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Dynamics of LINE-1 Retrotransposon Methylation Levels in Circulating DNA from Lung Cancer Patients Undergoing Antitumor Therapy

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Abstract—Malignant cell transformation is accompanied with abnormal DNA methylation, such as the hypermethylation of certain gene promoters and hypomethylation of retrotransposons. In particular, the hypomethylation of the human-specific family of LINE-1 retrotransposons was observed in lung cancer tissues. It is also known that the circulating DNA (cirDNA) of blood plasma and cell-surface-bound circulating DNA (csb-cirDNA) of cancer patients accumulate tumor-specific aberrantly methylated DNA fragments, which are currently considered to be valuable cancer markers. This work compares LINE-1 retrotransposon methylation patterns in cirDNA of 16 lung cancer patients before and after treatment. CirDNA was isolated from blood plasma, and csb-cirDNA fractions were obtained by successive elution with EDTA-containing phosphate buffered saline and trypsin. Concentrations of methylated LINE-1 region 1 copies (LINE-1-met) were assayed by real-time methylation-specific PCR. LINE-1 methylation levels were normalized to the concentration of LINE-1 region 2, which was independent of the methylation status (LINE-1-Ind). The concentrations of LINE-1-met and LINE-1-Ind in csb-cirDNA of lung cancer patients exhibited correlations before treatment ($r = 0.54$), after chemotherapy ($r = 0.72$), and after surgery ($r = 0.83$) ($P < 0.05$, Spearman rank test). In the total group of patients, the level of LINE-1 methylation (determined as the LINE-1-met/LINE-1-Ind ratio) was shown to increase significantly during the follow-up after chemotherapy ($P < 0.05$, paired t test) and after surgery compared to the level of methylation before treatment ($P < 0.05$, paired t test). The revealed association between the level of LINE-1 methylation and the effect of antitumor therapy was more pronounced in squamous cell lung cancer than in adenocarcinoma ($P < 0.05$ and $P > 0.05$, respectively). These results suggest a need for the further investigation of dynamic changes in levels of LINE-1 methylation depending on the antitumor therapy.

Keywords: circulating DNA, aberrant methylation, LINE-1 retrotransposons, antitumor therapy, prognosis, lung cancer

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INTRODUCTION

Abnormal DNA methylation is a frequent early event in malignant transformation [1–3]. The main features of aberrant methylation include the hypermethylation of tumor suppressor gene promoters and the hypomethylation of a significant portion of total DNA, which is associated with the oncogene and retrotransposon activation and leads to genomic instability [1, 4]. In patients with malignant tumors, circulating DNA (cirDNA) of blood plasma and cell-surface bound cirDNA (scb-cirDNA) accumulate fragments of tumor-specific abnormally methylated DNA, which can be considered to be potential tumor markers [5–7]. So far, hypomethylated tumor suppressor

Abbreviations: AC, adenocarcinoma; cirDNA, circulating DNA; csb-cirDNA, cell surface-bound circulating DNA; LC, lung cancer; NSCLC, non-small cell lung cancer; SCLC, squamous cell lung cancer; GE, genome equivalent.

Table 1. Clinico-morphological characterization of patients with NSCLC ($n = 16$)

Age, $n = 16$		Stage, $n = 16$	
≤60 years	6 (37%)	I-II	0 (0%)
>60 years	10 (63%)	III	16 (100%)
Sex		Smoking, $n = 16$	
Male	12 (75%)	Yes	12 (75%)
Female	4 (25%)	No	4 (25%)
Histological type, $n = 16$		Effect of chemotherapy, $n = 16$	
SCLC	10 (63%)	Partial regression	12 (75%)
AC	6 (37%)	Stabilization	4 (25%)
Prognosis, $n = 16$			
Favorable*		10 (63%)	
Unfavorable**		6 (37%)	

n, total number of patients; SCLC, squamous cell lung cancer; AC, adenocarcinoma; * no signs of tumor progression (relapse, distant metastases); ** tumor progression.

genes have been the principal targets of blood cirDNA analysis. However, since they are usually single-copy genes, it is a challenging task to detect their methylated alleles in cirDNA, which is present in the bloodstream in low amounts of several dozen nanograms on average [5, 6]. Therefore, it may be promising to investigate the methylation status of mobile genomic elements represented in the human genome by much higher copy numbers (from several to hundred thousand copies) and amount to nearly half of the total genome length [8].

