BAGE Hypomethylation, A New Epigenetic Biomarker for Colon Cancer Detection

Christoph Grunau,[1] Marie-Elisabeth Brun,[1] Isabelle Rivals,[2] Janick Selves,[3] Winfried Hindermann,[4] Magali Favre-Mercuret,[1] Guillaume Granier,[1] and Albertina De Sario[1]

[1]Institut de Génétique Humaine, Centre National de la Recherche Scientifique UPR 1142, Montpellier, France; [2]Ecole Supérieure de Physique et de Chimie Industrielles, Paris, France; [3]CHU Purpan, Toulouse, France; and [4]Institute for Pathology, Friedrich-Schiller University, Jena, Germany

Abstract

Early detection of colorectal cancer is a decisive step in the successful and complete cure of the disease. Epigenetic markers, in particular, those based on aberrant DNA methylation, can be used to diagnose cancer. *B melanoma antigens* (*BAGE*) are a family of genes and truncated genes located in the heterochromatic regions of several human chromosomes. Our previous work showed that *BAGE* loci (i.e., genes and truncated genes) were hypermethylated in normal tissues and hypomethylated in 98% of human cancers. In the present study, we analyzed DNA methylation of the *BAGE* loci in 54 colon cancers and in neighboring histopathologic normal tissue samples. Using a combined bisulfite restriction assay, we showed that *BAGE* loci were hypomethylated in 81% of carcinoma samples. Colon cancer could be diagnosed with 94% specificity, 83% sensitivity, and 89% accuracy. No correlation was found between DNA methylation of *BAGE* loci and age, gender of patients, nor with the tumor stage or site. Based on the hypothesis that during neoplastic transformation, hypomethylation occurs in juxtacentromeric CpG islands, we suggest that other genes located in the heterochromatic compartment should be tested. These new markers enrich the list of currently studied epigenetic alterations in colon cancer and could be associated with hypermethylation markers to develop reliable diagnostic tests. (Cancer Epidemiol Biomarkers Prev 2008;17(6):1374–9)

Introduction

Colon cancer is a major medical problem in the aging population of industrialized countries, and changing nutritional behavior in developing countries is equally putting the population in these regions at risk. The cumulative risk of colon cancer, and rectal and anal cancer incidence to age 69 is 1% to 2.5% worldwide (1). Colon cancer can be cured by a relatively simple surgical intervention, provided that early diagnosis detects the cancer before metastasis occurs. Current diagnostic tools include the fecal occult blood test, which is simple to use but has a relatively low positive predictive value of 5% to 26% (2-4), and colonoscopy which is highly sensitive but often requires hospitalization of the patient and is therefore costly. Methods based on medical imaging techniques are under development but are not routinely used. The need for a reliable diagnostic method for colon cancer that has the potential to be used noninvasively is therefore evident.

Aberrant DNA methylation is a hallmark of cancer development and was shown to serve as a diagnostic biomarker for the disease. Several groups have described hypermethylation or hypomethylation in specific loci of the DNA of colon carcinoma samples, and even changes in the methylation profile of DNA found in the stool of patients with colon cancer were evidenced (5). It would be useful to enlarge the basis of these studies and to find more markers.

We have recently shown that DNA methylation changes during neoplastic transformation in the *BAGE* loci (6, 7). *BAGE* is a family of full-length genes and of truncated nonfunctional genes located in the heterochromatic regions of several human chromosomes (8, 9). *BAGE* genes are silent in normal cells and are expressed in some tumors and cancer cell lines. We showed that *BAGE* loci (i.e., genes and truncated genes) are hypermethylated in normal tissues and hypomethylated in 98% of human cancers (6). Having hypothesized that these changes could be of diagnostic value for the detection of colon cancer, we decided to measure the degree of DNA methylation in *BAGE* loci in colon carcinoma tissues and normal mucosa.

Materials and Methods

DNA Samples. Biopsies of paired tumor and healthy mucosa were obtained from resected surgical samples with donor consent. Samples were divided into two parts: one was used for phenol chloroform DNA extraction and the other one for histopathologic inspection.

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Current address for C. Grunau: UMR 5244 CNRS/Université de Perpignan/EPHE, Perpignan, France.

Current address for G. Granier: C.H. Henri Duffaut, Avignon, France.

