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***RASSF1A* Polymorphism A133S Is Associated with Early Onset Breast Cancer in *BRCA1/2* Mutation Carriers**

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Abstract

The tumor suppressor gene *RASSF1A* regulates cell cycle progression, apoptosis, and microtubule stability and is inactivated by promoter methylation in ~50% of breast cancers. It has been shown previously that the polymorphism A133S in *RASSF1A* reduces its ability to regulate cell cycle progression and this polymorphism is associated with an increased risk of breast cancer. We analyzed the frequency of *RASSF1A* A133S in 190 Caucasian women without breast cancer and 653 patients with breast cancer including 138 *BRCA1* and *BRCA2* (*BRCA1/2*) mutation carriers, 395 non-*BRCA1/2* mutations carriers, and 120 untested for *BRCA1/2* mutations. Patients with breast cancer had a higher frequency of A133S than the controls [$P=0.017$; odds ratios (OR), 1.71; 95% confidence intervals (95% CI), 1.10–2.66]. There is also a higher frequency of A133S in patients with higher familial breast cancer risk ($P=0.029$; OR, 1.76; 95% CI, 1.06–2.92) and patients carrying *BRCA1/2* mutations ($P=0.037$, OR, 1.82; 95% CI, 1.04–3.18). Importantly, we found that the co-occurrence of a *BRCA1* or *BRCA2* mutation and A133S in *RASSF1A* was associated with earlier

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onset of breast cancer compared with those individuals with either a *BRCA1/2* mutation or the A133S polymorphism alone (36.0 versus 42.0 years old, $P = 0.002$). Our data suggest that the presence of the *RASSF1A* A133S polymorphism is associated with breast cancer patho-genesis in general and modifies breast cancer age of onset in *BRCA1/2* mutations carriers. Our results warrant a large-scale study to examine the effect of the A133S polymorphism in the development of breast and other types of cancers.

Introduction

Five percent to 10% of all breast and ovarian cancers are due to a genetic predisposition (1). The most common form of familial breast and ovarian cancers are associated with germ line mutations in *BRCA1* or *BRCA2* (2,3). Inherited mutations in other genes including *CHEK2*, *PTEN*, *TP53*, *ATM*, *NBS1*, *RAD50*, *BRIP1*, and *PALB2*, although rare, have been associated with an increased risk of developing breast cancer (4,5). Together, mutations in these genes account for ~25% of familial cases of breast cancer, leaving the majority of cases without an explanation. In addition, there is substantial variability in the incidence of breast cancer among mutation carriers, and the age of onset also varies widely (6). These observations suggest that unknown genetic factors contribute to the development of familial breast cancer.

We and others have shown that *RASSF1A* is a tumor suppressor gene located on chromosome 3p21.3, an area with frequent loss of heterozygosity (LOH) in lung and breast cancers (7). *RASSF1A* is expressed in all nonmalignant epithelial cells, but not in more than half of breast cancers due to hypermethylation in the promoter region (8,9). Additionally, we have shown that *RASSF1A* plays an important role in cell cycle control by preventing cyclin D1 protein accumulation (10), whereas others have shown that *RASSF1A* plays important roles in mitosis, apoptosis, and microtubule stabilization (11–14).

Mutations in *RASSF1A* are rare. However, several polymorphisms have been identified. One polymorphism, Ala133Ser (A133S), is located close to a putative *ATM* substrate site (S131) in the microtubule-binding domain. We have shown that ectopic expression of wild-type *RASSF1A* inhibits the accumulation of cyclin D1 during G₁-S phase progression and this effect was impaired in *RASSF1A* A133S cells (10). Thus, the A133S variant seems to be defective in cell cycle control.

Recently, Schagdarsurengin et al. showed a higher frequency of the A133S polymorphism in 141 Caucasian females with breast cancer compared with 70 individuals without breast cancer (15). However, the *BRCA1/2* mutation status and the family history of breast cancer in these patients were not reported. In this study, our goal was to determine the contribution of *RASSF1A* A133S in breast cancer development in a larger patient population, with a particular focus on the subgroup of breast cancer patients with *BRCA1/2* mutations.

Materials and Methods

Study population

Breast cancer patients and normal controls were all non-Ashkenazi Caucasian females. Informed consent was obtained from the controls and patients according to the University of Texas Southwestern Medical Center Institutional Review Board and national standards. Controls were women with no personal or family history of breast cancer. The majority of the controls were recruited in a population-based study (Genetic Epidemiology of Metabolic Syndrome, University of Texas Southwestern Medical Center). The rest of the controls were volunteers registered at the University of Texas Southwestern Medical Center. A self-administered questionnaire was used to collect information on individual's personal and family

history of breast cancer. All of the control individuals fitting the criteria of no personal and family history of breast cancer were included in the study.