LINE-1 is the largest class of autonomous retrotransposons that constitute up to 20% of mammalian genomes [9]. Under normal conditions, retroelements are inactive due to the high level of CpG dinucleotide methylation, but can acquire oncogenic potential if this level decreases, i.e, in the case of hypomethylation [8]. An analysis of DNA methylation patterns in tumor tissues showed that the development and progression in lung cancer (LC) tumors was accompanied with significant changes in the levels of LINE-1 retrotransposon methylation [10, 11]. LINE-1 hypomethylation was also observed in plasma cirDNA of patients with melanoma and hepatocellular carcinoma [12, 13]. Our previous work showed that the level of methylated LINE-1 fragments in blood cirDNA of patients with LC was lower than in healthy subjects [14]. However, there is currently no information concerning the changes in retrotransposon methylation in blood cirDNA in response to antitumor therapy. Therefore, it appears worthwhile to analyze the levels of LINE-1 methylation in blood cirDNA of cancer patients before and after treatment.

The aim of the present study was to determine the levels of methylation of LINE-1 retrotransposons in blood cirDNA of LC patients before treatment and at several subsequent stages of antitumor therapy.

EXPERIMENTAL

Subjects. The study involved 16 patients with non-small-cell lung cancer (NSCLC; $T_{1-3}N_{0-3}M_0$) aged 48–65 years who were undergoing treatment in the Tomsk Institute of Cancer Research (Table 1). The diagnosis was verified morphologically. All patients received a combination treatment including two courses of neoadjuvant chemotherapy according to the following protocol: day 1 consisted of paclitaxel intravenously (175 mg/m^2) and day 2 consisted of carboplatin (AUC 6) intravenously, followed by a 3-week pause. At the second stage, surgical intervention (lung resection or pneumonectomy) was performed. The study was performed using venous blood collected 10–15 days before treatment; then, after 15–30 days, after the last course of neoadjuvant chemotherapy and, finally, 10–15 days after surgery. The effect of chemotherapy was evaluated using the RECIST1.1 scale. Patients were divided in two groups, one that exhibited partial tumor regression and one in which tumor growth was arrested. The period of post-surgical follow-up monitoring for eventual tumor relapses or distant metastases was at least 5 years. The study was conducted in agreement with international ethical guidelines and was approved by the Ethical Committee of Tomsk Cancer Research Institute.

Venous blood was collected in 0.05 M EDTA solution in phosphate buffered saline (PBS) in a 1 : 5 proportion. Blood specimens were separated into plasma and blood cell fractions; scb-cirDNA was obtained by treating cells sequentially with 5 mM phosphate buffer and 0.25% trypsin solution as described previously in [6]. DNA was isolated from 1 mL plasma in 3 mL PBS–EDTA and from 1 mL of the trypsin fraction using a Blood DNA Isolation Kit (BioSilica, Russia). DNA isolated from the PBS–EDTA fraction and the

Table 2. Correlation between concentrations of LINE-met and LINE-Ind fragments in blood scb-cirDNA of LC patients

Fragment	LINE-Ind before treatment	LINE-Ind after chemotherapy	LINE-Ind after surgery
LINE-met before treatment	0.543* $P = 0.045$	−0.231 $P = 0.448$	0.082 $P = 0.811$
LINE-met after chemotherapy	−0.121 $P = 0.649$	0.723* $P = 0.01$	0.139 $P = 0.701$
LINE-met after surgery	0.391 $P = 0.235$	−0.370 $P = 0.293$	0.827* $P = 0.002$

* Significant differences by Spearman's rank correlation test.

trypsin fraction was combined, and these specimens were designated as "scb-cirDNA." DNA was modified with sodium bisulfite and purified using a Bisulfite ssDNA Isolation Kit (BioSilica).

