DNA Methylation at Selected CpG Sites in Peripheral Blood Leukocytes Is Predictive Of Gastric Cancer

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Abstract. *Background/Aim: Recently, a set of studies addressed the question of the prevalence of aberrant methylation in surrogate tissues, such as peripheral blood leukocytes. Toward this aim, we conducted a case-control pilot study to investigate aberrant methylation in leukocytes of gastric cancer patients. Materials and Methods: The SNuPE combined with ion pair reverse phase HPLC (SIRPH method) was used to examine site-specific methylation status at selected CpG sites of the promoter regions of APC, ACIN1, BCL2, CD44, DAPK1, CDKN2A, RARB, TNFRSF10C HS3ST2 and of LINE-1, Alu repeats. Results: We observed that in the patients, tumor suppressor genes were slightly but significantly higher methylated at several CpG sites, while DNA repetitive elements were slightly less methylated compared to controls. This was found to be significantly associated with higher prevalence for gastric cancer. Conclusion: These results suggest that larger studies must be carried-out to explore the biological significance and clinical usefulness of leukocyte DNA as non-invasive detection tool for gastric cancer.*

Gastric cancer is one of the leading causes of cancer-related deaths worldwide with highest rates in Central and Eastern Europe, although the incidence has gradually decreased in many Western countries due to the recognition of certain risk factors (*e.g.* low vegetable and fruit intake, high salt intake, smoking, *Helicobacter pylori*) (1). Despite the acknowledgement of the risk factors, the current 5-year survival rate of individuals diagnosed with gastric cancer is only 20-30%, with this low rate being attributable to the fact that most cases are already in an advanced stage when diagnosed. Therefore, the identification of early, accurate, non-invasive biomarkers remains the promising approach for improving the survival rate for gastric cancer patients.

Almost all gastric cancers have epigenetic abnormalities that drive cancer development and progression in collaboration with genetic changes leading to gain-of-function in oncogenes and loss-of-function in tumor suppressor genes (2, 3). However, a large body of evidence shows that genetic alterations, particularly gene mutations, are relatively infrequent in gastric cancer (4).

Changes in the DNA methylation status, including global DNA hypomethylation and site-specific gene hyper-methylation, are concomitantly found in tumors and are the most common molecular alterations in human neoplasia (5). Most investigations of DNA methylation are based on the assessment of differences in methylation levels between the tumor and the adjacent tissues. In such cases access to the affected tissue by surgery is required, which is a major limitation for clinical utility.

There is growing evidence that methylation changes in cancer patients arise systematically and may be measured in surrogate tissues. Changes of methylation in leukocyte DNA may reflect methylation levels within other tissues (6). Methylation status could also be passed across generations that may be affected by environmental exposures and aging (7, 8). Therefore, studies investigating leukocyte DNA methylation levels and cancer risk are rapidly emerging. Although a large body of literature exists on DNA methylation changes at the tissue level, less is known about whether DNA methylation measured in leukocytes can be used as a biomarker for different health outcomes. As blood biomarkers can be measured repeatedly over time, they can be useful to understand how disease risk changes over the life course. At least ten studies measuring leukocyte DNA global

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methylation in different cancer types including stomach (9, 10), colon (11, 12), breast (13), bladder (14-16), head and neck (17), renal cell (18) and pancreatic cancer (10) have found an elevated risk for cancer between those in the lowest quantile of global DNA methylation compared to those in the highest quantile of risk. Only few studies on gene-specific methylation in leukocyte DNA and cancer risk have focused on specific genes for breast and colon cancers with their results supporting the potential of gene-specific methylation measured in leukocytes as a biomarker of disease risk (20-25). These results demonstrate that methylation status in leukocyte DNA may provide a useful biomarker for early detection of cancer and, unlike tumor DNA, can be obtained noninvasively and relatively inexpensively.

Therefore, the aim of the present pilot study was to determine whether selected tumor suppressor genes and genome-wide repetitive sequence methylation in peripheral blood leukocytes of subjects with gastric cancer and healthy controls are different. An additional aim was to look for the correlation of the methylation levels with the clinical and morphological features.

Materials and Methods

Ethic statement. The study was approved by the Lithuanian Bioethics Committee (reference number BE-2-17) and written informed consents were obtained from all patients.