Breast cancer patients, including those seeking genetic counseling for breast cancer, were recruited from three institutes: 591 breast cancer patients were recruited sequentially from 1994 to 2006 at the Clinical Cancer Genetics Clinic at the University of Texas, Southwestern Medical Center. All patients from the University of Texas, Southwestern Medical Center ($n = 591$) signed consent forms allowing their DNA to be used for genetic research. Four hundred and seventy-one patients had their DNA analyzed for *BRCA1/2* mutations and the probability for a *BRCA1/2* mutation was estimated using the BRCAPRO model. The remaining 120 patients declined DNA analysis for *BRCA1/2* mutations for various reasons including the financial cost or not wanting to know the result. Additional DNA from breast cancer patients with *BRCA1/2* mutations were collected from the Dana-Farber Cancer Institute ($n = 35$) and from the University of Chicago School of Medicine ($n = 27$). Specimens collected at the Dana-Farber Cancer Institute were sequential women seen in the Dana-Farber Cancer Risk and Prevention and Breast Oncology Clinic, with germ line mutations in known breast cancer susceptibility genes (*BRCA1/2*, *PTEN*, *TP53*, *LBK1*, etc), and women at increased risk of breast cancer for other reasons (therapeutic thoracic radiation exposure, lobular carcinoma *in situ*, and other lesions). Specimens collected at the University of Chicago School of Medicine were from individuals who presented to the Cancer Risk Clinic at the University of Chicago between 1992 and 2007.

DNA extraction

Genomic DNA was extracted from blood by phenol/chloroform extraction and Proteinase K method or by using QIAamp DNA Mini Kit (QIAGEN).

Mutation detection

BRCA1/2 mutations were mostly identified by sequencing performed by Myriad Genetics. The entire coding region was sequenced in most of the patients except in a few cases in which mutation in a single site was determined based on a known *BRCA1/2* mutation in other family members.

Genotyping

Fluorogenic 5'-nucleotidase assay in the TaqMan system (Applied Biosystems) was used to detect A133S polymorphism. Forward and reverse primers for amplifying *RASSF1A* are 5' GCCTGTGGAGTGGGAGACA and 5'GGGCATTGTACTCCTTGATCTTCT separately. Fam-labeled MGB probe 5'CCTTTCTCAAtCTGAG and Vic-labeled MGB probe 5'CCTTTCTCAAgCTGAG was used for detecting A133S and wild-type alleles separately. The assay was performed on a 7900HT Fast Real-Time PCR instrument. The reaction conditions were: 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, and 60°C for 90 s.

Statistical analysis

Analysis was performed using SAS 9.1.3 Service Pack 3. A two-sample t test was used for comparing age differences and a χ^2 test was used for comparing allele frequencies among groups. In the cases in which the χ^2 test could not be performed, Fisher's exact test was used. Kaplan-Meier survival curves were constructed and a log-rank test was used to compare age at onset of breast cancer between A133S patients and wild-type *RASSF1A* patients in the *BRCA1/2*-positive and *BRCA1/2*-negative groups, respectively. $P \leq 0.05$ was considered statistically significant.

Results

Frequency of *RASSF1A* A133S in patients with breast cancer

We designed a high-throughput fluorogenic 5'-nucleotidase assay to determine the frequency of the A133S polymorphism in the *RASSF1A* gene (Supplemental data Fig. S1). Six hundred and fifty-three breast cancer probands and 190 controls were analyzed for the presence of A133S (Table 1). As shown in Table 2, 149 (22.8%) of the 653 breast cancer patients were homozygous or heterozygous for A133S. In contrast, 28 (14.7%) of 190 controls were homozygous or heterozygous for A133S [$P = 0.017$; odds ratios (OR), 1.71; 95% confidence intervals (95% CI), 1.10–2.66]. Thus, there is a significant difference between the frequencies of A133S in breast cancer patients that we studied compared with the controls.

In order to define the breast cancer risk associated with A133S, we divided the breast cancer patients into three groups: breast cancer patients with ($n = 138$) or without *BRCA1/2* mutation ($n = 395$), and patients not tested for *BRCA1/2* mutation ($n = 120$). As shown in Table 2, there is a higher frequency of A133S in *BRCA1/2* mutation carriers compared with the normal controls (23.9% versus 14.7%, $P = 0.037$; OR, 1.82; 95% CI, 1.04–3.18).