LINE-1 methylation levels were determined by quantitative methyl-specific PCR. The concentration of methylated LINE-1 fragments (LINE-1-met, sequence fragment 241–361, GenBank X58075) was determined using TaqMan methylation-specific PCR as described previously in [15]. To normalize the methylation data, the concentration of LINE-1 region 2 (LINE-1 Independent, LINE-1-Ind, sequence fragment 162925–163131, GenBank AL162574.14)) was evaluated by real-time PCR using EvaGreen fluorescent dye (Biotium, United States) and the primers 5'-ttttggaataggtgtg-gtgt-3' (forward), 5'-acttacactcccacacaata-3' (reverse) as described in [16]. Since the LINE-Ind primer sequences do not contain CpG dinucleotides, the amplification of this fragment does not depend on the status of LINE-1 methylation.

The exact concentrations of LINE-1 fragments that represent regions 1 and 2 were determined using the calibration standard of completely methylated bisulfite-converted human DNA (Zymo Research, United States) with a known concentration. The concentrations of methylated fragments that correspond to the LINE-1 region 1 (LINE-met) and normalizing fragments of the LINE-1 region 2 (LINE-Ind) were expressed in genome equivalents per 1 mL blood (GE/mL). The methylation index was calculated as the percentage of methylated molecules using the formula (%) = 100 × (LINE-met/LINE-Ind). Statistical analysis of the data was performed using the Statistica 6.0 software package.

RESULTS AND DISCUSSION

In our previous work, we showed that the concentration of methylated LINE-1 region 1 fragments (LINE-met) in cirDNA associated with blood cells (scb-cirDNA) differed significantly between LC patients and healthy subjects, whereas plasma cirDNA did not exhibit significant differences between these groups [14]. Taking into account that scb-cirDNA is a more informative source for analyzing aberrant LINE-1 methylation in LC, in this work, we selected this method to study LINE-1 methylation in LC patients at different stages of the dynamic follow-up, i.e., after two courses of neoadjuvant chemotherapy and after subsequent surgery.

It is a conventional practice to determine the gene methylation index as the ratio between the number of methylated fragments and the total of both methylated and nonmethylated fragments [17] or as the ratio between the number of methylated fragments of the target gene to the number of reference fragments. For example, for the single-copy housekeeping gene *COL2A1*, the number of methylated copies was constant within and among the groups studied [15]. In our work, it was LINE-1-Independent, the concentration of which in patients' blood did not exhibit significant variations in the course of the follow-up (before treatment–after chemotherapy–after surgery), which served as the reference sequence.

To analyze the correlations, the concentrations of methylated LINE-met fragments and reference LINE-Ind fragments in scb-cirDNA specimens were calculated in genome equivalent (GE) units per 1 mL blood. These values were found to correlate before treatment ($r = 0.54$), as well as after chemotherapy ($r = 0.72$) and after surgery ($r = 0.83$) ($P < 0.05$, Spearman's rank correlation test), which suggests an association between the treatment-induced changes in these parameters (Table 2).

Figure 1 shows the mean values of the LINE-1 methylation index (LINE-met/LINE-Ind) determined in the patients before treatment, after chemotherapy, and after surgery. It can be seen that antitumor therapy caused a linear increase in the LINE-1 methylation index, i.e., it increased nearly twofold after chemotherapy ($P = 0.04$, paired t test), and another 1.4 times compared to the previous step after surgery ($P = 0.134$, paired t test). Altogether, the combination treatment resulted in a threefold increase in the LINE-1 methylation index in scb-cirDNA ($P = 0.03$, paired t test) (Fig. 1).

