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Aberrant methylation of *RASSF1A* in plasma DNA prior to breast cancer diagnosis in the Breast Cancer Family Registry

Hulya Yazici[1], **Mary Beth Terry**[2], **Yoon Hee Cho**[1], **Ruby T. Senie**[2], **Yuyan Liao**[2], **Irene Andrulis**[3,4,5], and **Regina M. Santella**[1]

[1]Department of Environmental Health Sciences, Mailman School of Public Health of Columbia University, New York, New York, USA

[2]Department of Epidemiology, Mailman School of Public Health of Columbia University, New York, New York, USA

[3]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Department of Laboratory Medicine and Pathobiology, University of Toronto

[4]Department of Molecular and Medical Genetics, University of Toronto

[5]Division of Research, Ontario Cancer Genetics Network, Cancer Care Ontario Toronto, Ontario, CANADA

Abstract

In addition to classical 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 prior to 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-ethnicity matched population-based controls. Using methylation specific PCR, we found 11/61 (18%) cases were positive for methylation of *RASSF1A* in their plasma DNA collected prior to diagnosis. Two of 10 healthy high risk sibling controls (20%) had plasma DNA positive for *RASSF1A* methylation in their plasma DNA compared to 0/29 (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.

Keywords

RASSF1A genes methylation; breast cancer; inherited early events

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

Corresponding author: Dr. Hulya Yazici, Dept of Environmental Health Sciences, Mailman School of Public Health, Columbia University, 630 West 168th St, New York, NY 10032, Phone 212 305-8158, Fax 212 305-5328, hulyayazici@yahoo.com.

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 demonstrated that overexpression of RASSF1A in cancer cells leads to cell cycle arrest and inhibition of tumor grown in nude mice [4]. Methylated *RASSF1A* is frequently found in breast tumors with frequencies ranging from 10–95% [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* methylation levels in DNA isolated from plasma or serum in the range of 23–55% [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 0–10% [11;13;18].

In this study, we examined *RASSF1A* promoter methylation; (1) determine the frequency of methylation in plasma collected up to seven years prior to breast cancer diagnosis; (2) determine whether unaffected siblings from high risk families also had detectable alterations; (3) compare levels of methylation between a second set of cases and population-based healthy controls; and (4) assess if methylation status correlates with clinicopathological factors in the patients.

Materials and Methods

Samples

The study was approved by the Breast Cancer Family Registry (http://epi.grants.cancer.gov/CFR/) and consisted of 100 women. Twenty eight breast cancer cases who provided blood prior to diagnosis and 10 of their unaffected siblings were selected from the New York site of the BCFR and 33 cases and 29 age–ethnicity matched population-based controls were recruited from Ontario site of the BCFR. The time interval between blood collection and diagnosis ranged from 2 weeks to 83 months. For 12 of the NY cases, a 10 micron tumor tissue section was used for isolation of DNA.

Methylation Specific PCR (MSP)

Plasma DNAs were isolated using QIAmp UltraSense Virus Kits (Qiagen, Valencia, CA). Tumor DNA was extracted from microdissected samples by proteinase K treatment. Bisulfite modification was performed using EZ-modification Kits (Zymo-Research, Alameda, CA) according to the manufacturer's protocol. Methylation analysis was performed using MSP. 2μl modified plasma DNA was amplified with methylated and unmethylated specific *RASSF1A* primers RASSF1AFM:GTGTTAACGCGTTGCGTATC, RASSF1ARM:AACCCCGCGAACTA AAAACGA, (PCR product size: 105 bp), RASSF1AFUM:TTTGGTTGGAGTGTGTTAATGTG, RASSF1ARUM:CAAACCCCACAAACTAAAAACAA, (PCR product size: 93 bp)]. PCR reactions were performed on PTC-100 thermocyclers (MJ Research, Watertown, MA). 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-square tests and Fisher's exact test.

Results

The overall frequency of plasma DNA methylation for *RASSF1A* was 18% (11/61) and slightly higher in cases from the NY 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) prior to diagnosis. Negative bloods were collected 3 weeks to 71 months (Median 22 months, Mean 27 months) prior to diagnosis. A sample was positive as much as 6.9 years prior to diagnosis. Among the 10 high risk siblings of the NY cases, 20% were positive for *RASSF1A* methylation while none of the 29 Ontario population-based controls were. For 12 of the NY 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 and PR 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 do 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 – 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 23–55% [11;13–15;18]. In our previous studies of hepatocellular cancer, we demonstrated a high frequency of *RASSF1A* methylation (35/50, 70%) in plasma DNA collected up to 9 years prior to diagnosis; for *p16* and *p15* the values were 22/50 (44%) and 12/50 (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 1/10, 0/14 and 0/10 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 to 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 demonstrate epigenetic changes in plasma DNA of patients who gave blood years prior to breast cancer diagnosis. *RASSF1A* promoter methylation was more frequent in cases and their unaffected siblings compared to population based controls. Larger prospective studies will be needed to determine the association between epigenetic events measured in plasma DNA and breast cancer risk.

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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).

Table 2

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

Characteristics	N	N positive (%)
Years prior to diagnosis		
<1	15	2 (13)
1–2	17	3 (18)
>2	29	6 (21)
		p=0.91
Age at blood collection		
<40	7	1 (14)
40–49	16	1 (6)
50–59	22	6 (27)
>=60	16	3 (19)
		p=0.42
Age at diagnosis		
<40	6	1 (17)
40–49	16	1 (6)
50–59	16	4 (25)
>=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=.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	21	2 (10)
Never	18	0
Premenopausal	16	2 (13)
Postmenopausal	20	0
All Cases	61	11 (18)
Ever	32	7 (22)
Never	26	8 (31)
Premenopausal	21	3 (14)
Postmenopausal	35	6 (17)