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Circulating Tumor-Specific DNA: A Marker for Monitoring Efficacy of Adjuvant Therapy in Cancer Patients

Heidi Fiegl,[1] Simone Millinger,[1] Elisabeth Mueller-Holzner,[1] Christian Marth,[1] Christian Ensinger,[2] Andreas Berger,[3] Helmut Klocker,[3] Georg Goebel,[4] and Martin Widschwendter[1]

Departments of [1]Obstetrics and Gynecology, [2]Pathology, [3]Urology, and [4]Biostatistics and Documentation, Innsbruck Medical University, Innsbruck, Austria

Abstract

Adjuvant systemic therapy (a strategy that targets potential disseminated tumor cells after complete removal of the tumor) has clearly improved survival of patients with cancer. To date, no tool is available to monitor efficacy of these therapies, unless distant metastases arise, a situation that unavoidably leads to death. We analyzed *RASSF1A* DNA methylation in pretherapeutic sera and serum samples collected 1 year after surgery from 148 patients with breast cancer who were receiving adjuvant tamoxifen; 19.6% and 22.3% of patients with breast cancer showed *RASSF1A* DNA methylation in their pretherapeutic and 1-year-after serum samples, respectively. *RASSF1A* methylation 1 year after primary surgery (and during adjuvant tamoxifen therapy) was an independent predictor of poor outcome, with a relative risk (95% confidence interval) for relapse of 5.1 (1.3-19.8) and for death of 6.9 (1.9-25.9). Measurement of serum DNA methylation allows adjuvant systemic treatment to be monitored for efficacy: disappearance of *RASSF1A* DNA methylation in serum throughout treatment with tamoxifen indicates a response, whereas persistence or new appearance means resistance to adjuvant tamoxifen treatment. It remains to be seen whether modifications made in adjuvant therapeutic strategies based on detection of circulating nucleic acids will improve survival as well as quality of life. (Cancer Res 2005; 65(4): 1141-5)

Introduction

Breast cancer is the most frequent malignancy among women in the industrialized world. Although the presence or absence of metastatic involvement in the axillary lymph nodes is the most powerful prognostic factor available for patients with primary breast cancer (1), it is only an indirect measure reflecting the tendency of the tumor to spread. About 75% of breast cancers are hormone dependent, and the postoperative administration of tamoxifen reduces the risk of recurrence by 47% and reduces the risk of death by 26% (2). Tamoxifen is usually administered for 5 years to women with hormone receptor–positive breast cancers to target disseminated tumor cells. Recent evidence from large trials shows significant improvement of disease-free survival by administering letrozole or examestane, both aromatase inhibitors, after completing 5 or 2 to 3 years of standard tamoxifen treatment, respectively (3, 4); however, the absolute benefits are limited. For future secondary adjuvant treatment studies, a highly sensitive marker for tamoxifen-resistant circulating cells is urgently needed. Such a marker has to fulfill certain requirements: (*a*) absence in non–breast cancer patients, (*b*) easy availability and measurability in patients throughout follow-up period without discomfort or harm, (*c*) poor prognostic parameter in nonsystemically treated patients, and (*d*) identification of patients during adjuvant treatment who are nonresponsive to the endocrine therapy used.

In recent years, changes in the status of DNA methylation, known as epigenetic alterations, have turned out to be one of the most common molecular alterations in human neoplasia including breast cancer (5). In addition, numerous studies have shown tumor-specific alterations in DNA recovered from plasma or serum of patients with various malignancies, a finding that has potential for molecular diagnosis and prognosis (6–10). Very recently, we were able to detect a prognostic value for *APC* and *RASSF1A* methylation in pretherapeutic sera of patients with breast cancer (11). *RASSF1A* DNA methylation has consistently been shown to be a prognostic marker in patients who did not receive adjuvant therapy (11).

