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Investigation of Tumor-Derived Extracellular DNA in Blood of Cancer Patients by Methylation-Specific PCR

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ABSTRACT

The frequency of APC, RASSF1A, RARβ, CDH1 and CDH13 gene promoter methylation in samples of DNA isolated from breast and lung patient plasma was studied in order to develop the noninvasive tumor-specific DNA detection method. Methylation of at least one of genes was detected in extracellular DNA from most of the cancer blood specimens. The results obtained indicate that promoter hypermethylation of a number of marker genes represents a promising serum marker for early breast and lung cancer detection.

Key Words: Gene methylation; Cancer; Blood; PCR analysis.

Lung and breast cancers are among the leading causes of cancer- related death in the world. Surgery is the most effective treatment for these cancers, but this modality is

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limited because most of patients have advanced stages of the disease at the time of diagnosis. Therefore, it is vitally important to identify and develop reliable diagnostic and prognostic assays for the noninvasive detection of early stage in lung and breast cancers. Recent reports show that tumor-specific DNA is often detectable in the plasma and serum of cancer patients.[1] The serum of cancer patients contains, on average, about 4–40 times more free DNA compared with normal individuals.[2,3] Aberrant promoter methylation is the important mechanism of tumor suppressor gene inactivation, which is frequently observed in human cancers and seems to play an important role in the pathogenesis of different tumor types. So far, several known and putative tumor suppressor genes have been identified as being involved in the pathogenesis of lung and breast cancer and are frequently inactivated by methylation. Detection of cancer-specific DNA alterations in plasma of patients using methylation-specific PCR[4] seems to hold significant promise for early detection of lung and breast cancers.

In order to develop the noninvasive tumor-specific DNA detection method, based on MSP, we studied the frequency of APC, RASSF1A, RARβ, CDH1 and CDH13 gene promoter methylation in patient plasma. The sensitivity of the method under development should be high, as far as level of extracellular DNA is low in plasma from the most of lung cancer patients and many of breast cancer patients. According to our data, lung cancer patients are characterized with moderate plasma DNA concentration increase, ranging from 35 to 173 ng/ml, and breast patients, ranging from 10 to 1085 ng/ml.[5] To note, the proportion of tumor-derived DNA was shown to differ in plasma of cancer patients, varying from 90% to less than 10% of the total plasma DNA.[1] To define the sensitivity of MSP for methylated alleles, mixed DNA was used as a template for amplification, containing various amounts of methylated genomic DNA, added to fixed amounts of unmethylated DNA from normal leucocytes. Our data indicate, that no less than 0,1% of methylated DNA could be consistently detected, which was present in an otherwise unmethylated sample (data are not shown).

Thus, for samples with nanogram quantities of plasma DNA fixed amount of normal leucocyte DNA was added as a carrier before bisulfite modification. Bisulfite treatment of DNA was made according to the protocol by Herman et al[4] with modifications, using adapted guanidine thiocyanate/ activated glass-milk protocol[6] for DNA purification. The final modified DNA recovery was shown not to be less than 60% of the initial DNA amount.

Aberrant methylation of APC, RASSF1A, RARβ, CDH1 and CDH13 genes was estimated by MSP in plasma of breast cancer patients. Patients with the benign breast tumors (fibroadenoma and cystepythelioma) were also studied. Primer sequences of genes for both the methylated and unmethylated forms and MSP conditions were selected according to the references: APC 1A promoter,[7] RASSF1A,[8] RARβ,[9] CDH1[10] and CDH13.[4] The representative examples of MSP amplification products are shown at the Fig. 1.

Unmethylated products result from the amplification of plasma nontumor DNA and from normal leucocyte DNA added as a carrier. According to our data, most of breast cancer patients were characterized by at least one methylated DNA marker in plasma. The highest frequency of plasma DNA methylation was found for RASSF1A, which was detected in 47% of breast cancer patients (Table 1). High frequencies of