Requests for reprints: Albertina De Sario, Centre National de la Recherche Scientifique UPR 1142, 141 rue de la Cardonille, 34396 Montpellier, France. Phone: 33-4996-19977; Fax: 33-4996-19901. E-mail: Albertina.de-Sario@igh.cnrs.fr

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A first set of 14 patients was used for training (set 1 in Table 1). For one patient (22676/03), DNA was extracted from two independent tumors. A second independent set, 40 patients without microsatellite instability, was used as a test set (set 2 in Table 1). In the latter set, two tumor samples could not be analyzed, probably because of an inefficient bisulfite conversion. Both female and male patients were included in the two sets.

Table 1. Training and test sets

Samples	Sex	Age	TNM	Stage	Site*	Methylation, *Mbo*I (%) †	
						Cancer	Healthy
Set 1							
18947/03	M	73	$T_3N_0M_x$			41	49
18947/03	M	80	$T_3N_0M_x$			27	44
4	M	53	$T_4N_2M_0$	4		40	59
6	F	90	$T_3N_2M_0$	3		32	63
7	F	80	$T_3N_2M_1$	4		35	58
8	F	86	$T_3N_0M_0$	2		34	62
25824/03	F	74	$T_3N_2M_x$			42	41
22676/03 (I) ‡	M	76	$T_3N_0M_x$			6	18
(II)			$T_2N_0M_x$			17	
21846/03	F	75	$T_3N_0M_0$	2		66	70
25522/03	M	36	$T_3N_2M_x$			37	74
24214/03	F	91	$T_4N_0M_0$	3		23	58
25841/03	F	71	$T_4N_1M_1$	4		29	62
24184/03	F	74	$T_3N_0M_0$	2		21	54
25473/03	M	55	$T_3N_1M_x$			35	53
Set 2							
1	F	71	$T_3N_0M_0$	2	1	55	77
2	F	50	$T_3N_2M_1$	4	2	16	63
3	M	72	$T_3N_0M_0$	2	1	21	72
4	F	38	$T_2N_0M_0$	1	2	55	85
5	M	61	$T_1N_0M_0$	1	2	51	93
6	M	72	$T_2N_0M_0$	1	1	16	74
7	M	79	$T_4N_0M_0$	2	0	22	73
8	F	75	$T_3N_2M_0$	3	2	25	60
9	M	59	$T_3N_0M_0$	2	2	19	69
10	F	55	$T_1N_0M_0$	1	1	20	69
11	F	71	$T_2N_0M_0$	1	2	85	79
12	F	75	$T_4N_0M_0$	2	2	17	81
13	M	62	$T_3N_1M_0$	3	2	37	80
14	M	75	$T_3N_2M_1$	4	2	58	74
15	F	67	$T_3N_0M_0$	2	0	33	46
16	M	89	$T_4N_0M_0$	2	0	10	54
17	F	73	$T_2N_0M_0$	1	2	35	78
18	M	58	$T_3N_1M_1$	4	1	48	70
19	F	82	$T_4N_0M_0$	2	0	3	40
20	M	68	$T_3N_0M_0$	2	1	23	78
21	F	78	$T_4N_0M_0$	2	1	37	71
22	M	65	$T_3N_1M_0$	3	2	10	46
23	M	60	$T_2N_1M_0$	3	3	N.D.	43
24	M	52	$T_3N_0M_0$	2	0	35	59
25	M	81	$T_4N_1M_1$	4	1	9	77
26	F	62	$T_3N_0M_0$	2	0	20	51
27	M	75	$T_4N_1M_0$	3	1	7	80
28	M	72	$T_2N_0M_0$	1	1	15	52
29	F	84	$T_3N_2M_0$	3	0	29	79
30	M	68	$T_2N_0M_0$	1	2	40	61
31	M	77	$T_2N_0M_0$	1	2	50	77
32	M	77	$T_4N_1M_1$	4	2	27	68
33	F	72	$T_3N_0M_0$	2	2	10	71
34	M	84	$T_3N_1M_0$	3	0	67	86
35	M	66	$T_3N_2M_1$	4	1	13	79
36	F	64	$T_2N_1M_0$	3	2	31	75
37	M	84	$T_2N_0M_0$	1	2	14	93
38	M	81	$T_4N_0M_0$	2	2	29	75
39	M	84	$T_3N_0M_0$	2	0	N.D.	60
40	M	62	$T_3N_2M_1$	4	1	14	78

Abbreviations: TNM, tumor-node-metastasis; N.D., not determined.
*0, right colon; 1, left colon; 2, rectum; 3, multiple sites.
† Means of duplicates.
‡ Two tumors from the same patient.