This study now shows that methylated *RASSF1A* DNA in serum is a surrogate marker for circulating breast cancer cells and that this cancer-specific DNA alteration allows monitoring of adjuvant therapy in patients with cancer: Disappearance of *RASSF1A* DNA methylation in serum throughout treatment with tamoxifen indicates a response, whereas persistence or new appearance means resistance to adjuvant tamoxifen treatment.

Materials and Methods

Patients. We studied pre- and posttherapeutic serum samples of 148 patients with breast cancer. Serum samples from our serum bank were recruited from all patients diagnosed with breast cancer between September 1992 and February 2002 who met all the following criteria: primary breast cancer without metastasis at diagnosis, tamoxifen treatment for a total of 5 years or upon relapse, availability of serum samples before treatment and 1 year after treatment (a time when the patient has received at least six monthly adjuvant treatments with tamoxifen 20 mg/d) and no relapse after 1 year (Supplementary Data). Patients were 37 to 88 years old (median age at diagnosis, 62 years). After a median follow-up (after the second serum draw) of 3.6 years (range, 0.2-9.7 years) and 4.0 years (range, 0.5-9.8 years), seven (4.7%) and eight (5.4%) patients had relapsed or died, respectively. Throughout the entire observation period, 13 (8.8%) and 15 (10.1%) patients relapsed or died, respectively. Hormone receptor status was determined by either radioligand binding assay or immunohistochemistry. In addition, serum samples from 154 patients with benign condition of the breast and from 93 patients with cervical cancer have been analyzed (Supplementary Data).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Martin Widschwendter, Department of Obstetrics and Gynecology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria. Phone: 43-512-504-81055; Fax: 43-512-504-23112; E-mail: Martin.Widschwendter@uibk.ac.at.

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Serum Samples, DNA Isolation, and MethyLight Analysis. Patients' blood samples were drawn before or 1 year after therapeutic intervention. Blood was centrifuged at 2,000 × *g* for 10 minutes at room temperature, and 1-mL aliquots of serum samples were stored at −30°C.

DNA isolation, bisulfite modification, and MethyLight analysis was done as described recently (11).

Laser Capture Microdissection. The PixCell II LCM System (Arcturus Engineering, Mountain View, CA) was used for laser capture microdissection of paraffin-embedded tissues. Ten-micrometer-thick sections of 13 breast cancer patients with a ductal carcinoma *in situ* were used. For each analyzed fraction, 1,000 cells were "laser captured". DNA extraction was carried out using the Arcturus Pico Pure DNA extraction kit according to the manufacturers' instructions. DNA bisulfite modification and MethyLight analysis was done as described (11).

Statistics. We used Pearson's χ^2 or, in the case of low frequencies per cell, Fisher's exact method to test associations between categorically clinicopathologic features and methylation measures. The Mann-Whitney *U* test was used to assess differences between nonparametric distributed variables. Relapse-free and overall survival were calculated from the date of second serum draw (1 year after diagnosis) to the date of relapse or death or last follow-up. Relapse-free and overall survival curves were calculated with the Kaplan-Meier method. Univariate analysis of overall survival according to clinicopathologic factors [tumor stage, grading, nodal status, menopausal status, and hormone receptor status (estrogen and/or progesterone receptor positivity)], and pretherapeutic and 1-year-after serum *RASSF1A* DNA methylation was done using a two-sided log rank test.

Multivariate Cox proportional hazards analysis was used to estimate the predictive effect of methylated serum *RASSF1A* DNA.

A *P* value <0.05 was considered a statistically significant difference. All statistical analyses were done using SPSS Software 10.0 (Chicago, IL).

