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Evidence for Association of the rs17822931-A Allele in *ABCC11* with a Decreased Risk of Estrogen Receptor-negative Breast Cancer

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The rs17822931 SNP of the human *ABCC11* gene determines earwax types, and is also associated with some functions of apocrine glands, including the mammary gland. Nevertheless, whether the *ABCC11* polymorphism is correlated with estrogen receptor (ER)α status of breast cancer (BC) remains unclear. To investigate the correlation between rs17822931 and BC, we screened the genotypes in a total of 276 and 295 histological BC samples collected from Japanese and Ukrainian BC patients, and 269 and 264 ethnically-matched healthy individuals, respectively, using TaqMan™ PCR. Genotype frequencies at rs178229131 in Japanese and Ukrainian BC patients were not significantly different from those in their respective control populations. Consistently, no correlation between rs178229131 and the susceptibility to BC was found. The AA genotype, which corresponds to dry earwax, occurred less frequently in ERα-negative BC in Japanese [odds ratio, 0.48; 95% confidential interval, 0.29-0.80] but not in Ukrainian patients although a similar correlation was weakly observed. Our results indicate that the rs178229131-A allele may be important in reducing the risk of ERα-negative BC development.

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Introduction

The *ABCC11* gene product (MRP8) is a member of multidrug resistance protein (MRP) family of ATP-binding cassette transporters. MRP8 functions as a transmembrane efflux pump and is able to transport a large range of diverse substrates, including cyclic nucleotides, monoanionic bile acids, and steroid sulfates.[1-2] The *ABCC11* transcript is expressed in various tissues[3-7] and, of particular interest, in apocrine glands in the external auditory canal, axillary region, and the breast. It is also highly expressed in breast cancer cells.[5]

We have recently revealed the physiological function of MRP8,[8] showing that a single nucleotide polymorphism (SNP) at nucleotide 538 (c.538G/A, rs17822931) of *ABCC11* determines the human earwax type in a way that individuals with GG and GA genotypes have wet earwax, and AA homozygotes have dry earwax. A variant form of MRP8 protein (MRP8-180R, c.538A results in Arg180 instead of Gly180) has deficient transport activity compared to the wild type (MRP8-180G, c.538G results in Gly180).[8] Indeed, functions of two other apocrine glands, i.e., axillary and mammary glands, are related to the genotype at rs17822931[6,9-10] as well. Axillary osmidrosis, a condition with excessive

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axillary odor, has been demonstrated to strongly associate with wet earwax[9], and similarly, decreased volume of colostrum 24 hrs after delivery was more frequently seen in parturient women with dry-type earwax[10].

Despite evident connection between the type of earwax and apocrine gland functioning, the role of rs17822931 in susceptibility to breast cancer (BC) remains controversial.[11-13] Two most recent genotype analyses also showed discrepant results.[14,15] Ota et al.[14] claimed that the wild-type allele, rs17822931-G, is associated with BC in the Japanese, irrespective of the estrogen receptor (ER)α status and other clinicopathological features. However, Beesley et al.[15] found no evidence for association of rs17822931 with BC in Caucasian women. Therefore, additional genotyping studies are essential to shed light on an existing disagreement. Whether the *ABCC11* polymorphism correlates with the ER status of breast cancer cells, an important factor in cancer prognosis and therapy, also remains unclear.

Here, we analyzed genotypes at rs17822931 in patients with ERα-positive (ERα+) and ERα-negative (ERα-) BC, and compared genotype frequencies between cancer patients and control females in the Japanese and Ukrainian populations.

Patients and Methods

Samples

The study protocol was approved by Committee for Ethical Issues on Human Genome and Gene Analysis of Health Sciences University of Hokkaido. Of a total of 480 breast cancer (BC) surgical specimens used for analysis, 185 BC cases from Nagasaki prefecture were provided by the Department of Pathology, Atomic Bomb Disease Institute, Nagasaki University, Japan, 121 Ukrainian cases by Regional Diagnostic Center, Dnepropetrovsk, Ukraine, and 174 by the Department of Genetic Diagnostics, State Institute of Genetic and Regenerative Medicine, Kiev, Ukraine. Information on ERα status of BC samples (positive/negative) was obtained from these institutions. ERα was evaluated by H-score immunohistochemistry. The samples were stored as paraffin blocks in pathological archives of the institutions. Several 5-μm sections of each sample mounted on microscope slides were provided for the current study. Albeit not sampled in this study, genotype data for another series of 91 BC patients from Nagasaki prefecture with known ERα status were provided by Department of Human Genetics of Nagasaki University Graduate School of Biomedical Science.