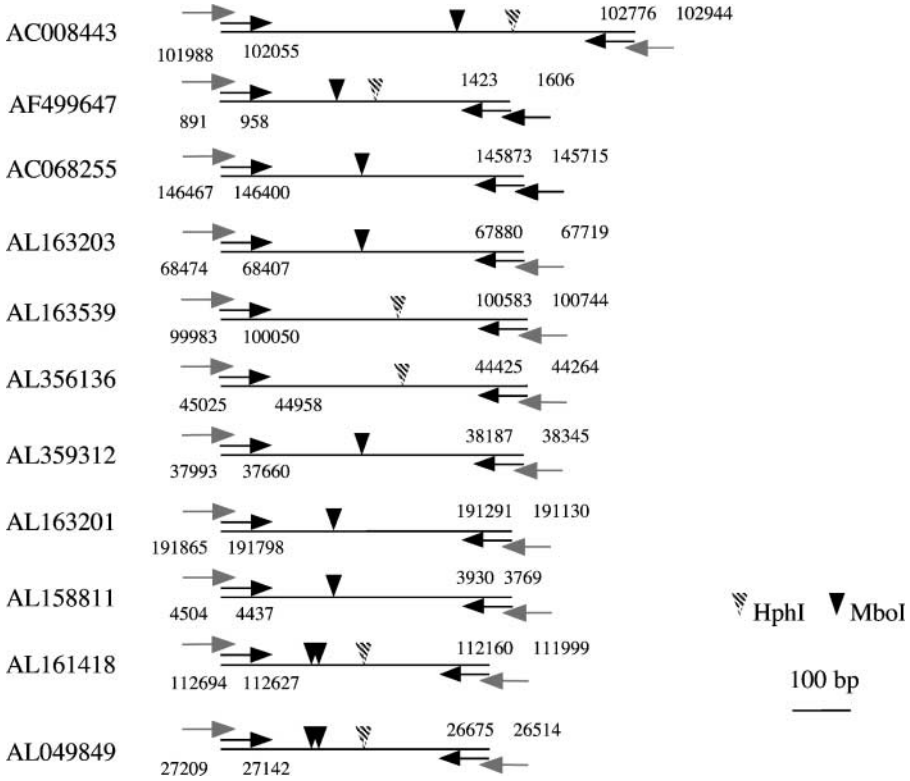

Figure 1. Map of the primers used for the first PCR (*gray arrows*) and the nested PCR (*black arrows*). Forward (*left*) and reverse (*right*) primers. Genomic sequences containing *BAGE* loci are indicated by their accession numbers. *Numbers in the map,* positions of the first nucleotide of the primer in the corresponding genomic sequence. *Triangles,* restriction sites used for the COBRA assays.

DNA Methylation Analysis. We analyzed DNA methylation in *BAGE* loci according to a previously described combined bisulfite restriction assay (COBRA) protocol (6). Briefly, genomic DNA was treated with sodium bisulfite, then *BAGE* loci were amplified with two nested PCR reactions (Fig. 1). Nested PCR does not change the ratio methylated/unmethylated DNA. The purified PCR products were digested either with *Mbo*I or with *Hph*I enzymes. Band intensities were measured and methylation percentages were calculated. All the experiments were done in duplicate.

Statistical Analysis. The normality of the methylation percentages and of the methylation differences were tested in normal and cancer tissues with Shapiro-Wilk normality tests (10). The differences in methylation between normal and cancer tissues were considered Gaussian, but the methylation percentages in normal and cancer tissues were significantly non-Gaussian in the second set ($P_{normal} = 5.4 \times 10^{-2}$, $P_{cancer} = 1.9 \times 10^{-2}$) and in the pooled data ($P_{normal} = 3.0 \times 10^{-2}$, $P_{cancer} = 9.9 \times 10^{-3}$). Thus, to establish the equality of two means, or the nullity of a mean difference, we systematically used the nonparametric Wilcoxon test; to establish correlations, we used the Spearman correlation coefficient of the ranks and the corresponding test (11).

The diagnostic test was characterized in terms of specificity [true normal / (true normal + false cancer)], sensitivity [true cancer / (true cancer + false normal)], and accuracy [(true cancer + true normal) / total].