Results

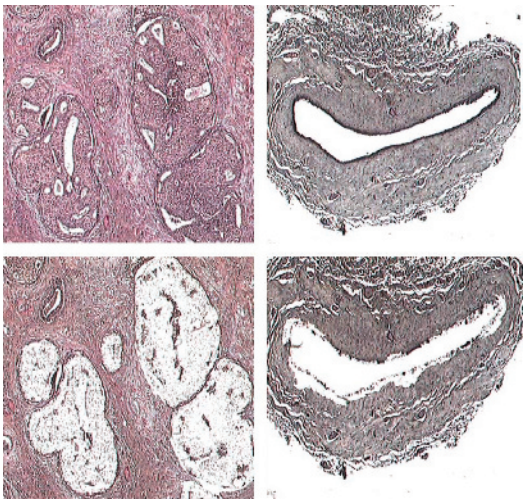
***RASSF1A* DNA Methylation in Laser Capture Microdissected Breast Cancer Cells.** The rationale for supposing *RASSF1A* methylation as a DNA-based marker for breast cancer cells was based on our previous finding that 98.6% of 148 analyzed breast cancer specimens showed *RASSF1A* DNA methylation (12) and that *RASSF1A* methylation in pretherapeutic serum samples of patients with breast cancer who did not receive any systemic adjuvant therapy was an independent poor prognostic marker (11).

To determine whether *RASSF1A* DNA methylation acts as a DNA-based marker solely for breast cancer cells but not for other breast- and/or tumor-associated cells, we did laser-assisted microdissection of 13 paraffin-embedded specimens that had been removed due to hormone receptor–positive carcinoma *in situ*. *RASSF1A* methylation was detected in all cancer cell fractions, whereas most of the underlying stroma, the nonneoplastic breast epithelium, or the adjacent stroma were negative for *RASSF1A* methylation (Fig. 1).

***RASSF1A* DNA Methylation in Serum of Non–Breast Cancer Patients.** To assess whether *RASSF1A* DNA methylation in serum is a breast cancer–specific marker, we analyzed pretherapeutic sera from non–breast cancer: *RASSF1A* DNA methylation was detectable in pretherapeutic serum samples from only 11 (7.1%) of 154 and 5 (5.4%) of 93 patients with benign conditions of breast and primary cervical cancer, respectively. The majority of control patients with *RASSF1A* methylation in their serum either were postmenopausal or had an advanced cervical cancer (Supplementary Data). These findings substantiate the conjecture that *RASSF1A* methylation in serum is a specific marker for circulating breast cancer cells.

***RASSF1A* DNA Methylation in Serum of Patients with Primary Breast Cancer Who Received Adjuvant Tamoxifen Treatment.** In this retrospective approach we used prospectively collected serum samples from patients who received tamoxifen for adjuvant treatment due to primary nonmetastatic breast cancer, who had pretherapeutic as well as serum samples drawn 1 year after diagnosis (i.e., >6 months after start of tamoxifen therapy) and who showed no relapse within the first year after diagnosis or at second serum draw. A total of 19.6% and 22.3% of patients showed *RASSF1A* DNA methylation in their pretherapeutic and 1-year-after serum samples, respectively. Pretherapeutic *RASSF1A* methylation showed nearly the same associations with clinicopathologic parameters as described earlier for a different set of patients (11) and was correlated with tumor size, menopausal status (Supplementary Data), and age [median age: *RASSF1A* unmethylated (59.7 years; range, 36.9-88.4); *RASSF1A* methylated (67.6 years; range, 45.8-85.3; P = 0.006)]. *RASSF1A* DNA methylation at second serum draw after 1 year (Supplementary Data) was associated only with age [median age: *RASSF1A* unmethylated (61.3 years; range, 37.8-86.1); *RASSF1A* methylated (67.4 years; range, 45.2-89.6; P = 0.047)].

A.



B.

Pat.ID	RASSF1A methylation			
	Tumor Epithelium	Tumor Stroma	Non-neoplastic Epithelium	Non-neoplastic Stroma
3167	+	–	–	–
2914	+	n.d.	–	–
217	+	–	n.d.	n.d.
2668	+	–	–	n.d.
1724	+	–	n.d.	n.d.
3161	+	–	+	–
673	+	–	–	–
3248	+	–	n.d.	–
3527	+	n.d.	–	–
3591	+	–	+	–
202	+	n.d.	–	n.d.
3610	+	–	–	–
1649	+	+	–	–

Figure 1. *RASSF1A* methylation in microdissected cells. *A,* tumor and nonneoplastic epithelial cells before and after microdissection. Original magnification, ×40. *B,* overview of *RASSF1A* methylation status in tumor and nonneoplastic tissue. +, *RASSF1A* methylated; –, *RASSF1A* not methylated; *n.d.,* not determined, because no DNA could be extracted.