As control samples from the Ukrainian population, fingernail clippings were collected from 177 Ukrainian volunteers after obtaining informed consent. Also, 45 surgical specimens from patients with diseases other than BC were provided by Regional Diagnostic Center, Dnepropetrovsk, Ukraine. As controls from the Japanese population (Nagasaki prefecture), 100 surgical specimens from patients with diseases other than BC were provided by the Department of Pathology, Atomic Bomb Disease Institute, Nagasaki University, Japan. In addition, our previous genotyping data for 42 Ukrainian and 169 Japanese controls from the same district/prefecture were available.[8,16]

Cumulatively, the study included 276 Japanese and 295 Ukrainian BC patients aged 55.8±11.4 years (28-88 years old, range) and 52.3±11.1 years (21-87 years old, range) at diagnosis, respectively, and 269 Japanese and 264 Ukrainian controls aged 58.2±12.3 (17-90 years old, range) and 55.2±9.6 years (19-82 years old, range) at sampling, respectively.

DNA extraction and genotyping

DNA was extracted from paraffin-embedded BC samples using DEXPAT reagent (TaKaRa Bio Inc., Otsu, Japan) according to the supplier's protocol. DNA was further precipitated with EtOH, and purified DNA (2 μl) was used as a template in genotyping reactions.

After washing with PBS/0.5% SDS solution and with 70% EtOH, the nail clippings from control individuals were frozen in liquid nitrogen and crushed into fine powder with Multi-beads Shocker (Yasui Kikai, Osaka, Japan). The nail powder was treated with ISOHAIR (Nippon Gene, Tokyo, Japan) at 55°C overnight. DNA was extracted with phenol/chloroform, precipitated with EtOH, incubated in 200 μl 1xTE/0.5%SDS containing 3 μl of 10mg/ml proteinase K at 55°C overnight and then again subjected to phenol/chloroform extraction and EtOH-precipitation.

BC and control samples were genotyped by TaqMan™ PCR using hydrolyzing probes and a set of amplifying primers, according to our recent work.[16] Reaction was performed in the TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), with the following thermal cycling conditions: after initial incubation at 50°C for 2 min, followed by incubation at 95°C for 10 min, PCR was cycled 50 times at 92°C for 15 sec, and at 60°C for 1 min. PCR was run on Rotor-Gene Q (QIAGEN, Tokyo, Japan). Four replicas of each sample were processed in one reaction. Genotypes were assessed by automated allelic discrimination analysis, and by comparison with external controls with known genotypes.

Statistical analysis

Association of the rs17822931 with breast cancer risk was estimated using multivariate logistic regression analysis for the co-dominant, log-additive, dominant and recessive models, and odds ratios (OR) and 95% confidence intervals (CI) were calculated. Similar analysis was done to assess correlation between the polymorphism and the ERα status of BC. A conventional level of significance of $P<0.05$ was chosen. Statistical analyses were performed using an on-line software package.[17]

The study had a power of >83% at less than 0.05 level of type I error to detect on OR of 1.75 in Japanese cases, taking into account the wet phenotype frequency of 23% among Japanese.[16] In Ukrainian cases, the study had a power of >80% to detect per-allele OR of 1.81 with the A allele frequency of 0.163, however less power to detect recessive effects.

Results

The distribution of genotypes at rs17822931 among cases and controls, and in patients grouped according to ER status is shown in Table 1. The A allele frequencies correspond to values of 0.125 and 0.890 represented in the NCBI SNP database for European and Japanese groups, respectively (build 132, September 2010).