Results

***BAGE* Loci are Hypomethylated in Colon Cancer.** We first analyzed DNA methylation of *BAGE* loci in the training set, using a COBRA assay and *Mbo*I digestion (6). In 12 out of 14 patients, *BAGE* loci were less methylated in colon cancer than in healthy mucosa (mean difference of 21.2%, Wilcoxon's test: $P = 6.8 \times 10^{-4}$). Only two female patients (21846/03 and 25824/03) showed similar DNA methylation in the tumor and in the normal tissue in the investigated loci. Thus, DNA methylation of the *BAGE* loci seemed to be highly informative for colon cancer detection.

To corroborate this finding, we then analyzed the independent test set consisting of 40 samples of paired colon cancers and healthy mucosa. Again, *BAGE* loci were less methylated in cancer tissues than in normal mucosa, and the difference was even larger (mean difference of 41.8%; Wilcoxon's test, $P = 4.4 \times 10^{-8}$). In both analyses (training set and test set), the methylation differences were higher than the variations due to SEs (for a discussion of SEs, see ref. 6).

We thus asked whether the mean DNA methylation was different in the two data sets. We independently compared cancers, healthy tissues, and the difference between paired tissues in the two data sets (Fig. 2). DNA methylation of normal mucosa and the methylation difference between paired cancer and normal mucosa were significantly different in the two sets (Wilcoxon's test, $P_{normal} = 8.8 \times 10^{-4}$ and $P_{difference} = 2.8 \times 10^{-4}$).

In our previous study of *BAGE* hypomethylation in various normal and cancer tissues, we designed a second COBRA assay based on digestion with the restriction *Hph*I enzyme (6). Samples used in this study were also digested with *Hph*I. However, having found that this site was almost always as methylated in colon cancer as in the paired healthy mucosa (data not shown), we

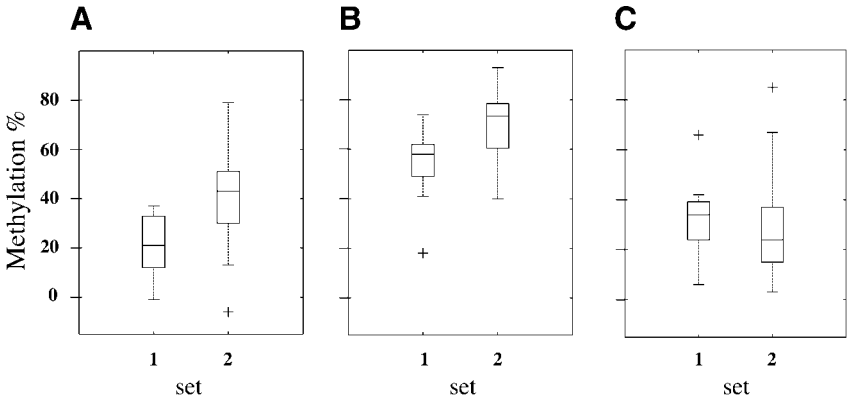

Figure 2. *BAGE* methylation in two independent sets of tissues. **A,** difference of methylation between paired healthy mucosa and cancer; **B,** methylation in healthy mucosa; **C,** methylation in colon cancer.

concluded that *Hph*I was not informative for colon cancer diagnosis.

***BAGE* Hypomethylation Can Be Used as a Tumor Marker.** To use DNA methylation of *BAGE* genes as a tumor marker, we must define a decision threshold, i.e., a value below which a tissue is considered cancer. An optimal threshold of 42% DNA methylation was found on the training set (Fig. 3A), i.e., tissues having ≤42% DNA methylation were considered cancer, and tissues having >42% methylation were considered healthy. This threshold led to 85.7% specificity, 93.3% sensitivity, and 89.7% accuracy.

Interestingly, although the methylation distribution was different in the two sets, this 42% threshold was also quite optimal for the test set (Fig. 3B), as it led to 95.5% specificity, 78.9% sensitivity, and 88.5% accuracy. Some justification is still needed to pool the two sets (Fig. 3C); however, when doing so and using the 42% threshold, the performance on the pooled data was 94.4% specificity, 83.0% sensitivity, and 88.8% accuracy.