Gene selection. We selected genes of different metabolic pathways, as certain genes, which are involved in cellular pathways such as signal transduction, apoptosis, cell-to-cell communication, cell cycles and cytokine signaling, are down-regulated in cancers and may be considered as potential tumor suppressor genes (26). Therefore, we investigated, *CDKN2A* (cyclin-dependent kinase inhibitor 2A; cell cycle regulation) (27) and *APC* (Adenomatosis polyposis coli; signal transduction) (28) as they have been shown to be methylated at variable frequencies in gastric cancer (29) and apoptotic genes: *DAPK1* (Death-associated protein kinase 1), *HS3ST2* (heparan sulfate (glucosamine) 3-O-sulfotransferase 2; cell growth, adhesion and migration) (30), *RARB* (Retinoic acid receptor beta; DNA binding, activation transcription) (31), *TNFRSF10C* (Tumor necrosis factor receptor superfamily member 10C), *BCL2* (B-cell leukemia/lymphoma 2) (32-34), *CD44* (CD44 antigen molecule; adhesion of cells to the extracellular matrix) (35) and *ACIN1* (apoptotic chromatin condensation inducer 1). The latter genes (*TNFRSF10C, BCL2, CD44, ACIN1*) were selected as they are frequently methylated in other human cancers (29, 32-34). For surrogate markers of global methylation, we selected long-interspersed elements (*LINE-1*) and the most abundant short-interspersed elements in the human genome (*Alu*). Both of these repetitive sequences account for 28% of the human genome and normally they are heavily methylated.

DNA samples. This study included 24 gastric cancer patients diagnosed at the Department of Surgery (Hospital of Lithuanian University of Health Sciences Kauno Klinikos, Lithuania) between August 2005 and January 2007. All diagnoses were based on histo-pathological evidence. The clinical and pathological characteristics of these gastric cancer patients based on the Union for International Cancer Control TNM classification (36) are summarized in Table I. Patients had neither been submitted to chemotherapy or radiotherapy prior to surgery, nor did they have any other diagnosed cancer. Blood samples were taken immediately prior to surgery. The control population consisted of 49 patients with benign diseases (12 with inguinal hernias, 8 with primary ventral hernias and 29 with cholelithiasis). There were no changes of inflammatory status in control individuals.

DNA extraction. Five milliliters corresponding to peripheral venous blood were available from each out of twenty-four patients with gastric cancer. Forty-nine peripheral venous blood samples were obtained from the control population. Genomic DNA was extracted from the peripheral blood by DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The DNA concentration was measured using NanoDrop1000 (Thermo Scientific, Wilmington, USA).

Bisulfite modification, polymerase chain reactions and site-specific methylation by SIRPH analysis. Two hundred ng of genomic DNA was subjected to bisulfite conversion with the EpiTect Bisulfite kit (Qiagen) according to manufacturer's recommendation. Methylation-specific polymerase chain reactions (PCR) with primers covering the site of interest were performed essentially as previously described (19). The site-specific quantitative methylation analysis at selected CpG sites were done by using single-nucleotide primer extension (SNuPE) and separation by ion-pair reverse-phase (IP RP) high-performance liquid chromatography (SIRPH method) as previously described by El-Maarri (37, 38). SNuPE primers used in this study are as previously described (19).

Data analysis. Descriptive analyses were performed on age, gender between cases and controls and on clinical characteristics in gastric cancer case group. Differences in methylation level values between cases and controls were analyzed by the Mann-Whitney test. The binary logistic regression analysis was used to determine associations of the methylation status with various clinical and pathological features. To evaluate the effects of site-specific CpG methylation levels on case control status, while controlling for inter-variable confounding, logistic regression was performed to determine odds ratios (OR) and their associated 95% confidence intervals (CI). In this analysis, site-specific CpG relative methylation level was broken into tertiles based on the distribution in controls. $p<0.05$ was considered as significant. Data were analyzed using the SPSS statistical software, version 17.0.0 (SPSS Inc., Chicago, USA). The hierarchical clustering as well as the principle component analysis (PCA) was done using the Qlucore Omics Exlorer version 2.3. (Qlucore, Lund, Sweden).

