SIGNIFICANCE OF MIR-146A QUANTIFICATION IN HUMAN BLOOD PLASMA FOR THE DIAGNOSIS OF COLORECTAL CANCER

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Colorectal cancer (CRC) is one of the most common cancer types in the world. Timely diagnosis of CRC and adenomatous polyps aided by effective screening techniques can considerably reduce mortality from this disease. MicroRNAs constitute a new class of promising biomarkers for a range of human diseases including cancer. The following article assesses the diagnostic significance of miR-146a concentrations in the blood plasma of patients with colorectal cancer. The main group included patients with stages I to III colorectal cancer (n = 102); the control group comprised patients with chronic colitis, nonspecific ulcerative colitis and Crohn's disease (n = 58) and healthy individuals (n = 42). MicroRNA levels were quantified by reverse-transcription real-time PCR, revealing significantly higher miR-146a concentrations in the samples of patients with CRC than in the controls ($p < 0.0001$). The optimal diagnostic sensitivity determined by ROC analysis was 47.3 %, specificity was 91.5 %, with AUC = 0.79 ± 0.018. Our findings demonstrate that the studied approach does not have sufficient specificity, but still suggest that miR-146a can be included into screening tests based on quantification of other microRNAs with improved specificity.

Keywords: cancer research, colorectal cancer, screening, biomarker, microRNA, miR-146a, cel-238, polymerase chain reaction, reverse transcription

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ОЦЕНКА ЗНАЧИМОСТИ ОПРЕДЕЛЕНИЯ КОЛИЧЕСТВА MIR-146A В ПЛАЗМЕ КРОВИ ЧЕЛОВЕКА ДЛЯ ДИАГНОСТИКИ КОЛОРЕКТАЛЬНОГО РАКА

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Колоректальный рак (КРР) — один из наиболее распространенных видов рака в мире. Эффективные методы скрининга для своевременного выявления КРР и аденоматозных полипов могут значительно снизить смертность от этого заболевания. МикроРНК — новый класс потенциальных биомаркеров для широкого круга заболеваний человека, включая онкопатологии. В статье оценивается диагностическая значимость концентрации микроРНК miR-146a в плазме крови человека с КРР. В опытную группу включили пациентов с колоректальным раком стадий I–III (n = 102), а в контрольную — пациентов с хроническим колитом, неспецифическим язвенным колитом и болезнью Крона (n = 58) и условно здоровых людей (n = 42). Количество микроРНК определяли при помощи ПЦР с обратной транскрипцией (ОТ-ПЦР) с детекцией результатов в «реальном времени». Было показано, что концентрация miR-146a статистически значимо выше в образцах плазмы крови пациентов с КРР в сравнении с пациентами контрольной группы ($p < 0,0001$). Оптимальное значение диагностической чувствительности, определенное с помощью ROC-анализа, составило 47,3 %, специфичности — 91,5 %, AUC = 0,79 ± 0,018. Исследуемый подход обладает недостаточно высокой специфичностью, но показано, что miR-146a в будущем может быть включена в состав диагностических профилей на основе нескольких микроРНК с улучшенной специфичностью.

Ключевые слова: онкология, колоректальный рак, скрининг, биомаркер, микроРНК, miR-146a, cel-238, полимеразная цепная реакция, обратная транскрипция

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Colorectal cancer is one of the most prevalent cancer types and the most common intestinal malignancy. Survival rates in timely diagnosed patients with stage I who receive adequate treatment are as high as 90 % vs. 6 % in individuals with stage IV disease. Therefore, early diagnosis is crucial for patient's survival. Today, one of the most promising areas of research is discovery of novel noninvasive molecular biomarkers that may be present in blood, feces and other human biological materials.

In 2008 Tewari et al. noticed that some microRNAs circulating in human blood plasma have a remarkably stable form protecting them from endogenous RNase [1]. This discovery inspired further studies of the diagnostic potential of extracellular microRNA. This nucleic acid has been found in blood plasma, saliva, urine, bile, breast milk and other human biological fluids. A wealth of information has been accumulated about specific changes in its expression in pathology, including cancer, cardio-vascular and inflammatory diseases, aging, etc. [2–5].

Both specific and nonspecific changes in microRNA expression profiles are observed in tumors of various origins. For example, most tumors are associated with increased miR-21 and reduced let-7 expression. There are also specific changes associated with the histological type of a tumor, gene expression in tumor cells or TNM stage.

A number of research works describe the role of tissue-specific (miR-21, miR-9, miR-155, miR-17, miR-19, let-7 and miR-24) and circulating (miR-181b, miR-21, miR-183, let-7g, miR-17 and miR-126) microRNAs in the development and progression of colorectal cancer [6]. The first study on microRNA expression in colorectal cancer conducted in 2003 revealed that tumor-suppressing miR-143 and miR-145 are expressed at reduced levels in adenomatosis and malignancies [7]. So far, a few dozens of microRNA have been described whose expression changes in CRC [8].