The good quality of the classifiers corresponding to the training set, to the test set, and to the pooled data is further illustrated by their receiver operating characteristic curves (Fig. 4A) and by the accuracy (Fig. 4B) obtained when varying the decision threshold. The area under the receiver operating characteristic curve, which represents the probability that a randomly chosen cancer tissue is considered as more likely to be cancer than normal (12), was indeed close to 1: this was equivalent to 0.88 in the training set, 0.95 in the test set, and 0.93 in the pooled data.

DNA Methylation of *BAGE* Loci is Not Correlated with Age, Gender nor with the Tumor Stage. To evaluate the possible effects of individual factors such as age, gender, and tumor stage (we used four groups according to the Duke system) of patients, for each set, we tested the correlation between each factor and the methylation difference between cancer and normal, as well as the correlation between each factor and DNA methylation in cancer or in healthy mucosa. No significant correlation was found.

DNA Methylation of *BAGE* Loci and Tumor Site. We also asked whether there was a relation between DNA methylation and the tumor site. Because the tumor site was not known for the patients of the first set, the analysis was done on the second set only. Patient no. 23, the only one showing multiple tumors, was excluded from the analysis. The other patients were divided into three groups having a tumor either in the left colon, or in the right colon, or in the rectum. An ANOVA led to the conclusion that the tumor location did not influence the methylation of cancer tissues nor the difference of methylation between normal and cancer tissues ($P_{\text{cancer}} = 3.5 \times 10^{-1}$ and $P_{\text{difference}} = 1.3 \times 10^{-1}$), but that there might be an effect on normal tissues ($P_{\text{normal}} = 2.0 \times 10^{-2}$). Two-by-two Wilcoxon tests established that only the difference in methylation

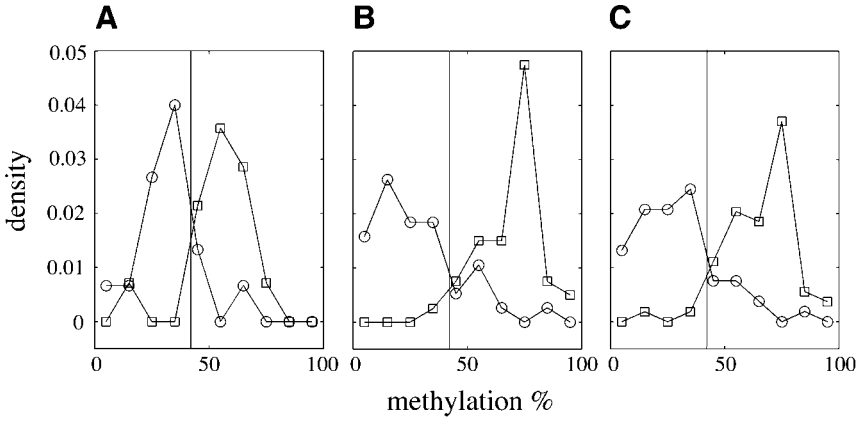

Figure 3. Distribution of DNA methylation in set 1 (**A**), in set 2 (**B**), and in the two pooled sets of tissues (**C**). Colon cancer tissues (○), healthy mucosa (□), and the 42% decision threshold (*vertical line*).

between right colon and rectum might be significant ($P = 3.8 \times 10^{-2}$), methylation being lower in the right colon than in the rectum.

Discussion

We showed earlier that hypomethylation in the *BAGE* loci family is a faithful tumor marker for several types of cancer, which is different from colorectal carcinoma (6). Here, we give evidence that this observation is also true for the colon carcinoma. A threshold value of 42% for DNA methylation was established on the training set and also proved to be optimal on the test set. Using such a threshold, we calculated that 44 out of 53 carcinoma samples (83%) showed hypomethylation in *BAGE* loci compared with the normal mucosa. Thus, colon cancer could be diagnosed with 94% specificity, 83% sensitivity, and 89% accuracy on the pooled data.

We detected strong *BAGE* hypomethylation in only one histopathologically normal mucosa (patient 22676/03). Two more patients had a slight hypomethylation in normal mucosa, the percentages of methylation (40% and 41%) being very close to the cutoff value that we defined. Thus, the overall frequency of false-positives was very low.

Our study was done on two independently collected colon cancers and paired healthy mucosa samples. In both data sets, *BAGE* loci were significantly less methylated in colon cancers than in healthy mucosa. However, DNA methylation in healthy tissues was significantly lower in the first set than in the second one. Two confounding factors can account for this: first, patients having microsatellite instability were excluded from the second set of samples; second, the two sets were collected in two different geographic areas (Germany and France). In any case, the interesting point is that the epigenetic *BAGE* marker was informative for colon cancer diagnosis, irrespective of the criteria we used to recruit patients.