To determine whether the A133S polymorphism is associated with familial breast cancer independent of *BRCA1/2* status, we further divided the non-*BRCA1/2* mutation carriers into two groups [higher familial breast cancer risk group ($n = 223$) and lower familial breast cancer risk group ($n = 172$)] using BRCAPRO, a computer model designed to calculate the predisposition of an autosomal dominant familial breast cancer phenotype (16,17). As shown in Table 2, A133S is more frequent in patients in the higher familial breast cancer risk group (23.3%, $P = 0.029$; OR, 1.76; 95% CI, 1.06–2.92) compared with the controls. These results suggest that the presence of A133S is associated with familial breast cancer in general, and is independent of *BRCA1/2* mutation status.

The presence of *RASSF1A* A133S is associated with a younger age at diagnosis in *BRCA1/2* mutation carriers

BRCA1/2 mutation carriers develop breast cancer at an earlier age than average breast cancer patients without *BRCA1/2* mutations. In our data, the median age of onset of breast cancer for all breast cancer cases was 43.0 years, which was 41.0 for *BRCA1/2* mutation carriers. It is noted that the mean age at onset of breast cancer in our study group is significantly younger than the average for all breast cancer patients because of a study population bias toward patients with a family history of breast cancer. However, when we compared the age at onset among the *BRCA1/2* mutation carriers with or without A133S, we found that A133S carriers were diagnosed 6 years earlier than those with wild-type *RASSF1A* (36.0 versus 42.0 years old, $P = 0.002$; Table 3; Fig. 1). In contrast, when we compared the age at onset among non-*BRCA1/2* mutation carriers with or without A133S, we found that there was no significant age difference (42.0 versus 43.0 years old, $P = 0.701$; Table 3; Fig. 1). Thus, the presence of an A133S polymorphism along with a *BRCA1/2* mutation in the same individual is associated with earlier onset of breast cancer.

Discussion

In this study, we examined whether there was an association between the frequency of the *RASSF1A* A133S polymorphism and the incidence of sporadic and familial breast cancers. We show that there is higher frequency of *RASSF1A* A133S in breast cancer patients compared with the controls, which is consistent with prior work (15). In addition, we show that there is a higher frequency of *RASSF1A* A133S in familial breast cancers and in breast cancer patients carrying *BRCA1/2* mutations. Our most significant finding is that patients with both A133S

and *BRCA1/2* mutations were diagnosed with breast cancers 6 years earlier than patients with the *BRCA1/2* mutation only.

LOH and promoter hypermethylation are the two main mechanisms for *RASSF1A* inactivation in breast cancer. The results of this study suggest that the presence of a polymorphic allele of *RASSF1A* is another mechanism involving *RASSF1A* in breast cancer development. A simple hypothesis is that women carrying the A133S allele already have one inherited inactivation event (haploinsufficiency) for *RASSF1A*. This hypothesis is consistent with the previously published data showing loss of *RASSF1A* function with the A133S polymorphism reported by ourselves and others (10,18). The phenotype of *Rassf1a* knockout mice also supports this hypothesis in which both *Rassf1a*^{-/-} and *Rassf1a*^{+/-} mice showed increased tumor multiplicity and tumor size relative to control animals in response to carcinogen treatment (19). Thus, comprehensive studies including LOH, promoter methylation, and now, A133S polymorphism are needed to further understand how loss of *RASSF1A* contributes to cancer development.

Reports on *BRCA1/2* modifier genes are scarce and controversial (6). To our knowledge, there are no genes that have been confirmed to modify *BRCA1/2* mutation risk with respect to early onset of breast cancer. We report here that there is an increase of *RASSF1A* A133S in patients with *BRCA1/2* mutations. Moreover, the average age at breast cancer diagnosis for *BRCA1/2* mutation carriers with A133S is 6 years earlier than in patients with wild-type *RASSF1A*. Our study supports the hypothesis that *RASSF1A* modifies the risk of breast cancer in *BRCA1/2* mutation carriers.