We have shown that the levels of LINE-1 methylation significantly increased after the course of chemo-

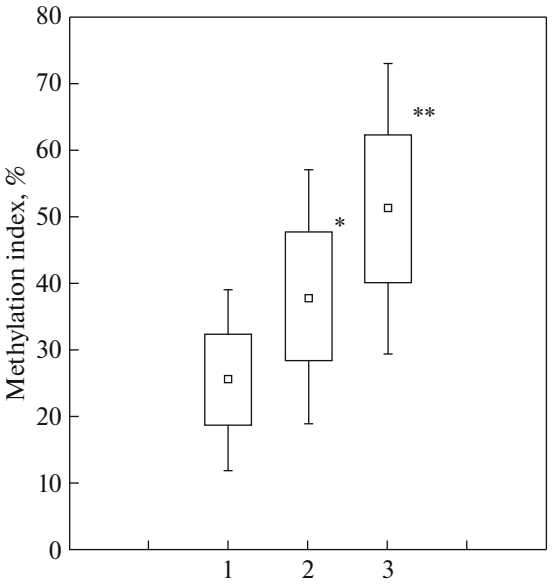

Fig. 1. LINE-1 methylation index in scb-cirDNA of patients with NSCLC before and after combination therapy. 1, Before treatment; 2, after chemotherapy; 3, after surgery. * Indicates significant difference between the pre-treatment and the post-chemotherapy levels; ** indicates significant difference between pre-treatment and post-surgery levels. □ is the mean; ⊓ is the mean ± standard error; ⌶ is the mean ± 1.96* standard error.

therapy, and increased further after surgery was performed 15–30 days later. It was previously found that carboplatin did not affect the levels of methylation of LINE-1 repeats or the CMV promoter in a tumor cell culture [18]. Exposure to a combination of chemotherapeutical drugs paclitaxel (an antimicrotubolar agent) and carboplatin (an alkylating agent) blocks mitosis in dividing tumor cells and induces apoptosis; as a result, the tumor mass decreases or stabilizes [18, 19]. We suppose that these events lead to a decrease in the blood levels of circulating tumor DNA fragments with hypomethylated LINE-1 sequences and, consequently, to an increase in the methylation index of LINE-1 in cirDNA.

An analysis of the relationships between the LINE-1 methylation index in blood scb-cirDNA and the histological type of the tumor showed that patterns of treatment-associated changes in LINE-1 methylation levels differed between patients with lung adenocarcinoma (AC) and those with squamous cell lung cancer (SCLC) (Table 3). In the case of SCLC, the index of LINE-1 methylation increased monotonously in the sequence before treatment–after chemotherapy–after surgery (17–27–57%), while in AC, the most significant change occurred at the chemotherapy stage, while surgery did not lead to an increase of the LINE-1 methylation index (24–49–37%).

These results may be related to the fact that the two histological types of lung cancer (SCLC and AC) differ significantly in their pathogenesis and clinical behavior [20]. It was described previously that SCLC and AC differ in the levels of LINE-1 methylation in tumor tissues [21]. In particular, the authors observed that LINE-1 hypomethylation in tumor DNA was more pronounced in SCLC than in AC. Characteristic changes in the gene copy numbers and DNA methylation patterns are specific for each histotype [22]. One of the possible reasons is the fact that, compared to SCLC, AC tumors exhibit a higher level of cellular and genetic heterogeneity. Some carcinogenic factors, such as tobacco smoke, tend to trigger a specific type of malignant transformation; therefore, smoking subjects are more likely to develop SCLC than AC tumors [23, 24].

It was previously reported that changes in the methylation levels of LINE-1-type retrotransposons in tumor tissues of cancer patients, e.g., in colorectal cancer, were associated with tumor response to therapy [25]. Therefore, in this work, we undertook to evaluate the patterns of treatment-induced changes in LINE-1 methylation levels in cirDNA of patients with NSCLC. Depending on the tumor response to neoadjuvant chemotherapy, the patients were divided in two

Table 3. Levels of LINE-1 methylation in blood scb-cirDNA of patients with NSCLC before and after antitumor treatment depending on the histological tumor type and on the tumor response to chemotherapy