To analyze DNA methylation in *BAGE* loci, we previously designed two COBRA assays, based on digestion with *Mbo*I and *Hph*I. Here, we show that *Mbo*I digestion alone was informative. The *Hph*I site was generally as methylated in colon cancer as in healthy mucosa. This result is not without precedent: in our previous study, we found that methylation in the *Hph*I site depended on the cancer type and was, for instance, high in breast cancer.

In a previous work on different cancers, we showed that *BAGE* hypomethylation was not correlated with the hypomethylation of repetitive satellite sequences (6). In this work, we analyzed DNA methylation of LINE sequences in the training set. LINE sequences were hypomethylated in colon cancers as well as in 31% of adjacent normal mucosa (data not shown) and this result is consistent with previously published data (13).

No correlation was found between DNA methylation of *BAGE* loci and age, gender, or with the tumor stage. This latter result suggests that loss of methylation in *BAGE* loci is an early event in tumor progression. Accordingly, Bariol *et al.* (14) observed both aberrant CpG island methylation and global DNA hypomethylation in the precursor lesions of colon cancer. Because it is very likely that loss of methylation at *BAGE* loci is a consequence of the demethylating wave that occurs in tumor cells, the epigenetic marker *BAGE* may also be informative to detect adenomas.

Our data shows that normal tissues in the vicinity of tumors are significantly less methylated in the *BAGE* loci in the right colon. This is surprising because the majority of colorectal carcinomas occur in the left colon and in the rectum. However, our findings are consistent with earlier studies which showed that aberrant hypermethylation of CpG islands is more frequent in the right-sided colon than in the left-sided colon or the rectum of patients with cancer (15-17). It was proposed that epigenetic alterations predominantly occur in right-sided colon carcinomas, whereas left-sided carcinomas are due to mutations (15).

Thus far, epigenetic cancer biomarkers have been identified by two complementary approaches: the investigation of candidate genes with known changes in expression patterns in cancer tissues (e.g., tumor suppressor genes) and genome-wide identification of anonymous loci using methylation-sensitive restriction enzymes. Here, we present a new approach that is based on the hypothesis that during neoplastic transformation, hypomethylation occurs in juxtacentromeric CpG islands. Other genes located in the heterochromatic compartment could undergo loss of methylation as suggested by our preliminary results (7). These new markers enrich the list of currently studied epigenetic alterations in colon cancer and could be associated with

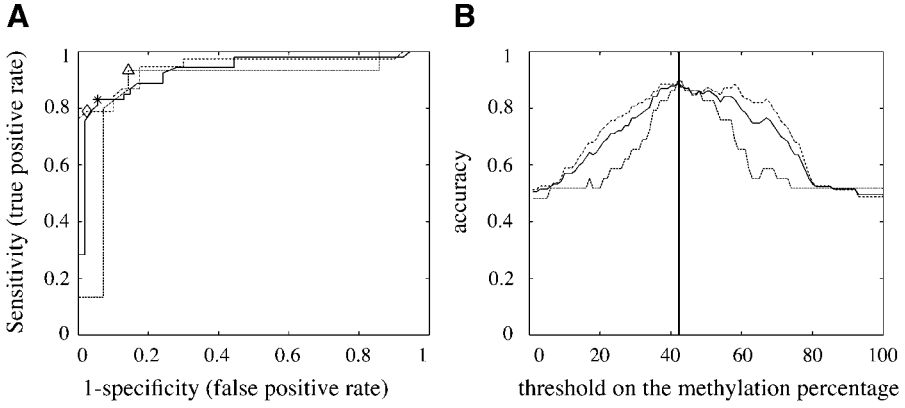

Figure 4. A, receiver operating characteristic curves; **B,** accuracy obtained when varying the decision threshold on set 1 (*dashed line*), on set 2 (*dash-dotted line*), and on the pooled data (*continuous line*). The markers (△, set 1; □, set 2; and *, pooled data in **A**) and the vertical line in (**B**) correspond to the 42% decision threshold.

hypermethylation markers to develop reliable diagnostic tests.

Finally, to develop a noninvasive tumor test to monitor the general population, it will be interesting to assess whether *BAGE* hypomethylation can be detected in fecal DNA from patients with colon cancers compared with healthy controls.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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