Results

In the present study, we investigated the locus-specific methylation at selected CpGs at *Alu* and *LINE-1* repeats and at promoter regions of nine tumor suppressor genes. We measured methylation at three CpG sites in the *Alu* consensus sequence, and at two CpG sites at *LINE-1*. For tumor suppressor genes we measured one CpG site in the *APC, BCL2, DAPK1, TNFRSF10C, CD44* promoter region, and two

Table I. *Clinical characteristics of patients included in this study.*

		Cases		Controls	
		n	%	n	%
Age (years)	<65	16	66.7	30	61.2
	>=65	8	33.3	19	38.8
Gender	Female	9	37.5	30	61.2
	Male	15	62.5	19	38.8
Cancer stage*	I+II	10	41.7		
	III+IV	14	58.3		
pT category	pT1+pT2	11	45.8		
	pT3+pT4	13	54.2		
Lymph node involvement	Negative	4	16.7		
	Positive	20	83.3		
Distant metastases	Negative	19	79.2		
	Positive	5	20.8		
Invasion into blood vessels	Negative	9	37.5		
	Positive	15	62.5		
Invasion into lymph vessels	Negative	4	16.7		
	Positive	20	83.3		
Perineural spread	Negative	8	33.3		
	Positive	16	66.7		
Cell differentiation grade	G1+G2	7	29.2		
	G3+G4	17	70.8		
Hystological type	Tubular	8	33.3		
	Poor diff., signet, mucinous	16	66.7		

*According to UICC 2009.

Table II. *The comparison of peripheral blood-derived DNA mean methylation in cases and controls.*

Site	Primer	Control group		Gastric cancer group		
		Mean	SD	Mean	SD	*p*-Values*
LINE-1	SN-1	51.99	0.70	50.50	1.70	<0.0001
	SN-8	61.65	0.98	60.36	2.19	0.0036
Alu	SN-1	30.02	2.57	26.93	1.23	<0.0001
	SN-3	34.53	1.41	31.18	3.33	<0.0001
	SN-4	36.95	1.30	35.29	1.02	<0.0001
CDKN2A	SN-1	5.14	1.02	4.53	1.34	0.0629
	SN-2	24.24	5.66	29.05	15.81	0.4884
APC	SN-1	5.35	.96	7.32	2.32	0.0008
HS3ST2	SN-1	10.96	2.86	10.88	2.52	1.0000
	SN-2	21.00	6.30	20.74	3.56	0.7691
DAPK1	SN-3	11.93	3.01	14.19	3.77	0.0132
ACIN1	SN-1	8.85	0.50	12.26	1.58	<0.0001
	SN-3	16.39	2.68	16.27	1.02	0.1659
BCL2	SN-1	3.66	1.45	5.94	2.58	<0.0001
CD44	SN-2	3.33	0.41	4.23	1.08	<0.0001
RARB	SN-1	7.59	4.49	7.24	1.69	0.3878
	SN-2	5.38	4.09	6.63	1.71	0.2665
TNFRSF10C	SN-1	4.92	3.59	5.27	0.40	<0.0001

*Mann-Whitney test.

CpG sites were measured in the *CDKN2A, HS3ST2, ACIN1* and *RARB* promoter regions. Control patients used in this study were matching the cases in both age and gender distribution (Table I).

Peripheral blood-derived DNA methylation analysis. The average methylation levels at each CpG site for both cases and controls blood derived DNA are summarized in Table II. Mean methylation level at *Alu* and *LINE-1* repeats in patients was slightly lower than the mean level in the controls. Methylation difference was significant ($p<0.01$) for all investigated CpGs. The opposite methylation phenomenon was observed at tumor suppressor genes. Mean methylation level was significantly ($p<0.05$) higher in patients than in controls at CpGs of *APC, DAPK1, BCL2, CD44, TNFRSF10C* and at one of two (*i.e.* SN1) investigated CpGs of *ACIN1* gene.

