCC11 fluorescence intensity from normal (N) to cancer (C) tissue was observed in each patient irrespectively 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 type, and associates with some functions of apocrine glands. Individuals with the AA genotype are characterized by the reduced cerumenous secretion [14] and a nearly complete loss of axillary odor [11] as compared to those homozygous or heterozygous for wild-type G allele. However, as the results reported here reveal, the *ABCC11* polymorphism does not seem to influence the localization of the ABCC11 protein in the mammary gland. The ABCC11 expression pattern was similar in the mammary glands of different *ABCC11* genotype carriers, suggesting that non-functional ABCC11 is not degraded but incorporated into the cellular membrane. Similar observations were previously made in the sweat glands [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.

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