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Table 1. Results of univariate analysis for relapse-free and overall survival

A. For relapse-free survival

Variable	No. of patients who relapsed/ total no.	Relative risk of relapse (95% CI)	*P*
Size of tumor			<0.001
T1	2/92		
T2/3/4	11/56	10.0 (2.2-45.3)	
Tumor grade			0.04
I	2/47		
II/III	11/97	4.3 (0.9-19.7)	
Lymph node metastases			0.003
Negative	3/88		
Positive	10/51	5.8 (1.6-21.0)	
Menopausal status			0.89
Premenopausal	3/30		
Postmenopausal	10/118	1.1 (0.3-4.0)	
Hormone receptor status			0.68
Negative	1/7		
Positive	12/141	0.7 (0.1-5.1)	
Pretherapeutic *RASSF1A* methylation			0.53
Negative	10/119		
Positive	3/29	1.5 (0.4-5.8)	
"One-year-after" *RASSF1A* methylation			0.005
Negative	6/115		
Positive	7/33	4.2 (1.4-12.5)	

B. For overall survival

Variable	No. of patients who died/ total no.	Relative risk of death (95% CI)	*P*
Size of tumor			0.02
T1	5/92		
T2/3/4	10/56	3.4 (1.2-10.0)	
Tumor grade			0.06
I	3/47		
II/III	12/97	3.2 (0.9-11.3)	
Lymph node metastases			0.03
Negative	5/88		
Positive	9/51	3.2 (1.1-9.7)	
Menopausal status			0.34
Premenopausal	2/30		
Postmenopausal	13/118	2.0 (0.5-9.2)	
Hormone receptor status			0.72
Negative	1/7		
Positive	14/141	0.7 (0.1-5.2)	
Pretherapeutic *RASSF1A* methylation			0.28
Negative	11/119		
Positive	4/29	1.9 (0.6-6.1)	
"One-year-after" *RASSF1A* methylation			0.002
Negative	7/115		
Positive	8/33	4.7 (1.6-13.6)	

Abbreviation: 95% CI, 95% confidence interval.

Prognostic Significance of Clinicopathologic Features and Pretherapeutic *RASSF1A* DNA Methylation in Serum. Tumor size as well as lymph node metastasis were poor prognostic parameters for relapse-free as well as for overall survival, whereas tumor grade had a statistically significant effect on relapse-free survival (Table 1A and B). Neither menopausal status, hormone receptor status, nor pretherapeutic *RASSF1A* DNA methylation in serum had an impact on prognosis (Table 1A and B).

Early Identification of Patients Who Are Nonresponsive to Adjuvant Tamoxifen. About 1 year (1.04 ± 0.11 years) after primary diagnosis of breast cancer (after patients were on tamoxifen 20 mg daily for at least 6 months), a second serum draw was done. Serum *RASSF1A* DNA methylation at that time indicated poor relapse-free as well as overall survival (Table 1A

Table 2. Results of multivariate analysis for relapse-free and overall survival

A. For relapse-free survival

Variable	Relative risk of relapse (95% CI)	*P*
Size of tumor		
T2/T3/T4 vs. T1	4.7 (1.0-24.4)	0.05
Tumor grade		
II/III vs. I	3.6 (0.6-20.2)	0.15
Lymph node metastases		
Positive vs. negative	2.3 (0.5-10.3)	0.27
Menopausal status		
Postmenopausal vs. premenopausal	1.7 (0.3-11.1)	0.59
Hormone-receptor status		
Positive vs. negative	0.5 (0.04-6.0)	0.57
Additional chemotherapy		
Yes vs. no	3.1 (0.5-19.3)	0.22
"One-year-after" *RASSF1A* methylation		
Positive vs. negative	5.1 (1.3-19.8)	0.02

