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Early diagnostic of lung cancer based on methylation of mononuclear cell fraction: Method development

Relevance: *According to the International Agency for Research on Cancer (IARC), lung cancer (LC) today ranks first in cancer incidence worldwide [1]. In the Republic of Kazakhstan, about 3800 new cases of LC and more than 2000 deaths from LC are registered each year (one-year mortality exceeds 49.4%) [2]. This supports the relevance of early LC diagnostics. The study of DNA methylation in human peripheral blood mononuclear cells (PBMC) suggests its use as an early diagnostic and prognostic marker for LC before detecting a malignant neoplasm by visual diagnostic methods.*

The purpose of the study *was to find specific diagnostic and prognostic markers by DNA methylation profiling of PBMC in patients with LC.*

Results: *Methylation markers of blood mononuclear fraction were detected in CG islets associated with genes ICAM5, mir138, SYNE1, and KLK4 in 97% of plasma samples from patients with LC and were absent in healthy people. The usability of these markers to differentiate LC from 16 other cancers using NCBI GEO and TCGA methylation data was demonstrated with a specificity level of 0.96 and a sensitivity of 0.84.*

Conclusion: *The specificity and sensitivity of the method of LC early diagnostics and prognosis based on the methylation of blood mononuclear cells (detection of methylation of CG islets associated with the ICAM5, mir138, SYNE1, and KLK4 genes in PBMC) are enough to use it in screening for LC.*

Keywords: *lung cancer, epigenetics, DNA methylation, blood mononuclear cells, early diagnostics, biomarker.*

Introduction. According to the International Agency on Research for Cancer (IARC), as of today, lung cancer (LC) ranks first in incidence among other cancers worldwide. In 2018, 2 094 million new LC cases were registered, accounting for 11.6% of all new cancer cases, and 1.8 million deaths from LC were reported (18.4% of all deaths from cancer) [1]. The 5-year survival rate was 23% with non-small cell lung cancer (NSCLC) and only 6% – with small cell lung cancer [3].

The LC statistical data analysis of the Republic of Kazakhstan revealed the patterns of morbidity and mortality depending on the gender and age of patients [2]. In Kazakhstan, the LC ranks second (11.6%) in cancer incidence in both sexes. It was first in male cancer incidence (20.8%) and 7th in female cancer incidence (4.3%) [4].

In Kazakhstan, about 3 800 patients are diagnosed with LC every year; of these, over 2 000 (more than 49.4%) die within one year. Noteworthy is the following statistics provided by Kaidarova D.R. et al. [4]: out of all 3548 patients with LC registered in 2012 by the National Cancer Registry, only 246 patients were still alive by 2018. In other words, the 5-year survival rate was only 6.9%. The main reason for high mortality from LC was its late detection. Notwithstanding the high awareness about LC, in 2018, most patients (72.5%) had stage III-IV disease when the treatment was started. This evidences the urgency of the problem of early diagnostics of primary LC and its relapses.

Although the genetic changes are responsible for most body processes, it is becoming increasingly apparent that occasional gene expression changes are due to epigenetic transformations. Higher epigenome velocity is manifested in the methylation of cytosine residues in DNA CG-dinucleotides. It is assumed that the immunological changes in the body resulting from MN development lead to epigenetic DNA alterations in peripheral blood cells [5, 6]. The available results of the study on DNA methylation in peripheral blood mononuclear cells make it possible to attribute them as a diagnostic and a prognostic LC marker within the frames of minimally invasive diagnostics [7-10].

The findings of the number of studies evidence that DNA methylation of peripheral blood mononuclear cells has correlation differences between healthy people and individuals with various malignant neoplasms [8, 9]. In a study with 100 patients with LC involvement, the scientists found a significant correlation between p53 methylation and LC's development in the body. In another study with DNA methylation in T-lymphocytes in the course of LC, within the frames of a "case-control" study, the authors assumed that DNA methylation changes in peripheral blood reflect the changes in the lung itself [11- 13].