MicroRNA was shown to be a feasible nononvasive tool for colorectal cancer diagnosis in 2008–2009 [9, 10]. Studies in this field are still very relevant [11, 12].

Previously we analyzed the data collected under the SysCol project (Systems Biology of Colorectal cancer) [13] on microRNA profiles obtained by next generation sequencing (NGS). Our analysis demonstrated that miR-146a (ENSG00000253522) had a significantly higher expression in colorectal adenocarcinoma tissue than in control samples (logFC = 1.742, adjusted p = $5.57E^{-79}$). The aim of the present work is to assess significance of miR-146a levels circulating in human blood plasma for the diagnosis of colorectal cancer.

METHODS

The study was carried out in patients of City Clinical Hospital No. 1 (Novosibirsk), the Center of New Medical Technologies (Novosibirsk) and City Hospital No. 40 (St. Petersburg) who presented with different conditions of the large bowel. The main group consisted of 102 patients with stages I-III adenocarcinoma of the colon; the control group included 58 patients with inflammatory diseases of the bowel (chronic colitis, nonspecific ulcerative colitis and Crohn's disease) and 42 healthy individuals. The patients with inflammatory conditions were included into the control group because tumor progression is always accompanied by inflammation in the adjacent tissues. Detailed information about both groups is provided in Tables 1 and 2. The study was approved by the Ethics Committees of the Center of New Medical Technologies (Novosibirsk, Protocol No. 18 dated October 24, 2014). All patients gave their informed consent.

Samples of peripheral blood (10 ml) were collected into EDTA-containing Vacutainer tubes, then mixed thoroughly but gently and centrifuged for 10 min at 1,600 g and room temperature. The obtained plasma (4–5 ml) was carefully collected without disturbing the pellet and transferred into 15 ml conical-bottom tubes. The samples were centrifuged again, plasma was transferred to new 1.5 ml tubes, frozen and stored at –80 °C. The samples were pooled for further experiments (10 samples of 50 µl per group).

MicroRNA was isolated from frozen blood plasma. Prior to extraction, synthetic microRNA cel-238 (the internal control) was added to each sample in the amount of 5 x 10^7 copies per sample. Internal controls help to assess the quality of extraction

Table 1. The group of patients with colorectal cancer (n = 102)

Disease stage	Sex	Age. years	Number of patients
I–II	M	54.3 ± 16.4	13
	F	62.1 ± 11.2	8
III	M	59.3 ± 14.2	49
	F	58.9 ± 14.0	32

Table 2. The control group (no malignancies detected, n = 100)

Disease	Sex	Age, years	Number of patients
Chronic colitis	M	36.5 ± 19.3	10
	F	38.4 ± 10.8	16
Nonspecific ulcerative colitis	M	26.5 ± 8.3	4
	F	42.2 ± 11.7	20
Crohn's disease	M	31.5 ± 6.3	4
	F	28.4 ± 4.8	4
No bowel pathologies detected	M	46.5 ± 17.1	15
	F	49.4 ± 19.2	27

and polymerase chain reaction (PCR), e. g. to ensure that there are no amplification inhibitors in the samples. The number of cel-238 copies was taken as a normalizing coefficient to calculate the number of the analyzed microRNA copies in the sample.

To quantify microRNA, we performed poly(A) tailing by poly(A) polymerase and real-time reverse-transcription PCR (RT-PCR). Primers for reverse transcription had 5 or 6 nucleotides at their 3'-ends complementary to the 3'-end of microRNA, a sequence of 11 thymines and binding sites for the fluorescently labeled hydrolyzable probe and the universal reverse primer (Table 3).

cDNA molecules yielded by reverse transcription and diluted 5-fold to avoid inhibition were amplified by real-time PCR using specific forward and universal reverse primers and a universal probe. PCR was performed in the CFX96 thermocycler equipped with an optical unit for fluorescence detection (Bio-Rad, USA). The protocol was as follows: initial denaturation for 15 min at 96 °C; amplification (x40): denaturation for 10 sec at 96 °C, primer annealing for 20 sec at 56 °C, elongation for 10 sec at 72 °C, signal recording for 10 sec. The reaction mixture (20 µl) contained 65 mM Tris-HCl (pH 8.9), 3 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 0.2 mM dNTP, 300 nM of primers and 100 nM of the hydrolyzable fluorescently labeled probe, 0.5 un. of hot-start Taq-polymerase (Biosan, Institute of Chemical Biology and Fundamental Medicine SB RAS), and 2 µl of cDNA.

