

# Aberrant Methylation of *RASSF1A* in Plasma DNA Before Breast Cancer Diagnosis in the Breast Cancer Family Registry

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## Abstract

In addition to classic genetic mechanisms such as deletions and mutations, growth regulatory genes can be inactivated via methylation of cytosine-residues in their promoter regions. Hypermethylation of promoter CpG islands is now recognized as an important and early event in carcinogenesis. Detection of methylated DNA in serum or plasma has been suggested to be a marker for early cancer development. We examined methylation changes in *RASSF1A*, a growth regulatory gene in plasma DNA from blood collected before diagnosis from women with breast cancer and from controls. Samples were from two sets of subjects, 28 women with breast cancer and 10 of their unaffected siblings, and 33 women with breast cancer and 29 age- and ethnici-

ty-matched population-based controls. Using methylation specific PCR, we found 11 of 61 (18%) cases were positive for methylation of *RASSF1A* in their plasma DNA collected before diagnosis. Two of 10 healthy high-risk sibling controls (20%) had plasma DNA positive for *RASSF1A* methylation in their plasma DNA compared with 0 of 29 (0%) population-based controls. Tumor tissue was available for 12 cases and all were positive for *RASSF1A* methylation. These results, if replicated, suggest that aberrant promoter hypermethylation in serum/plasma DNA may be common among high-risk women and may be present years before cancer diagnosis. (Cancer Epidemiol Biomarkers Prev 2009;18(10):2723-5)

## Introduction

Different types of genetic lesions including gene amplifications or deletions, point mutations, loss of heterozygosity, chromosomal rearrangements, aneuploidy, and global and promoter methylation have been associated with cancer. Hypermethylation of CpG islands in promoter regions is now recognized as an important and early event in carcinogenesis (1, 2).

The RAS-association domain factor 1A gene (*RASSF1A*) is a tumor suppressor gene in the RAS pathway that can regulate proliferation, induce apoptosis, and stabilize microtubules (3). Both *in vitro* and *in vivo* studies showed that overexpression of *RASSF1A* in cancer cells leads to cell cycle arrest and inhibition of tumor growth in nude mice (4). Methylated *RASSF1A* is frequently found in breast tumors with frequencies ranging from 10% to 95% (5-11).

Detection of methylated DNA in serum or plasma has been suggested to be a marker for early cancer development (12). Several studies have reported *RASSF1A* meth-

ylation levels in DNA isolated from plasma or serum in the range of 23% to 55% (11, 13-18). All of these studies used blood collected from cases at the time of diagnosis. Methylation levels in controls including healthy women and patients with inflammatory breast disease were in the range of 0% to 10% (11, 13, 18).

In this study, we examined *RASSF1A* promoter methylation to (a) determine the frequency of methylation in plasma collected up to 7 years before breast cancer diagnosis; (b) determine whether unaffected siblings from high-risk families also had detectable alterations; (c) compare levels of methylation between a second set of cases and population-based healthy controls; and (d) assess if methylation status correlates with clinical/pathologic factors in the patients.

## Materials and Methods

**Samples.** The study was approved by the Breast Cancer Family Registry<sup>6</sup> and consisted of 100 women. Twenty-eight breast cancer cases who provided blood before diagnosis and 10 of their unaffected siblings were selected from the New York site of the Breast Cancer Family Registry, and 33 cases and 29 age- and ethnicity-matched population-based controls were recruited from Ontario site of the Breast Cancer Family Registry. The time interval between blood collection and diagnosis ranged from 2 weeks

Received 12/31/08; revised 7/10/09; accepted 7/15/09; published OnlineFirst 9/15/09.

**Grant support:** National Cancer Institute under RFA # CA-06-503 and through cooperative agreements with members of the Breast Cancer Family Registry and P.L.S. The content of this manuscript does not necessarily reflect the views or policies of the National Cancer Institute or any of the collaborating centers in the Cancer Family Registry, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government or the Cancer Family Registry.

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doi:10.1158/1055-9965.EPI-08-1237

<sup>6</sup> <http://epi.grants.cancer.gov/CFR>

**Table 1. Frequency of RASSF1A methylation in breast cancer cases and controls**

Sites	Subjects	n	n positive (%)
All	All cases	61	11 (18)
New York	Cases	28	7 (25)
	Sibling controls*	10	2 (20)
Ontario	Cases	33	4 (12)
	Population-based controls†	29	0 (0)

\*Unaffected siblings from high-risk families.

†Population based healthy controls (age and race matched).

to 83 months. For 12 of the NY cases, a 10- $\mu$ m tumor tissue section was used for isolation of DNA.

**Methylation-Specific PCR.** Plasma DNAs were isolated using QIAmp UltraSense Virus kits (Qiagen). Tumor DNA was extracted from microdissected samples by proteinase K treatment. Bisulfite modification was done using EZ-modification kits (Zymo-Research) according to the manufacturer's protocol. Methylation analysis was done using methylation-specific PCR. Modified plasma DNA (2  $\mu$ l) was amplified with methylated and unmethylated specific RASSF1A primers [RASSF1AFM: GTGTTAACGCGTTGCGTATC, RASSF1ARM: AACCCCGCGAACTA AAAACGA (PCR product size, 105 bp), RASSF1A FUM:TTTGGTTGGAGTGTGTTAATGTG, RASSF1ARUM:CAAACCCACAAAC-TAAAAACAA, (PCR product size, 93 bp)]. PCR reactions were done on PTC-100 thermocyclers (MJ Research). Conditions for amplification were 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, with a final extension cycle of 72°C for 5 min.