The prerequisite for that study was the necessity to determine the LC diagnostic tumor biomarkers before its detection by visual diagnostic methods. At the beginning of the last century, the Nobel Prize winner Paul Ehrlich, one of the immunology founders, suggested that the body's immune system plays an important role in carcinogenesis. It is known that in the case of colorectal cancer, melanoma, esophageal and ovarian can-

cer, the presence of CD8+T-cells correlates with a better prognosis. The DNA methylation profile and tumor transcriptomes reflect the specific immune script depending on the tumor subtype [13, 14]. However, the tumor location-related specificity of peripheral blood immune cells has never been discussed. Since the immune system plays a key role in tumor growth control, the differences in tumor progression and response to treatment are associated with the differences in the molecular programming of immune cells. In particular, certain peculiarities of peripheral blood immune cells' DNA methylation profiling registered in LC could serve as the earliest biomarkers of the disease.

It is assumed that the immunological changes occurred in the body in cancer lead to epigenetic DNA alterations in peripheral blood mononuclear cells [5, 6]. The progressive accumulation of genetic and epigenetic changes, including the point mutations and recombination of chromosome regions, is one of the known reasons for LC development. The findings of studies related to DNA methylation in peripheral blood mononuclear cells make it possible to determine its role as a predictive marker [7].

The study of Dr.Ponamareva showed that the patients with LC had a significantly higher level of RARB2 gene methylation in the DNA of circulating tumor blood cells versus healthy donors ($p<0.05$). The RARB2 gene methylation level in a series of healthy donors was determined in the amount of 1057 ± 211 copies/ml, with elevation in patients with chronic obstructive pulmonary disease up to 4853 ± 606 copies/ml and up to 7524 ± 939 copies/ml in patients with LC. The methylation index of other genes, such as RASSF1A, in DNA of circulating tumor blood cells, also has been significantly elevated in patients with LC versus the patients with chronic obstructive pulmonary disease and healthy donors ($p<0.05$). Simultaneously, in patients with LC of the IIIrd stage, the level of RARB2 gene methylation was statistically significantly higher than in patients of stage I-II ($p<0.05$). It is also of interest the facts witnessing that after the tumor resection, that indicator significantly declined statistically versus the indicator before the treatment or elevated in case of progression or recurrence of the disease, which was directly linked with identification of the disease development clinical signs [15]. Budak et al. concluded that the methylated PTPRF, HOXD3, HOXD13, and CACNA1A genes could be the potential biomarkers for lung adenocarcinoma diagnosis and treatment [11]. In that situation, the epigenetic analysis is the only preferred method to diagnose the potential cell pathology. The important difference between the genetic and epigenetic changes is that the epigenetic changes could be corrected by treatment with the help of drugs that are completely powerless in genetic mutations.

There is a known method for LC detection with the application of the LC specific biomarker, which can detect the methylation of PCDHGA12 gene in the 5'UTR region or exon 1 in cells of LC patients, as well as its use as a biomarker for identification of the disease progression stage. This diagnostic kit allows a more accurate and fast early detection of LC compared to conventional methods. The kit is also used for LC prognosis, monitoring, and staging [12]. However, this and other similar studies intend to increase the sensitivity and specificity of LC early diagnostics. The timely detection of the disease at an early stage is the main organizational medical measure of practical public health, which will improve patients' survival rates [4].

Many authors have established that epigenetic methods have enormous diagnostic and therapeutic capacity. The epigenetic method belongs to minimally invasive methods since only a sample of the patient's blood plasma is taken for testing [16-19]. The aspects of minimally invasive diagnostics for LC by DNA methylation profiling in peripheral blood mononuclear cells stand high on the agenda on national and international levels.

The purpose of the study was to find specific diagnostic and prognostic markers by DNA methylation profiling of PBMC in patients with LC.

Materials and Methods: The search for LC markers based on methylation of peripheral blood mononuclear cells was carried out among 50 patients with NSCLC, who underwent examination and specialized antitumor treatment in the facilities of Kazakh Research Institute of Oncology and Radiology JSC, Almaty, Kazakhstan. The study was carried out in accordance with the STP Protocol No.AP05131940 "Possibilities for identification of T-lymphocyte markers in early diagnostics and prognosis of LC and breast cancer" within the framework of the budget program 217 "Science development," subprogram 102 "Grant funding of scientific studies"; treatment - according to international protocols of NSCLC treatment. The diagnosis was histologically verified in all studied patients.