In conclusion, our results warrant a large-scale study to validate the effect of A133S in the development of breast and other types of cancers. If confirmed, *RASSF1A* A133S could be developed as one of the molecular biomarkers for refining cancer risk estimates for women at higher risk for breast cancer. For individuals with a *BRCA1/2* mutation, the confirmation of the association of A133S with earlier onset of breast cancer may indicate that breast cancer surveillance should start at an earlier age if they carry the A133S allele in *RASSF1A*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Kaplan-Meier plots comparing age at diagnosis for breast cancer patients with A133S or wild-type *RASSF1A*. *A*, breast cancer patients with *BRCA1/2* mutations. *B*, breast cancer patients with wild-type *BRCA1/2* gene. *Red line*, fraction of patients with A133S not diagnosed with breast cancer at the ages indicated on the X-axis. *Black line*, fraction of patients with wild-type *RASSF1A* not diagnosed with breast cancer at the ages indicated on the X-axis. *P* values are based on log-rank test.

Table 1

Clinical variables for controls and patients with breast cancer

Cases and controls	No. (%)	Median age (range)	Primary breast cancer only (%)	Bilateral and multiple breast cancer (%)	Breast cancer and other cancers (%)
Control	190 (100)	46.0 (19–72)			
All cases	653 (100)	43.0 (22–83)	515 (78.9)	75 (11.5)	63 (9.7)

Table 2
High prevalence of A133S in patients with breast cancer compared with normal controls

	G/G (%)	G/T (%)	T/T (%)	G/T and T/T (%)	P*	OR (95% CI)
Control (n = 190)	162 (85.3)	27 (14.2)	1 (0.5)	28 (14.7)		
All cases (n = 653)	504 (77.2)	138 (21.1)	11 (1.7)	149 (22.8)	0.017	1.71 (1.10–2.66)
BRCA1/2 mutation tested positive (n = 138)	105 (76.1)	30 (21.7)	3 (2.2)	33 (23.9)	0.037	1.82 (1.04–3.18)
BRCA1/2 mutation tested negative (n = 395)	310 (78.5)	79 (20.0)	6 (1.5)	85 (21.5)	0.053	1.59 (0.99–2.53)
BRCA1/1 tested negative, BRCA1/2 ≤ 5 (n = 172) [†]	139 (80.8)	31 (18.0)	2 (1.2)	33 (19.2)	0.260	1.37 (0.79–2.39)
BRCA1/2 tested negative, BRCA1/2 > 5 (n = 223) [‡]	171 (76.7)	48 (21.5)	4 (1.8)	52 (23.3)	0.029	1.76 (1.06–2.92)
BRCA1/2 mutation not tested (n = 120)	89 (74.2)	29 (24.2)	2 (1.7)	31 (25.8)	0.016	2.02 (1.14–3.57)

NOTE: G/G, homozygous for wild-type codon 133; G/T, heterozygote for codon 133; T/T, homozygous for codon A133S. Values in boldface indicate $P < 0.05$.

* Cases compared with the controls.

[†] Patients with lower risk of familial breast cancer based on BRCA1/2 calculations.

[‡] Patients with higher risk of familial breast cancer based on BRCA1/2 calculations.

Table 3

Category	Median age (range)	A133S		Wild-type		Age difference*	<i>P</i> value, <i>t</i> test
		No.	Median age (range)	No.	Median age (range)		
Control (<i>n</i> = 190)	46.0 (19–72)	28	46.0 (28–62)	162	46.0 (19–72)	0	0.577
All cases (<i>n</i> = 653)	43.0 (22–83)	149	42.0 (23–76)	504	43.0 (22–83)	−1.0	0.064
BRCA1/2 mutation tested positive (<i>n</i> = 138)	41.0 (23–66)	33	36.0 (23–52)	105	42.0 (25–66)	−6.0	0.002
BRCA1/2 mutation tested negative <i>n</i> = (395)	43.0 (22–78)	85	42.0 (24–75)	310	43.0 (22–78)	−1.0	0.701
BRCA1/1 tested negative, BRCAPRO ≤ 5 (<i>n</i> = 172)	43.0 (26–72)	33	43.0 (26–60)	139	43.0 (28–72)	0.0	0.511
BRCA1/2 tested negative, BRCAPRO > 5 (<i>n</i> = 223)	42.0 (22–78)	52	42.0 (24–75)	171	42.0 (22–78)	0.0	0.928
BRCA1/2 mutation not tested (<i>n</i> = 120)	43.5 (24–83)	31	42.0 (26–76)	89	46.0 (24–83)	−4.0	0.600

NOTE: Letters in boldface indicate $P < 0.05$.

* Age difference is the value of the median age at diagnosis for A133S minus the age for wild-type.