B. For overall survival

Variable	Relative risk of death (95% CI)	*P*
Size of tumor		
T2/T3/T4 vs. T1	2.8 (0.7-10.9)	0.14
Tumor grade		
II/III vs. I	3.8 (0.8-16.9)	0.09
Lymph node metastases		
Positive vs. negative	2.9 (0.7-12.1)	0.14
Menopausal status		
Postmenopausal vs. premenopausal	2.8 (0.4-22.1)	0.30
Hormone receptor status		
Positive vs. negative	0.3 (0.02-4.2)	0.37
Additional chemotherapy		
Yes vs. no	0.7 (0.2-3.3)	0.70
"One-year-after" *RASSF1A* methylation		
Positive vs. negative	6.9 (1.9-25.9)	0.004

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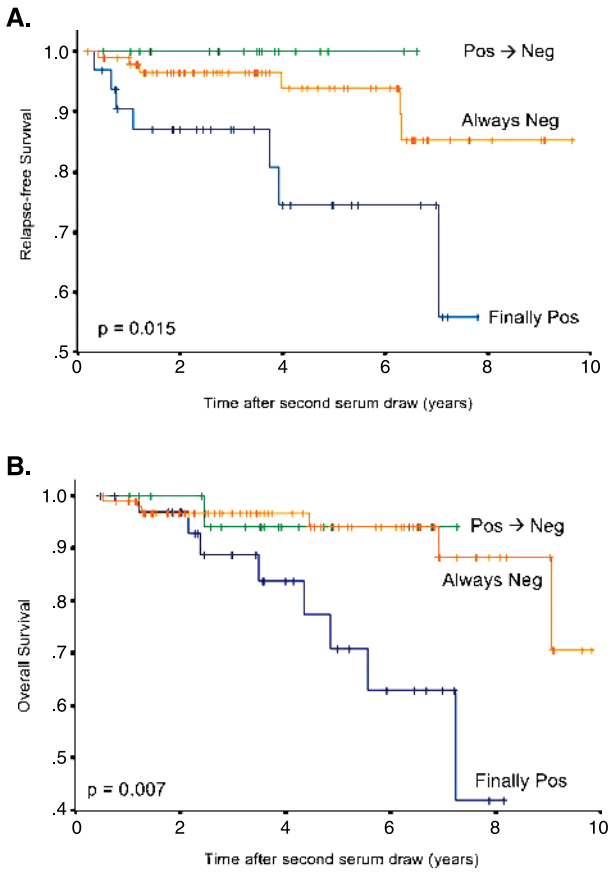

C.

Characteristic	Pos –> Neg	Finally Pos	Fishers' Exact Test
	no. of patients	no. of patients	
Size of tumor			0.78
T1	11	19	
T2/3/4	10	14	
Tumor grade			0.07
I	3	13	
II/III	17	20	
Lymph node metastases			1.00
Negative	10	18	
Positive	8	13	
Menopausal status			1.00
Premenopausal	1	3	
Postmenopausal	20	30	
Hormone-receptor status			0.39
Positive	20	33	
Negative	1	0	
Adjuvant radiation therapy			0.76
No	7	13	
Yes	14	20	
Additional Chemotherapy			0.26
No	15	18	
Yes	6	15	
Type of surgery			0.59
BE	9	17	
ME	12	16	

Figure 2. Survival and changes in *RASSF1A* DNA methylation status. Relapse-free (*A*) and overall survival (*B*) according to *RASSF1A* methylation status in serum that switched from positive to negative, stayed always negative, or was finally positive after 1 year of tamoxifen treatment. *C,* characteristics of those patients according to the *RASSF1A* methylation status.