Association of peripheral blood relative methylation with prevalence of gastric cancer. As we observed significant differences in blood methylation between patients and healthy controls, we performed binary logistic regression analysis to examine the association between relative methylation level at each of the investigated regions (gene promoter regions and repetitive elements) and the presence of gastric cancer. In this model, methylation was categorized into three groups divided at the 33rd and 66th percentiles, with the highest tertile of relative methylation serving as reference for *Alu* and *LINE-1*. The lowest tertile served as the referent for promoters of tumour suppressor genes *CDKN2A, APC, 30ST2, DAPK1, ACIN1, BCL2, CD44, RARB, TNFRSCF10C*. We observed that cases in the lowest tertile of *LINE-1* SN1 (OR 6.7, 95%CI 1.7-26.6) relative methylation had a significantly higher prevalence of gastric cancer (Figure 1). Similar findings were observed in the lowest tertile of *Alu* CpGs: SN1 (OR 10.3, 95%CI 2.2-48.6), SN3 (OR 8.6, 95%CI 2.0-37.3) and SN4 (OR 10.3, 95% CI 2.2-48).

The highest relative methylation tertile of six tumor suppressor genes promoter regions were associated with the increased prevalence of gastric cancer (Figure 1). Single investigated CpG of *APC* SN1 (OR 3.6, 95% CI 1.1-12.6), *DAPK1* (OR 3.9, 95% CI 1.1-13.9), *ACIN1* SN1 (OR 30.9, 95% CI 3.6-265.4), *BCL2* (OR 8.0, 95% CI 2.0-32.6), *CD44* (OR 10.9, 95% CI 2.3-51) and *TNFRSF10C* (OR 8.1, 95% CI 1.9-33.6) were associated with gastric cancer as well as the middle relative methylation tertile of *RARB* SN2 (OR 8.9, 95% CI 1.8-43). However, there were no associations between methylation levels of *CDKN2A, HS3ST2* and prevalence of gastric cancer. It should be noted that methylation levels of the latter two genes also were not significantly different, when comparing between patients and controls.

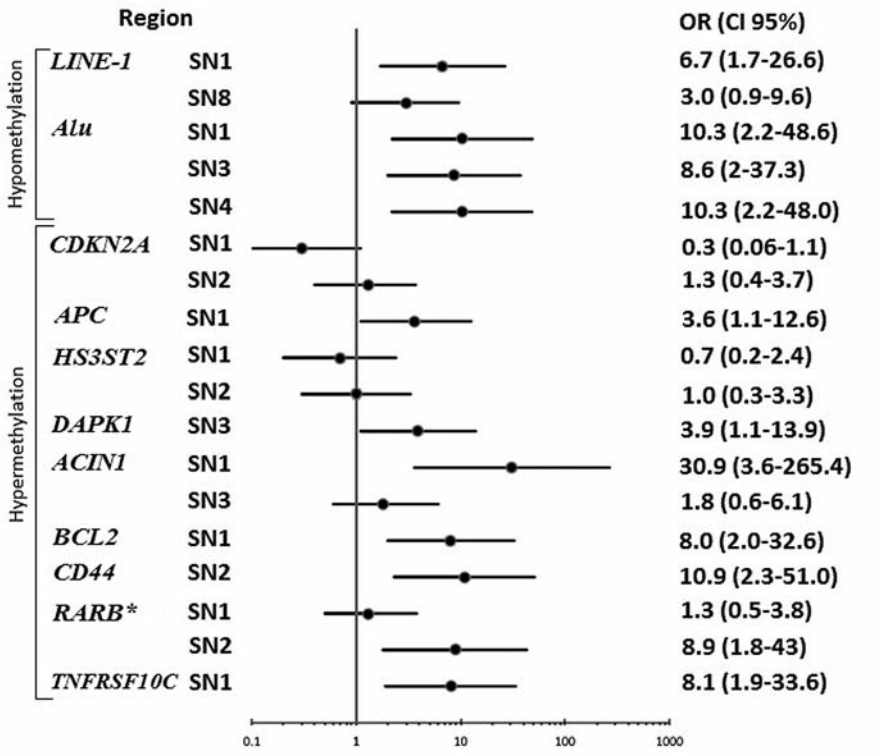

Figure 1. *The association of blood-derived DNA high methylation level at specific CpG sites and low methylation level at repetitive DNA elements with prevalence of gastric cancer. Dots indicate odds ratio (value) and whiskers 95% CI. Lowest tertile is set as reference (1.0) trend line for gene specific CpG sites. Highest tertile is set as reference trend line for repetitive DNA elements. Forrest plot presented in logarithmic scale. RARB*-middle tertile.*