Withdrawal criteria: any identified inflammatory disease (bacterial or viral infection, diabetes, asthma, autoimmune disease, exacerbation of thyroid disease, which may change the characteristics of immunity indicators).

The control group included 40 healthy volunteers from Kazakhstan. The methylation data from the NCBI GEO and TCGA databases for LC and 16 other types of cancer were used for comparison analysis. The data of studied patients with LC divided by gender, age peculiarities, and spread of the tumor process are reflected in Table 1.

The first blood sampling was carried out during the initial diagnostics; the second blood sampling was performed after the antitumor treatment. 15.0 ml of blood were collected using a vacuum blood collection system and sterile VenoSafe EDTA tubes. Before molecular genetic analysis, the peripheral blood samples were stored in a freezer at -20°C. First, lymphocytes and whole blood plasma were isolated and cryopreserved using ficoll.

The upper plasma layer, which does not contain cells, was used to isolate the circulating DNA, and the lymphocyte layer was used to isolate the genomic DNA. The plasma DNA was extracted with the application of an internal "cell-free protocol" of DNA extraction (HKG Epitherapeutics, Hong Kong). The extracted DNA was converted to bisulfite with the use of the EZ DNA Methylation-Direct kit (Zymo Research, Calif., USA) and stored at -20°C until the start of further processing. The amount of isolated double-stranded nuclear DNA had to be at least 20 ng.

Table 1 - Patients data including gender, age, and tumor staging

No.	Patient ID	Age, years	Ethnicity	Sex	Smoker	TNM staging	Stage	Histological verification
1	el1	61	Kazakh	male	yes	T3N0M0	II	adenocarcinoma
2	el2	70	Kazakh	female	no	T2aN0Mo.	IB	adenocarcinoma
3	el3	71	Kazakh	male	yes	T3NxM0.	III	adenocarcinoma
4	el4	71	Russian	male	yes	T2bN0M0	II	adenocarcinoma
5	el5	64	Kazakh	female	no	T3N2M0	III	adenocarcinoma
6	el6	61	Kazakh	female	no	T4NxM1a	IV	adenocarcinoma
7	el7	29	Kazakh	male	yes	T3N0M0	IIB.	sarcoma
8	el8	65	Kazakh	male	yes	T3N1M0	III	squamous
9	el9	56	Russian	female	no	T3N0M0	II	squamous
10	el10	65	Russian	male	yes	T3N1M0	III	squamous
11	el11	56	Kazakh	male	yes	T2N0M0	II	squamous
12	el12	46	Kazakh	male	no	T3N2M1	IV	squamous
13	el13	61	Kazakh	female	no	T3N2M1	IV	adenocarcinoma
14	el14	63	Kazakh	male	yes	T4N2M1	IV	small cell
15	el15	58	Kazakh	male	yes	T2N0M0	II	squamous
16	el16	55	Korean	male	yes	T4NxM1	IV	adenocarcinoma
17	el17	58	Kazakh	male	yes	T4NxM0	III	small cell
18	el18	54	Russian	male	yes	T3N2M0	III	adenocarcinoma
19	el19	64	Russian	male	yes	T4N0M0	III	adenocarcinoma
20	el20	51	Kazakh	female	no	T3N1M0	III	squamous
21	el21	49	Kazakh	male	yes	T3N2M0	III	squamous
22	el22	43	Russian	male	yes	T4N1M1	IV	adenocarcinoma
23	el23	47	Kazakh	female	no	T2bN2M0.	III	adenocarcinoma
24	el24	71	Kazakh	male	yes	T3N2M0.	III	squamous
25	el25	43	Kazakh	female	no	T4N2M0.	III	adenocarcinoma
26	el26	36	Russian	male	no	T2bN2M0.	III	squamous
27	el27	64	Kazakh	male	no	T2N2M0.	III	adenocarcinoma
28	el28	39	Korean	female	no	T2N0M0.	II	adenocarcinoma
29	el29	47	Azerbaijani	female	no	T2N0M0.	II	squamous
30	el30	34	Azerbaijani	male	yes	T4NxM1.	IV	squamous
31	el31	30	Kazakh	female	no	T2N1M0.	III	squamous
32	el32	42	Azerbaijani	male	yes	T3N0Mo	III	adenocarcinoma
33	el33	63	Kazakh	male	yes	T2N0Mo	II	adenocarcinoma
34	el34	60	Russian	male	yes	T3NxMo	III	squamous
35	el35	45	Kazakh	male	yes	T3N1Mo	III	adenocarcinoma
36	el36	47	Kazakh	male	yes	T3NxMo	III	squamous
37	el37	45	Kazakh	female	no	T2NxMo	II	squamous
38	el38	48	Kazakh	male	yes	T2NxMo	II	squamous
39	el39	55	Kazakh	male	yes	T3N2M1	IV	small cell
40	el40	80	Kazakh	male	no	T2NxM0	II	squamous
41	el41	39	Ukrainian	female	no	T2N0M0	II	squamous
42	el42	47	Kazakh	male	no	T2N1M0	II	adenocarcinoma
43	el43	33	Kazakh	male	no	T3N0M0	II	squamous
44	el44	71	Russian	female	yes	T4N2M0	III	squamous
45	el45	54	Kazakh	male	yes	T2N1M0	II	adenocarcinoma
46	el46	43	Kazakh	male	no	T3N0M0	II	adenocarcinoma
47	el47	41	Kazakh	female	no	T4N1M0	III	adenocarcinoma
48	el48	53	Russian	male	no	T3N1M0	III	squamous
49	el49	47	Kazakh	male	no	T2N1M0	II	adenocarcinoma
50	el50	51	Kazakh	male	no	T2aN0M0	IB	adenocarcinoma