and B). To test whether serum *RASSF1A* DNA methylation is an independent predictor of nonresponsiveness to tamoxifen, we used Cox multiple-regression analysis that included tumor size, grade, lymph node metastasis, menopausal status, hormone receptor status, and additional adjuvant chemotherapy. Besides tumor size, methylated *RASSF1A* serum DNA was strongly associated with poor outcome, with a relative risk for relapse of 5.1 (Table 2A). The only predictor for poor overall survival was *RASSF1A* serum DNA methylation, with a relative risk for death of 6.9 (Table 2B). To assess which patients might profit from adjuvant tamoxifen treatment and which should be offered an alternative therapy to prevent relapse and/or death from breast cancer, we grouped patients into three categories according to *RASSF1A* DNA methylation in pretherapeutic and 1-year-after serum: (*a*) primary positive that switched to negative after 1 year, (*b*) always negative, and (*c*) positive after 1 year, irrespective of primary methylation status. Despite no difference in the follow-up period or any other clinicopathologic feature or treatment modality, 0% and 21% of patients relapsed and 5% and 24% of patients died in the "Pos → Neg" and "Finally Pos" groups, respectively (Fig. 2). With regard to survival, no statistically significant difference between the "Pos → Neg" and "Always Neg" groups was observed (Fig. 2).

Discussion

To date there has been no target to assess whether a patient will truly profit from adjuvant therapy after tumor removal. We therefore sought a simple tool to indicate "tumor activity" that is nonresponsive to a patient's current systemic therapy. To our knowledge, no systemic marker for monitoring adjuvant treatment in patients with breast cancer has yet been established.

During recent years, some studies have reported cell-free DNA in serum/plasma of patients with breast cancer at diagnosis (7, 11, 13, 14). This article shows that *RASSF1A* DNA methylation is present in nearly all breast cancer cells and is rare in serum of patients with nonneoplastic breast conditions or patients with other invasive cancers, such as cervical cancer. Recently, *RASSF1A* methylation was reported to never be observed in serum of non–cancer patients (9, 10). This slightly discrepant finding may be because we studied a highly selected group of patients who had either a cervical cancer (and 4 of 5 patients with *RASSF1A* methylation in their serum had advanced disease) or a nonneoplastic lesion of the breast, conditions that are associated with a higher lifetime risk of developing breast cancer (15), especially in the age group of patients who showed *RASSF1A* methylation in their serum. In light of these data, we speculate that our "false-positive" controls may indicate cancer predisposition or a cancer not yet evident clinically.

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We assume that serum *RASSF1A* DNA methylation is a surrogate marker for circulating breast cancer cells and disappearance indicates a response, whereas persistence or reappearance means resistance to adjuvant tamoxifen treatment.

Adjuvant endocrine therapy is one of the keys to improving breast cancer–specific survival. Recently, a prospective, placebo-controlled trial showed beneficial effects of the aromatase inhibitor letrozole, a drug that reduces local production of estradiol, after discontinuation of tamoxifen therapy (4). Of the 2,582 patients treated in the letrozole arm, only 29 women profited from this treatment by developing no distant metastases as compared with the placebo group. This means that 100 patients have to be treated to prevent distant metastasis in one patient. Because aromatase inhibitors are potentially harmful (e.g., osteoporosis) and cause discomfort (e.g., arthralgia, myalgia) to patients as well as giving economic strain to the health system, tools to identify patients likely to profit from this treatment are acutely needed. Serum *RASSF1A* DNA methylation is an easy means of detecting patients undergoing adjuvant tamoxifen treatment who need secondary adjuvant therapy. We were able to detect *RASSF1A* methylation in about 20% of patients with breast cancer 1 year after treatment commencement. It is plausible to speculate that only these patients will benefit from further adjuvant treatment (e.g., switch to aromatase inhibitors). The ability to detect such patients would have a great effect on cost-effectiveness and on preventing side effects in patients otherwise "overtreated" with adjuvant treatment.

In conclusion, we here describe a DNA methylation–based surrogate marker for circulating tamoxifen-resistant cells that can be easily measured in serum.

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