The association of peripheral blood methylation with clinical and morphological features. Next, we searched for relationship between clinical and morphological features (Table I) and hypomethylation or hypermethylation of all studied loci. For this analysis, we grouped the highest and the middle tertiles for the repetitive elements as well as the lowest and the middle tertile for genes in the CpG island loci into one variable. High level of *DAPK1* SN3 methylation was significantly associated with higher cancer pT category (OR 21, 95%CI 1.9-227.2) and higher stage according to UICC (OR 9, 95% CI 1.3-63.9). High level of *RARB* SN2 methylation was significantly associated with cancer cell low-grade of differentiation (OR 0.03, 95% CI 0.02-0.34).

Hierarchical clustering and principle component analysis of peripheral blood samples. In total 24 and 49 blood samples corresponding to gastric tumor patients and healthy controls, respectively, were used for the hierarchical cluster analysis. When taking all the methylation data as input in an unsupervised hierarchical clustering we could distinguish one major cluster group containing 13 gastric patient blood samples (or about 54%) (Figure 2A and B); four others small clusters (of two to three) corresponding to the gastric patients could also be distinguished. However, differential methylation analysis to distinguish the blood of gastric patients from healthy controls (at *t*-test *p*-value of 1.5×10^{-5} and false discovery rate (FDR) of $<3.3\times10^{-5}$) could distinguish a major cluster containing 20 (83%) gastric patient blood samples and a two minor cluster of 2 samples each (Figure 2C and D). The PCA analysis could clearly distinguish both groups of healthy control blood and gastric cancer patients; however, the two groups appear continuous and close to each other and separated in the middle by an interface that contain mixed samples from both healthy and tumor groups that are quite close in space. Therefore, based only on 10 CpG sites, most of the samples could be efficiently clustered into two groups.

Discussion

In the present study, we examined peripheral blood DNA to determine whether changes in methylation at various CpG sites of tumor suppressor genes and repetitive elements are correlated with gastric cancer. We demonstrated highly