Sequencing and quantitative polymerase chain reaction (qPCR, PCR). The sequencing was performed by DNA methylation using the Infinium Methylation Epic bead array on a new generation Illumina MiSeq sequencer (Illumina, California, USA). For multiplex sequencing, two PCR cycles were performed. The first step was to examine four genes (ICAM5, mir138, SYNE1, and KLK4) in the genome containing CG, the methylation of which we found in all LC cells. The sequence, included in the primers for that amplification step, corresponded to the bisulfite-converted version of the target sequence that served as an anchor for the second PCR. During the second PCR, a primer for sequencing was added, which determined an index sequence that served as a unique identifier for each patient. The first cycle of PCR amplification was carried out in reaction mixtures containing 2 μl of bisulfite-converted DNA and Taq DNA polymerase (Thermo Scientific, CA, USA), with the use of reverse and direct primers: HKGepilung1 - for mir138, HKGepilung2 - for ICAM5, HKGepiLung3 - for SYNE with use of the standard amplification. The second PCR cycle was performed to label each amplicon by a specific barcode using several direct and reverse HKG barcode sets. The results of the second PCR were used as quality control.

The library containing the same dose of received amplicons was pooled according to their dsDNA concentrations determined with the use of the Qubit ™ HS dsDNA assay kit (Invitrogen, CA, USA), then purified twice with the use of the purification beads, and identified the amount with the use of the RealTime PCR (NEBNext® Library Quantification Kit for Illumina, New England Biolabs, MA, USA). Then, the sequencing was

performed on the Illumina platform using the MiSeq Reagent V2 Micro (Illumina, CA, USA). After FastQ files' sequencing, the methylation level was determined for each patient and each gene site.

The methylation was conducted in cooperation with the laboratory of HKG Epitherapeutics Limited (Hong Kong Science Park Shatin, Hong Kong), the bioinformatic analysis - in Professor Moshe Szyf laboratory (Canada) using the ChAMP package in R.

Integrated methylation analysis. For pairwise comparisons between groups, the Student's t-test was used for each biomarker. Freely available DNA methylation databases (Illumina 450K) were used for comparison purposes and to verify the sensitivity and specificity of the studied set of DNA methylation markers for LC: TCGA (cancer genome atlas), GSE40279, GSE61496, GSE76269, and GSE66836. In order to determine the functionally significant differences in DNA methylation in LC, the correlation analysis was performed with the determination of the direct and inverse relationship between the differences in methylation of the promoter and enhancer.