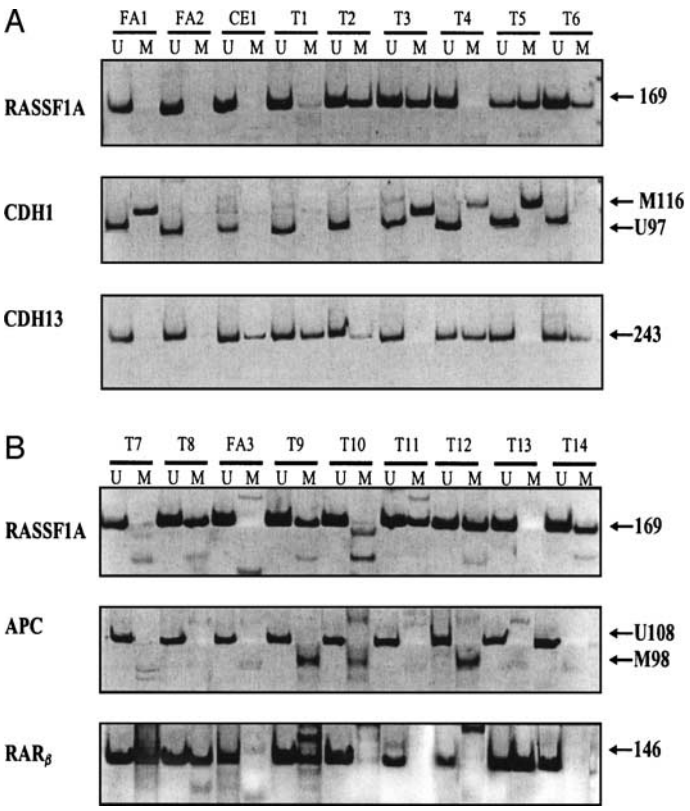

Figure 1. MSP analysis of plasma DNA from patients with breast tumors (T1–T14), breast fibroadenoma (FA1–FA3) and cystoepythelioma (CE1). PCR products are present in all of the *Unmethylated* (U) lanes, resulting from the amplification of plasma non-tumor DNA and from normal leucocyte DNA, which was used as carrier. (A) Results of testing for the methylated forms of *RASSF1A, CDH13* and *CDH1*. The presence of PCR product in *Methylated* lanes (M) of plasma DNA indicates the presence of methylated *RASSF1A* alleles in tumor patients (T2, T3, T5, T6) plasma only and presence of methylated *CDH13* and *CDH1* alleles both in tumor and nonmalignant patients (FA1, CE1). (B) Results of testing for the methylated forms of *RASSF1A, RARβ* and *APC*. Methylated-specific DNA markers are present in cancer patients (T7–T14) plasma only.

methylated form were found also for CDH1 and CDH13 genes. However, methylated forms of cadherin genes have been found also in the benign tumors (Fig. 1A). In contrast, neither of patients with benign tumors has shown methylation of APC, RASSF1A and RARβ genes in plasma DNA (Fig. 1B). MSP analysis of APC, RARβ and RASSF1A gene methylation in patient plasma was shown to provide high efficiency of noninvasive breast cancer diagnostics.

The frequency of APC and RASSF1A gene methylation was analysed in plasma DNA of lung cancer patients. APC and RASSF1A gene promoter methylation has been detected in plasma DNA of 44% and 30% of cancer patients, being practically undetected in plasma of patients with nonmalignant diseases (Table 1). MSP analysis of

Table 1. Gene promoter methylation in patients with breast and lung cancer and non-malignant diseases.[a]

	RASSF1A/total[b] (% positive)	RARβ/total (% positive)	APC/total (% positive)	CDH1/total (% positive)	CDH13/total (% positive)
Breast cancer	8/17 (47%)	5/15 (33%)	4/10 (40%)	4/9 (44%)	3/8 (38%)
Breast nonmalignant[c]	0/6 (0%)	0/6 (0%)	0/6 (0%)	1/6 (17%)	1/6 (17%)
Lung cancer	4/9 (44%)	ND	3/9 (30%)	ND	ND
Lung nonmalignant[d]	0/16 (0%)	ND	0/16 (0%)	ND	ND

[a]Blood samples of breast cancer and nonmalignant patients were obtained from National Novosibirsk Regional Oncology Dispensary. Blood samples of lung cancer and nonmalignant patients were obtained from Tomsk Regional Oncology Dispensary. Blood (2,4 ml) was collected into the tubes with PBS, 10 mM EDTA (0,6 ml) with the following fractionation into plasma and cellular frations.

[b]Detected genes/total samples (% positive).

[c]6 patients: 4 cases with fibroadenoma and 2 cases with cystoepythelioma.

[d]16 patients: 6 cases with pneumonia and 10 cases Chronic Lung Disease, nonmalignant occupational disease, acquired by the workers of Tomsk Plutonium Manufactory.

APC and RASSF1A gene methylation in patient plasma was shown to be promising for the accurate lung cancer detection.

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