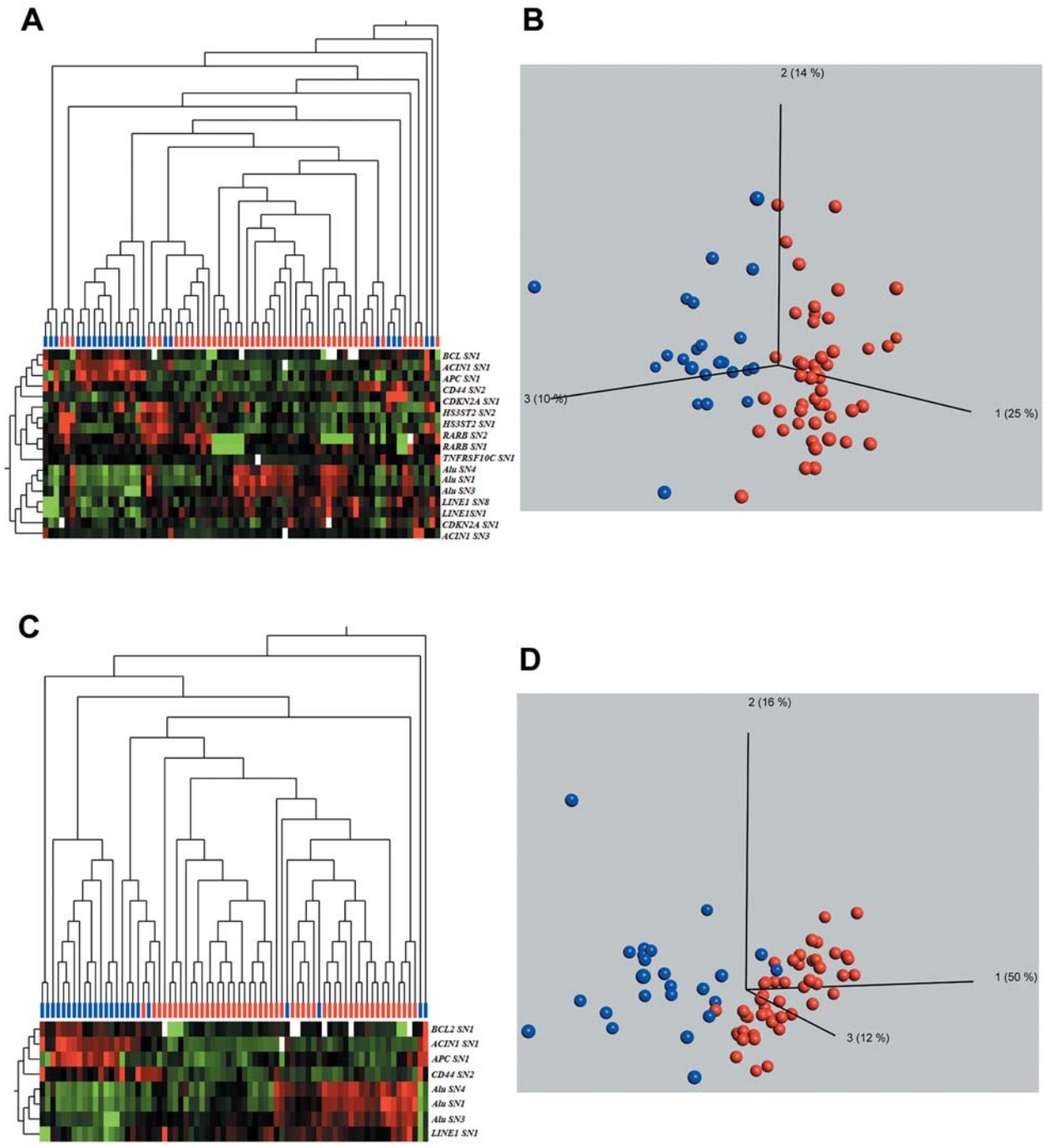

Figure 2. *Hierarchical clustering and PCA analysis: unsupervised hierarchical clustering (A) and PCA plot (B) of all CpG sites and all samples. Differential methylation analysis of blood samples of healthy and gastric sample patients at a t-test p-value of 1.5×10^{-5} and FDR of $<3.3\times10^{-5}$; C) hierarchical clustering of 10 CpG sites above the selected p-values; D) PCA plot showing clear separation.*

significant hypomethylation at repetitive DNA elements as well as significant hypermethylation at specific loci in leukocyte DNA of the patients with gastric cancer. Importantly, we found that methylation differences were significant across the clinical and morphological features of the disease, suggesting that the differentially-methylated CpG signatures may be useful for early diagnosis.

To date, only few cases–control studies have examined gastric cancer risk in relation to both *Alu* and *LINE-1* methylation. A Polish case–control study found gastric cancer

risk to be highest among those with lowest level of methylation in either Alu or *LINE-1* (10). The results from another study suggested that DNA methylation of *Alu*, but not *LINE-1*, in blood leukocytes might be inversely associated with risk of gastric cancer (9). In the present study, we found significant methylation differences between patients, suffering from gastric cancer and controls in all the 5 *LINE-1* and *Alu* studied CpGs. The association with gastric cancer prevalence was confirmed with all investigated *Alu* CpGs and with one *LINE-1* CpG. Taken together these three studies, the divergent associations with *Alu* and *LINE-1* raise the possibility that the roles of repetitive DNA methylation are different in gastric cancer pathogenesis. There were no statistical differences in the methylation levels by clinical and morphological features of gastric cancer. Therefore, our findings suggest that the loss of *LINE-1* and *Alu* methylation, in blood, may occur more significantly at the early onset of carcinogenesis and thus be rather the marker of tumor initiation than the progression of the disease. Another explanation, however, might be that the small number of cases in the subgroups did not allow us to detect more associations.