Table 1. Genotypes at rs17822931 and the A allele frequencies among study groups. P<0.05 in bold

Population, genotype	BC[a] cases n (%)	Controls n (%)	ER[b] positive BC n (%)	ER negative BC n (%)
Japan	n=276	n=269	n=167	n=83
GG	6(2.2)	6(2.2)	1(0.6)	3(3.6)
GA	85(30.8)	66(24.5)	47(28.1)	33(39.8)
AA	185(67)	197(73.2)	119(71.3)	47(56.6)
*P**	0.262		0.32	**0.016**
A, %	0.824	0.855		
Ukraine	n=295	n=264	n=193	n=102
GG	208(70.5)	181(68.6)	136(70.5)	72(70.6)
GA	82(27.8)	80(30.3)	53(27.5)	29(28.4)
AA	5(1.7)	3(1.1)	4(2.1)	1(1)
P	0.711		0.605	0.929
A, %	0.156	0.163		

[a] BC, breast cancer [b] ER, estrogen receptor [*] Chi-square test

We compared genotype (GG, GA, and AA), and earwax type (wet or dry) frequencies in BC patients with respective control values. As shown in Table 2, there was no evidence that would reject the null hypothesis of rs17822931 not being correlated with BC risk.

Table 2. Results of the logistic regression analyses of rs17822931 association with breast cancer risk in Japanese (Nagasaki prefecture) and Ukrainian populations according to different inheritance models

Population	Genotype	OR*(95% CI)	*P*
Japan, Nagasaki prefecture	GG	1.00[a]	0.262
	GA	1.29(0.40-4.18)	
	AA	0.94(0.30-2.96)	
	Risk per A allele[b]	0.79(0.57-1.10)	0.161
	GA + AA vs GG[c]	1.03(0.33-3.22)	0.964
	AA vs GG + GA[d]	0.74(0.51-1.07)	0.114
Ukraine	GG	1.00	
	GA	0.89(0.62-1.29)	0.711
	AA	1.45(0.34-6.15)	
	Risk per A allele	0.95(0.68-1.32)	0.738
	GA + AA vs GG	0.91(0.64-1.31)	0.617
	AA vs GG + GA	1.50(0.36-6.34)	0.579

[a] Co-dominant model of inheritance. [b] Log-additive model of inheritance. [c] Dominant model of inheritance. [d] Recessive model of inheritance. [*] Adjusted for age

BC samples with known ERα status (295 Ukrainian and 250 Japanese BC cases) were included into the analysis of association between c.538G/A, rs17822931 and two BC subtypes (i.e., ERα+ and ERα-). The results are summarized in Table 3.

Table 3. Association of genotypes at rs17822931 with breast cancer (BC) subtypes (i.e., estrogen receptor positive (ER+) and ER negative (ER-)) in 250 Japanese and 295 Ukrainian cases. *P*<0.05 in bold

Population, genotype	ER+ BC versus controls OR* (95% CI)	*P*	ER- BC versus controls OR (95% CI)	*P*
Japan				
GG	1.00a	0.284	1.00	**0.019**
GA	4.27(0.50-36.67)		1.00(0.24-4.25)	
AA	3.62(0.43-30.48)		0.48(0.12-1.98)	
Risk per A allele[b]	0.99(0.66-1.46)	0.942	0.54(0.35-0.85)	**0.008**
GA + AA vs GG[c]	3.79(0.45-31.74)	0.159	0.61(0.15-2.49)	0.485
AA vs GG + GA[d]	0.91(0.59-1.39)	0.653	0.48(0.29-0.80)	**0.005**
Ukraine				
GG	1.00	0.605	1.00	0.929
GA	0.88(0.58-1.33)		0.91(0.55-1.51)	
AA	1.77(0.39-8.06)		0.84(0.09-8.19)	
Risk per A allele	0.96(0.66-1.40)	0.838	0.91(0.57-1.46)	0.687
GA + AA vs GG	0.91(0.61-1.37)	0.662	0.91(0.55-1.50)	0.707
AA vs GG + GA	1.84(0.41-8.32)	0.421	0.86(0.09-8.38)	0.898

[a] Co-dominant model of inheritance. [b] Log-additive model of inheritance. [c] Dominant model of inheritance. [d] Recessive model of inheritance. [*] Adjusted for age

The dry-type earwax (AA genotype) was less frequently seen in ERα- BC in Japanese population (OR=0.48; 95% CI, 0.29-0.80, *P*=0.005). Hence, rs17822931-A may be associated with a decreased risk of the ERα- BC development (OR=0.54; 95% CI, 0.35-0.85, *P*=0.008). In Ukrainians, a similar correlation was observed, however it was not statistically significant given a very low frequency of "AA" homozygotes in the population.