MicroRNA quantity was calculated from a calibration curve and expressed in arbitrary units. The curve was constructed based on a series of four 4-fold dilutions of test cDNA, which in our case was a 5-fold concentration of cDNA obtained from 5 random samples (the lowest concentration was taken as 1 arb. unit). The coefficient of correlation between the expected and empirical values was at least 0.99. PCR efficiency calculated from the slope of the curve was 82 % for miR-146a and 93 % for cel-238. The measured values were within the linear section of the curve. To assess reproducibility of Ct values, cDNA samples were amplified with each pair of primers in duplicate. In general, we avoided taking measurements at Ct > 37.

Distribution of normalized microRNA concentrations was tested using the Anderson–Darling Normality Test. Significance of differences between the groups was estimated by the nonparametric Mann–Whitney U-test. ROC-analysis was done in the Web-based Calculator for ROC Curves [14].

RESULTS

In the course of our study, we extracted microRNA, synthesized cDNA and measured miR-146a and cel-238 concentrations in the individual and pooled samples of patients' blood plasma. The main group will be further referred to as T, the controls — as C.

The median of normalized miR-146a concentrations in the pooled samples was 9.7 arb. un. in group T vs. 4.65 in group C (Table 4, Fig. 1) and 7.6 vs. 2.5, respectively, in individual samples (Table 4, Fig. 2). Levels of mir-146a were reliably higher in both pooled and individual samples obtained from the main group, in comparison with the controls.

The diagnostic potential of the method was assessed by ROC analysis. The following values were obtained: AUC = 0.79, SD = 0.018, sensitivity of 47.3 %, specificity of 91.5 % at threshold sensitivity for miR-146a 4 set to 4 arb. un. (Fig. 3).

Comparison of healthy individuals (n = 42) and patients with granulomatous and ulcerative colites (n = 58) revealed that patients with inflammatory bowel disorders had elevated levels of miR-146a in their blood plasma (an average of 3.1 ± 1.61 vs. 2.33 ± 0.67, respectively); however, the differences were less marked and less significant (Mann–Whitney U, p = 0.01).

DISCUSSION

At the moment circulating microRNA are in the focus of the search for new methods for cancer diagnosis [15]. The potential of miR-146a as a biomarker of colorectal cancer has been investigated only once, with no satisfactory results [16]. However, there are reasons to believe that this microRNA should be regarded as a potential biomarker of CRC. A number of authors have shown that miR-146a is involved in the suppression of inflammation by inhibiting NF-κB signaling [17], at least via downregulating the expression of *TRAF6* and *IRAK1* [18]. Increased concentrations of miR-146a in blood plasma

Table 3. Nucleotide sequences of oligonucleotide primers and hydrolizable fluorescently labeled probes used in the study

MicroRNA	Oligonucleotide	Sequence
miR-146a	U	5'- ggctgagaactgaattccat-3'
	R	5'-gagcagggtccgaggt-3'
	Probe	5'-HEX-accaccgcaccacgcc-BHQ-3'
	RT	5'-gagcagggtccgaggtaaccaccgcaccacgccttttttttttttaaccca -3'
cel-238	U	5' -tttgtactccgatgcc–3'
	R	5'-gagcagggtccgaggt-3'
	Probe	5'-FAM-tcgcacgaccacccgc-BHQ-3'
	RT	5'-gagcagggtccgaggtatcgcacgaccacccgcttttttttttttctgaa-3'

Table 4. Analysis of miR-146a levels circulating in the blood plasma of patients with colorectal cancer (T) and controls (C)

Parameter	T (pool)	C (pool)	T	C
Sample size	10	10	102	100
Mean value	10.28	4.8	7.40	2.58
Standard deviation	3.98	2.23	3.04	0.97
Median	9.7	4.65	7.6	2.5
P-value (Mann—Whitney)	0.0019		< 0.0001	

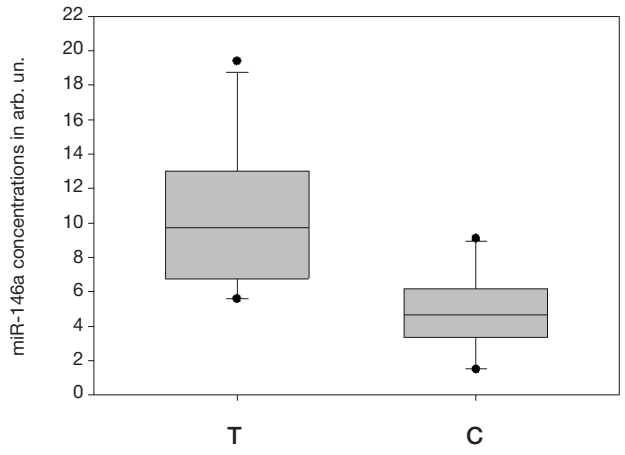

Fig. 1. Distribution of normalized miR-146a concentrations expressed in arbitrary units in 10 plasma pools obtained from patients with colorectal cancer (T) and controls (C)