Compliance with ethical principles during the implementation of the project. The study was carried out in accordance with the Law of the Republic of Kazakhstan, "On health protection of citizens in the Republic of Kazakhstan"; "Patent Law of the Republic of Kazakhstan," Law "On Copyright and Related Rights." The study protocol was approved by the institutional ethical committee of the Kazakh Research Institute of Oncology and Radiology (protocol of the IEC meeting No. 13/17 of 09/28/2017). Informed consent was obtained from each patient for inclusion in the study.

In 2019, the authors filed a patent application. The authors published the preliminary study findings in relevant abstracts of the XI Congress of oncologists and radiologists of the CIS and Eurasia [17].

Results and discussion: During the search of markers for detection of LC, we used the previously discovered facts that the CG islets associated with ICAM5, mir138, SYNE1, and KLK4 genes, methylated in tumor samples of LC patients, which is not typical for other tumors and blood samples of healthy individuals (Fig. 1).

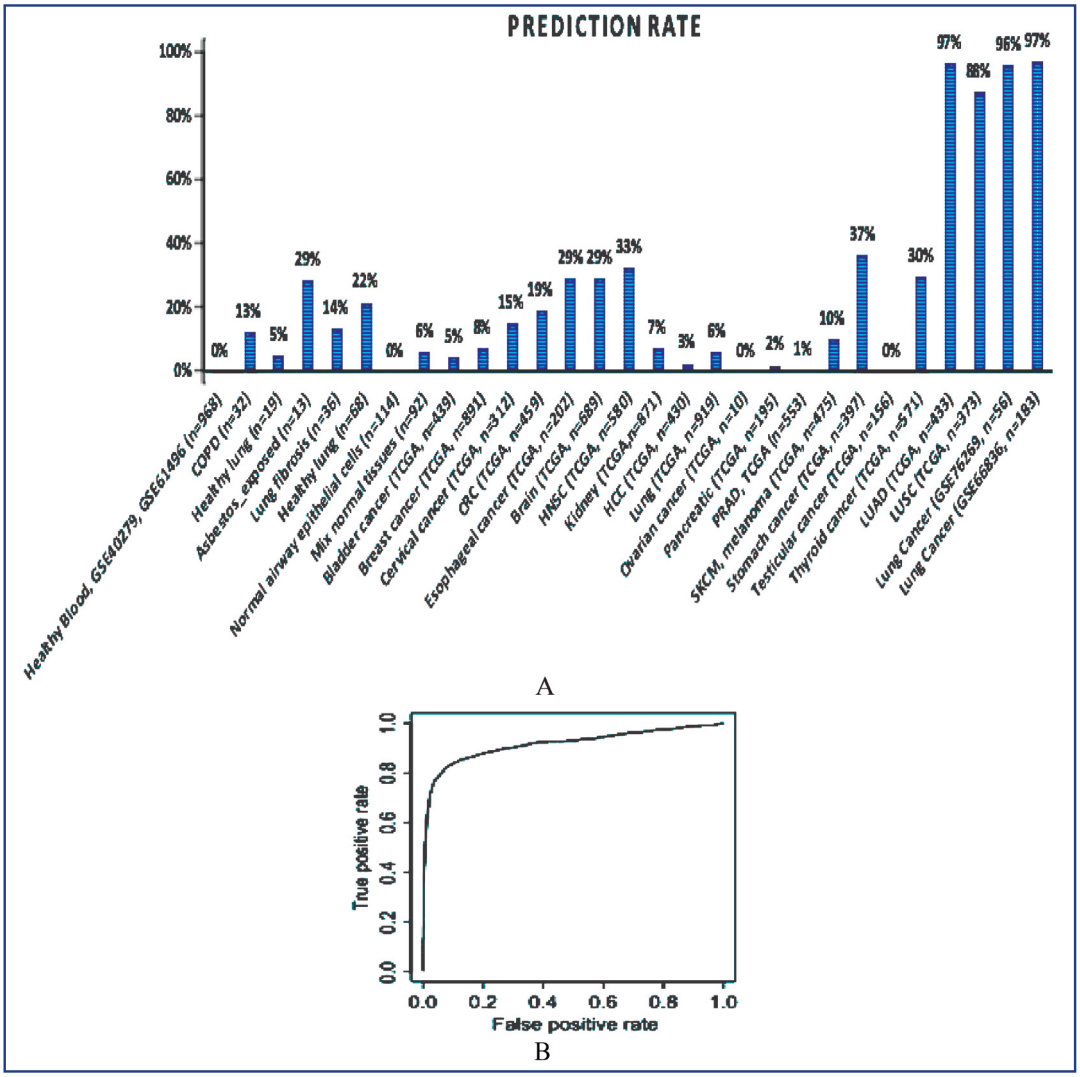

Figure 1 – LC methylation sensitivity and specificity:
A – Prognostic specificity for tumors of different localization (in %),
B – ROC-curve of prognostic specificity and sensitivity (direct and inverse correlation between indicators)