The few studies that exist on gene-specific methylation in leukocytes and cancer risks have mostly focused on selected genes for breast, colon and pancreatic cancer but they also support the potential for gene-specific methylation measured in leukocytes as a biomarker of risk (19, 21-24). Widschwendter *et al*. examined locus-specific methylation and found that particular DNA methylation patterns in peripheral blood may serve as surrogate markers for breast cancer (24). In a small cell lung cancer study, methylation profiling analysis in leukocyte DNA identified two CpG sites that discriminated cancer patients from control population (39). Pedersen *et al*. built a prediction model consisting of five CpG sites that discriminated pancreatic cancer patients from controls (23). Our previous study revealed that in the blood of patients with pancreatic ductal adenocarcinoma tumor suppressor gene sites were slightly but significantly higher methylated at several selected CpG sites (19). Also, there are published studies with contradictory results. The findings of the cross-sectional study by Cui *et al*. suggest that measurements of insulin-like growth factor-II (*IGF-II*) methylation status in lymphocytes could be used for the prediction of colorectal cancer risk (40). Controversially, another larger prospective study did not find any relationship between colon cancer risk and methylation levels at CpGs of *IGF-II* and thus does not support the hypothesis that colon cancer can be predicted from the different levels of methylation of the *IGF-II* gene from lymphocyte DNA (22).

However, available data are still very limited, and systematic or unbiased approach to the selection of genes has not been routinely employed (a large genome wide study is needed). We have selected genes of different metabolic pathways as they are considered potential tumor suppressor genes. Among these genes, at least five were reported to be associated with gastric cancer (29, 41-44). According to our results, methylation levels of various genes were slightly, but significantly higher in the blood of gastric cancer patients and associated with the presence of gastric cancer. High methylation levels of *DAPK1* and *RARB* in peripheral blood were found to be associated with a more advanced stage of the disease and with poor cancer cell differentiation grade, respectively. Interestingly, the results from studies on gastric cancer tissues are partly consistent with our findings from peripheral blood DNA. *DAPK1* promoter hypermethylation in cancer tissue was previously found to be associated with advanced stage of gastric cancer (41) and *RARB* promoter hypermethylation is a frequent finding in gastric cancerous tissue, but not in association with cancer cell differentiation grade (44-46).

It is important that future studies address the molecular mechanisms that lead to methylation changes in leukocytes DNA of cancer patients since it is not clear whether the observed methylation changes in leukocytes represent a cause or consequence of the disease. We also cannot completely rule-out the possibility that our observations may be biased by the impact of some other unknown to us concomitant chronic diseases in our patients. While this is an exciting area of investigation, clearly more input is needed to fully understand and appreciate the findings in leukocytes.

When interpreting our results, it is important to rule-out circulating tumor cell or free-cell DNA which could influence the methylation results, because both are increased in cancer patients (47). The peripheral blood samples used in this study were obtained after the diagnosis of gastric cancer before any specific treatment and before surgery. It is worth to note, that most cases were without distant metastases at the time of surgery. However, even in patients with metastatic gastric cancer, an average of 24 tumor cells out of 4.5 million white blood cells in 7.5 ml of peripheral blood are the circulating tumor cells (47). The possibility that the methylation differences we have detected in peripheral blood cell DNA might be influenced or originated from tumor cell DNA can be completely excluded. Moreover, the blood was centrifuged before DNA extraction, with only the isolated nuclei used in the DNA extraction. Therefore, the influence of free plasma DNA is also not relevant.

However, this study has certain limitations. First, we tested only one to two CpG sites per gene. The findings could indicate that not all CpG sites are equal in cancer risk prediction and that a larger number of CpGs that cover a more extensive area of the CpG island should be studied to provide higher predictive values. Second, we were unable to determine possible mechanisms influencing methylation differences between cases and controls, including genetics, nutrition, enviromental pollution and sub-population of leukocytes. Third, the clinical sample size is small. Larger studies are clearly

required to confirm the significance of aberrant methylation in peripheral blood leukocytes. Also, further investigations should be focused on the estimation whether the observed differences occur prior to, or as a result of, carcinogenesis and whether changes in leukocyte DNA methylation are associated with disease risk changes over the lifetime.

In conclusion, we showed that methylation levels and patterns could be used to distinguish most cases of gastric cancer from healthy controls. Although this pilot study is limited by both the number of patients and number of loci studied, it nevertheless shows that more studies should be undertaken in this direction. This could lead to more accurate and sensitive methods for non-invasive early detection of cancer.

Conflicts of Interest

The Authors declare that they have no competing interests

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