Discussion

The present study was conducted to test whether the *ABCC11* polymorphism correlated with a susceptibility to BC in humans. The proposed hypothesis that earwax type may associate with BC risk is based on the common origin of ceruminous and mammary glands.[7,13] The *ABCC11* gene product, MRP8 (multidrug resistance protein), together with other ABC transporters, showed an increased expression in BC patients who responded poorly to neoadjuvant chemotherapy in one study.[18] Such a resistance to anticancer treatments is likely attributed to MRP8 ability to transport 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), an active metabolite of the chemotherapy agent, 5-fluorouracil (5-FU).[19] Moreover, because MRP8 can transport glucuronidated and sulfated steroids,[1] the protein may play a role in regulatory cell responses to sex steroids in breast tissues, particularly to estrogens. Together, these MRP8 characteristics support the notion that the *ABCC11* SNP genotypes may be involved in mammary gland carcinogenesis.

We found that *ABCC11* genotype rs17822931 and earwax phenotype frequencies in BC patients did not significantly differ from those in the controls in allele-A rich Japanese, or allele-G rich Ukrainian populations (Table 2). The observed lack of association between rs17822931 and BC in Ukrainian women is consistent with the observation of Beesley et al.[15] who found no association in Caucasian patients. By contrast, in a recent report, a positive correlation of the allele-G with BC risk was detected in 270 samples collected in Yokohama district, neighboring the Tokyo metropolitan area, when compared with 273 control individuals.[14] Taking into consideration the stratification of Japanese map with regard to rs17822931-A frequency,[16] the control individuals should be chosen with particular caution, as demonstrated in this study. To evaluate the association between *ABCC11* polymorphism and BC risk nationwide, an increased cohort of patients proportionally from all prefectures and a comparison against all-Japan control will be required. The results in our study indicate that earwax type-determining SNP, rs17822931, in *ABCC11* does not display a strong association with BC.

We also analyzed the association between *ABCC11* genotype and two subtypes (ERα+ and ERα-) of human BC. The results revealed a correlation of earwax type with the BC subtype; patients with ERα- tumors were less likely to be "AA" homozygotes (Table 3). Moreover, risk per A-allele with an OR of 0.54 (95% CI, 0.35-0.85, *P*=0.008) suggests that the presence of mutant variant at rs17822931 may be important for reducing the risk of development of ERα- BC.

Our findings are somewhat contrast with a recent study in which GA/GG genotypes (wet earwax type) were associated with BC susceptibility in a Japanese population.[14] Nevertheless, our results are supported by the widely accepted associations between ethnicity and BC characteristics. Of note, it has been shown that BC mortality rates are positively correlated with wet earwax type,[13,20] suggesting that the higher rs17822931-A frequency in population, the longer BC-specific survival is. Concordantly, women of Asian ethnicity, and particularly Japanese Americans, the population that carries rs17822931-A with a high frequency, are more likely to develop ERα+/progesterone receptor positive (PR+) subtype of BC.[21] The lower mortality rates among women of Asian ancestry is thus consistent with the idea that patients with ERα+/PR+ tumors have the lowest mortality risk, compared to women with other BC subtypes.[22]

In the present study, we found an association of rs17822931-A with decreased risk of ERα- BC even analyzing the minimally sufficient number of subjects. We believe that expanding sample size of the Japanese BC patients will provide even stronger evidence for the association, and our results may be expediently used for the future meta-analysis of pooled data. At the same time, observation of a statistically significant correlation in Ukrainians or other European populations is less feasible due to the low frequency of the AA genotype among Caucasians.

In conclusion, our genotype analysis demonstrates that the SNP determining earwax type is not a factor affecting susceptibility to BC. However, AA genotype at rs17822931 occurs less frequently in ERα- tumors, particularly in patients of Asian ancestry, leading to a notion that it may possibly play a protective role in carcinogenesis of this subtype of BC.

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