The analysis was based on a weighted estimate of the LC DNA methylation-based on the a.m. genes and the threshold value detected in the LC diagnostics test in the "verification cohort." The "verification cohort" included the normalized DNA beta methylation values for Illumina 450K from GSE66836, GSE63704, GSE76269, and 919 LC patients from TCGA. With that weighted indicator, 88–97% of LC samples were identified as LC (Figure 1A).

We have demonstrated the possibility of using these markers to differentiate LC from 16 other cancers by methylation data from the NCBI GEO and TCGA databases (Figure 1A). The ROC-curve presented in Figure 1B shows that methylation indication offers the specificity of 0.96 and the sensitivity of 0.84 when differentiating LC from other types of cancers or healthy tissues.

The analysis of 50 blood plasma samples from patients with LC was carried out in an independent tissue cohort to answer the question, "Is it possible to use the new LC methylation markers to detect LC in the blood plasma of patients" (Table 1). As a control, we used a pool of plasma DNA from 40 healthy volunteers of corresponding sex and age.

By histological type, the group of LC (50 patients) included patients diagnosed with: adenocarcinoma – 24 (48%), squamous cell carcinoma – 22 (44%), small cell lung carcinoids – 3 (6%), lung sarcoma – 1 (2%). By tumor localization, the patients were divided as follows: central cancer – 21 (42%), peripheral cancer – 29 (58%). By the TNM stage, the patients were divided as follows: stage I – 2 (4%), stage II – 18 (36%), stage III – 22 (44%), and stage IV – 8 (16%). The age of patients varied from 29 to 80 years. 35 were men, 15 – women. 25 men and one woman were smokers (Tables 1, 2, 3).

Table 2 – LC patients' distribution by gender

Disease stage	Total no. of patients	Distribution by gender	
		Men	Women
I	2	1	1
II	18	13	5
III	22	16	7
IV	8	6	2
Total	50	35 (70%)	15 (30%)

Table 3 – LC patients' distribution by age

Disease stage	No. of patients	Age, years			
		below 40	40–49	50–59	over 60
I	2	-	-	1	1
II	18	4	6	4	4
III	22	2	7	4	9
IV	8	1	2	2	3
Total	50	7	15	11	17

DNA methylation plasma profiling was first made for 40 healthy individuals. A deviation from the healthy DNA methylation profile was assessed as cancer. The heatmap of those profiles for each patient and healthy controls is provided in Figure 2.

The error curve, or ROC-curve, obtained from the studied groups' plasma methylation data (shown in Figure 3), graphically characterizes the compared parameters of methylation profiles in the groups of LC and healthy individuals. The method specificity was equal to 1, the sensitivity – to 0.938.