Observation points	AC*		SCLC*		Patients with partial tumor regression*		Patients with tumor stabilization*	
Before treatment vs after chemotherapy	24 ± 7	$P = 0.095$	17 ± 3	$P = 0.333$	16 ± 3	$P = 0.294$	23 ± 7	$P = 0.074$
	49 ± 13		27 ± 6		28 ± 7		39 ± 11	
After chemotherapy vs after surgery	49 ± 13	$P = 0.535$	27 ± 6	$P = 0.078$	28 ± 7	$P = 0.395$	39 ± 11	$P = 0.850$
	37 ± 8		57 ± 15		44 ± 11		45 ± 10	
Before treatment vs after surgery	24 ± 7	$P = 0.123$	17 ± 3	$P = 0.049$	16 ± 3	$P = 0.184$	23 ± 7	$P = 0.061$
	37 ± 8		57 ± 15		44 ± 11		45 ± 10	

*LINE-1 methylation index (%) shown as mean ± standard error; significance of differences was assessed using the paired *t* test.

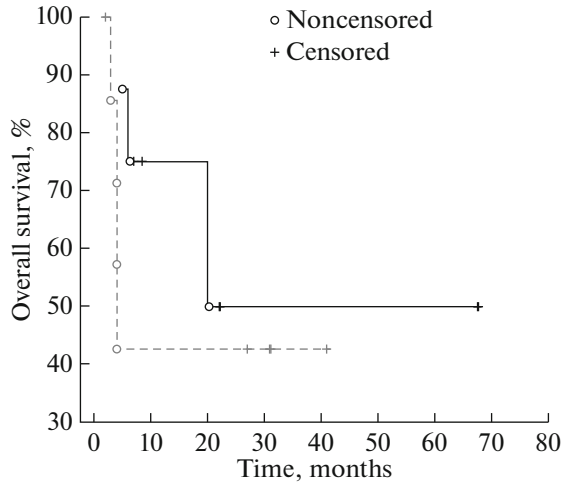

Fig. 2. Overall survival of patients with NSCLC depending on the threshold level of the pre-treatment LINE-1 methylation index in scb-cirDNA. Noncensored data: completed follow-up cases in which an event of tumor progression (relapse, metastasis) was registered; censored data: uncompleted follow-up cases without events of tumor progression. Group 1 (solid line): threshold methylation index higher than 18%; group 2 (dashed line): threshold methylation index lower than 18%.

groups: one with a positive response (partial regression of the tumor) and the other with less pronounced response (tumor stabilization or progression). It was found that the increase in the LINE-1 methylation index in scb-cirDNA across the treatment stages followed different patterns in these groups. In patients with moderate tumor response (tumor stabilization group), the methylation index showed a more substantial increase after chemotherapy, followed by a slight increase after surgery (23–39–45%). In patients with a positive tumor response (tumor regression group), the LINE-1 methylation index tended to increase across the treatment stages following a different (convex) pattern (16–28–44%).

After the antitumor therapy, the follow-up period continued for at least 5 years. The patients were divided in two groups, i.e., one included patients with an unfavorable prognosis (relapses, distant metastases; $n = 6$) and the other one included those with a favorable prognosis (no signs of tumor progression; $n = 10$). We performed a univariate analysis of the overall survival of patients with NSCLC depending on the Kaplan–Meier estimate of the threshold value (Fig. 2). It was found that the threshold value corresponded to the median of the pre-treatment LINE-1 methylation index in scb-cirDNA. Although the subgroups were characterized with somewhat different trends, we did not detect significant differences in the overall survival depending on the LINE-1 methylation index in scb-cirDNA (long-rank test, $P = 0.35$). Larger groups of LC patients need to be studied to establish the eventual significance of differences.

Thus, our results suggest that determining the LINE-1 methylation index in cirDNA can be informative for monitoring LC patients after combination antitumor therapy. It was found that the dynamics of the methylation index depended on the histological type of the tumor. In SCLC, changes in the methylation index were more pronounced after the treatment was completed, while in AC, the most substantial change in the LINE-1 methylation index occurred at the first stage of treatment (after chemotherapy). Changes in the LINE-1 methylation index in cirDNA exhibited different trends in the patient groups with different response to chemotherapy, which indicates a profound relationship between this serological marker and the pathological process in LC. There can be no doubt that LINE-1 methylation index in cirDNA should be studied in large groups of LC patients as a promising marker for predicting a tumor's response to treatment, evaluating the therapy effect, and the early detection of relapses.

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