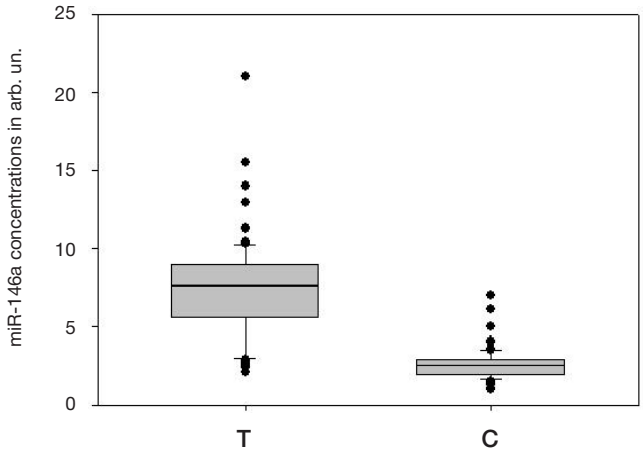

Fig. 2. Distribution of normalized miR-146a concentrations expressed in arbitrary units in patients with colorectal cancer (T) and controls (C)

are observed in a number of inflammatory conditions, such as sepsis [19]. This may be a result of hyperstimulation of molecular mechanisms that curb inflammation. However, we still do not know for sure which cell types or molecular mechanisms are implicated in the elevated miR-146a levels in the circulation in some cancers, including colorectal cancer. Association studies have shown that the polymorphism *rs2910164* in the miR-146a gene is associated with the risk of malignancies of the digestive tract [19, 20], which also underpins our choice.

In our study we have shown a statistically significant association ($p < 0.0001$) between miR-146a concentrations and large bowel cancer. Moreover, we have discovered that this association is also statistically significant, though not that strong, for pooled samples. Sample pooling is often used in initial screening in order to increase performance and reduce costs [21, 22], but few studies have validated such an approach so far. In our study we have demonstrated the feasibility of sample pooling for initial screening.

In spite of statistically significant increase in the levels of miR-146a in the blood plasma of patients with colorectal cancer in comparison with patients who did not have this disease, ROC-analysis yielded a relatively moderate AUC of 0.79 ± 0.018 and unsatisfactory diagnostic sensitivity of 47.3 %. Moreover, miR-146a concentrations were elevated in patients with inflammatory conditions of the bowel, which is unsurprising, considering the important role of miR-146a in the regulation of inflammation. Previously elevated miR-146 concentrations in blood plasma were observed in patients with autoimmune thyroiditis [24], sepsis [19] and other inflammatory conditions.

CONCLUSIONS

Based on our findings, we conclude that diagnostic quantification of miR-146a in blood plasma has low specificity in patients with colorectal cancer. Specificity and sensitivity of this method can be validated in prospective studies, which are not very popular at the moment. Still, the functional link between miR-146a, inflammation and development of colorectal cancer, as well as and the significant association between increased miR-146a concentrations in blood plasma and CRC, render this microRNA a potential candidate for inclusion into screening tests based on quantification of other microRNAs with improved specificity.

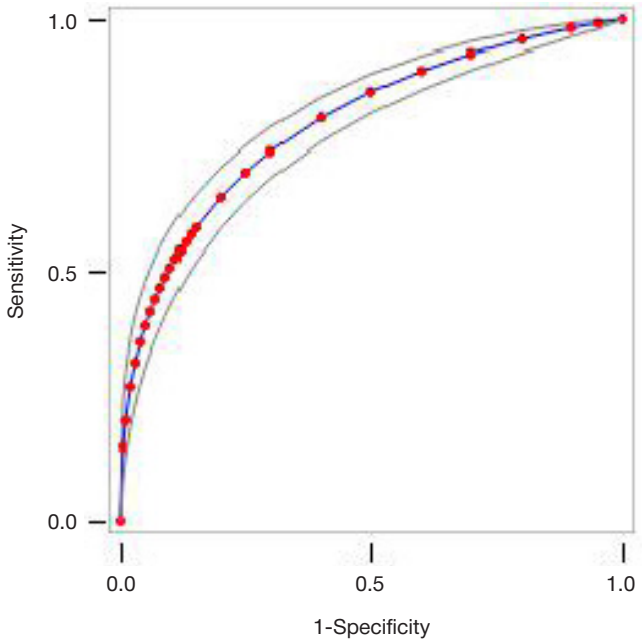

Fig. 3. The ROC-curve (shown in blue) constructed from the distribution of measured miR-146a concentrations expressed in arbitrary units for patients with and without colorectal cancer. Confidence intervals are represented by grey curves

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