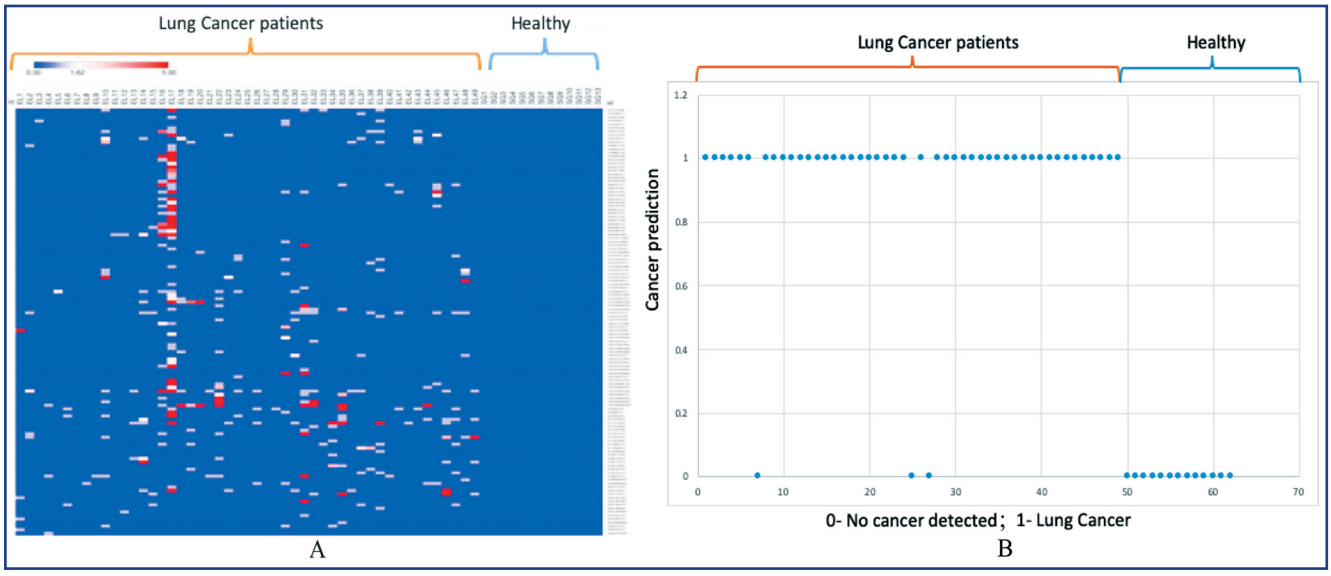

Figure 2 – Heatmap of DNA methylation plasma profiles of LC patients and healthy individuals

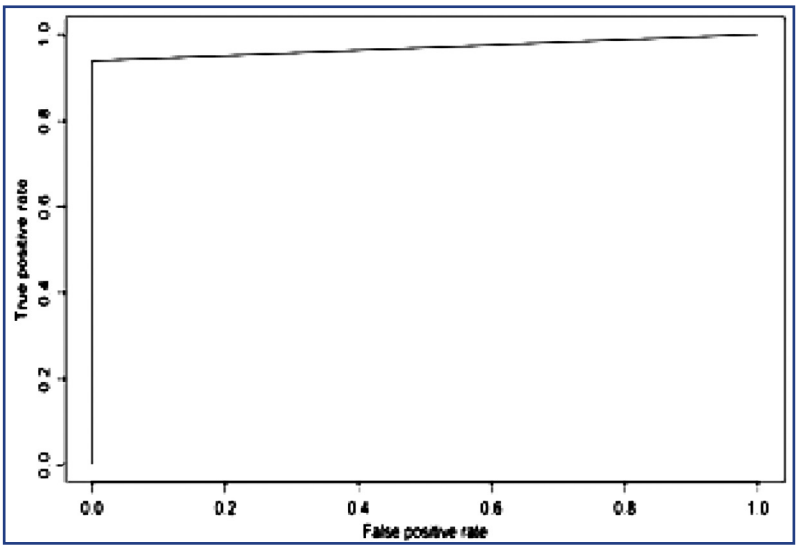

Figure 3 – ROC-curve of the method specificity and sensitivity in LC diagnostics

Conclusion: We established the CG combinations (associated with the ICAM5, mir138, SYNE1, and KLK4 genes) found both in tumor DNA and plasma of LC patients. These combinations were differentiated from the health individuals' circulating free plasma DNA. A specific tumor methylation profile against a huge amount of blood or other normal tissue DNA of a patient demonstrates the potential of DNA plasma methylation markers for non-invasive early LC detection.

This study's uniqueness is that it affords ground for further deeper study of epigenetic biomarkers as a prognostic factor for LC diagnostics and subsequent relapse control. The study results will help to determine the dynamics of DNA methylation profile in peripheral blood mononuclear cells after a specialized antitumor treatment. This is needed to verify the test prognostic value and reveal possible correlations between the biomarkers' expression and the clinical course. Further research with a larger amount of healthy and cancerous samples and a prospective clinical study are required to confirm these findings and prove the validity of conclusions made for further clinical application.

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