**Statistics.** We performed descriptive statistics using  $\chi^2$  tests and Fisher's exact test.

**Table 2. Distribution of methylated RASSF1A according to years before diagnosis, age, and hormonal status among breast cancer cases**

Characteristics	n	n positive (%)
Years before diagnosis		
<1	15	2 (13)
1-2	17	3 (18)
>2	29	6 (21)
		P = 0.91
Age at blood collection (y)		
<40	7	1 (14)
40-49	16	1 (6)
50-59	22	6 (27)
$\geq$ 60	16	3 (19)
		P = 0.42
Age at diagnosis (y)		
<40	6	1 (17)
40-49	16	1 (6)
50-59	16	4 (25)
$\geq$ 60	23	5 (22)
		P = 0.55
ER status		
Positive	14	2 (14)
Negative	4	2 (50)
		P = 0.20
PR status		
Positive	8	1 (13)
Negative	11	3 (27)
		P = 0.60

**Table 3. Frequency of RASSF1A methylation according to menopausal and smoking habits among cases and controls**

Subjects	n	n positive (%)
All controls	39	2 (5)
Ever Smoking	21	2 (10)
Never Smoking	18	0
Premenopausal	16	2 (13)
Postmenopausal	20	0
All cases	61	11 (18)
Ever Smoking	32	7 (22)
Never Smoking	26	8 (31)
Premenopausal	21	3 (14)
Postmenopausal	35	6 (17)

## Results

The overall frequency of plasma DNA methylation for RASSF1A was 18% (11 of 61) and slightly higher in cases from the New York site (25%) than cases from the Ontario site (12%; Table 1). Positive bloods had been collected 2 weeks to 84 months (median, 29 months; mean, 36 months) before diagnosis. Negative bloods were collected 3 weeks to 71 months (median, 22 months; mean, 27 months) before diagnosis. A sample was positive as much as 6.9 years before diagnosis. Among the 10 high-risk siblings of the New York cases, 20% were positive for RASSF1A methylation, whereas none of the 29 Ontario population-based controls were. For 12 of the New York cases, tumor DNA was available for analysis; all were positive for methylation. Of these cases with positive tumor samples, three also were positive in the plasma DNA.

The distribution of RASSF1A methylation among all cases combined by demographic and clinical characteristics is shown in Table 2. There were no associations of age at blood collection or age of diagnosis and frequency of methylation. Data on ER (Estrogen Receptor) and PR (Progesterone Receptor) status of the tumors were available only for a subset of the cases but no associations with methylation status were found.

Among the combined sibling and population controls, there were no significant differences in the frequency of methylation by smoking or menopausal status (Table 3). Similarly, among all cases combined, the frequency of methylation was not associated with menopausal status. There was no correlation of methylation with histology grade or BRCA 1 and 2 mutations status, but we had very limited power due to the small sample size (data not shown). There was a similar frequency of methylation detection (15%) in plasma from patients with invasive versus *in situ* cancers.

## Discussion

We investigated methylation levels of plasma DNA in RASSF1A in breast cancer cases, unaffected siblings, and age-matched population-based controls. Bloods from cases were collected 2 weeks to 83 months before clinical diagnosis. The frequency of methylation in cases was 18% for RASSF1A. Prior studies determining plasma/serum DNA methylation in blood samples collected at the time of diagnosis reported frequencies RASSF1A in the range of 23% to 55% (11, 13-15, 18). In our previous studies of hepatocellular cancer, we showed a high frequency of

*RASSF1A* methylation (35 of 50; 70%) in plasma DNA collected up to 9 years before diagnosis; for *p16* and *p15*, the values were 22 of 50 (44%) and 12 of 50 (22%), respectively (19). Thus, these genes are good candidates for use in a panel for early detection of cancer.

There is little prior data on plasma/serum DNA methylation in controls. *RASSF1A* methylation was observed in 1 of 10, 0 of 14, and 0 of 10 healthy women (13, 14, 18). However, a study of 32 healthy pregnant women found 56% of serum DNAs positive for *RASSF1A* methylation but only 1 of 10 healthy controls, compared with 8 of 10 patients with advanced breast cancer (20). Among the 50 controls in our prior study of hepatocellular cancer, promoter methylation was found in only 3 subjects (6%) for *RASSF1A* (19). In the present study, although none of 29 plasma samples from age- and race-matched population-based controls displayed *RASSF1A* promoter methylation, 20% of unaffected sibling controls at high risk for breast cancer were positive. This finding of high levels of methylated plasma DNA in high-risk siblings suggests that either some unknown inherited factors are affecting methylation levels or that these individuals may be in the early stages of breast cancer development. We are continuing to follow these subjects to determine if their breast cancer status changes but the small number of subjects in our study warrants caution in the interpretation of results.

The frequency of gene methylation did not differ among controls by smoking status but the number of subjects within each group was small. Prior studies have suggested that smoking may impact on promoter hypermethylation in sputum (21). There are no prior data on smoking and plasma/serum DNA methylation.

In conclusion, this is the first study to show epigenetic changes in plasma DNA of patients who gave blood years before breast cancer diagnosis. *RASSF1A* promoter methylation was more frequent in cases and their unaffected siblings compared with population based controls. Larger prospective studies will be needed to determine the association between epigenetic events measured in plasma DNA and breast cancer risk.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

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H. Yazici was the recipient of an Avon Foundation-AACR International Scholar Award in Breast Cancer Research. We thank the AVON Foundation and AACR. This work was supported by awards from Friends for an Earlier Breast Cancer Test and the Breast Cancer Research Foundation. Additional support was

obtained from NIH grants U01 CA69398, U01 CA69467, P30 CA13696, and P30 ES009089.

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*Cancer Epidemiol Biomarkers Prev* 2009;18:2723-2725. Published OnlineFirst September 15, 2009.

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