ISSN 0233–7657. Biopolymers and Cell. 2014. Vol. 30. N 2. P. 129–134 doi: http://dx.doi.org/10.7124/bc.00088B

UDC 577.218 + 577.133.4

Comparative analysis of epigenetic markers in plasma and tissue of patients with colorectal cancer

A. G. Kondratov[1], K. A. Nekrasov[1], L. V. Lototska[1], G. V. Panasenko[1], L. A. Stoliar[1], Y. V. Lapska[1], O. O. Kolesnyk[2], I. B. Shchepotin[2], A. V. Rynditch[1], V. I. Kashuba[1, 3]

[1]State Key Laboratory of Molecular and Cellular Biology,
Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680

[2]National Cancer Institute
33/43, Lomonosova Str., Kyiv, Ukraine, 03022

[3]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet
Nobels vag 16, Box 280, 171 77-Stockholm,

o.g.kondratov@imbg.org.ua

***Aim**. The work is devoted to the development of less invasive tools for the colorectal cancer (CRC) screening. **Methods**. Q-PCR and methylation-specific PCR techniques were used in the current work. **Results**. We have shown that the levels of cell-free plasma DNA are higher in the CRC patients compared with the healthy donors ($p < 0.01$). Hypermethylation of APC, FHIT, LRRC3B and HIC1 genes was studied in the tumor and plasma samples of CRC patients. Two-stage verification for CRC screening was proposed. **Conclusions**. We proposed and tested a novel approach for CRC screening based on the determination of cell-free DNA and methylated DNA fragments in the plasma.*

Keywords: colorectal cancer, cell-free DNA, DNA methylation, APC, FHIT, LRRC3B, HIC1.

Introduction. Colorectal cancer (CRC) is the third commonly diagnosed cancer that causes more than 600 000 deaths per year worldwide [1]. In most cases, CRC tumors grow slowly – approximately 1 cm per year without noticeable symptoms. The most sensitive modern diagnostic tool for the CRC detection is a colonoscopy. It allows detecting the tumors of less than 1 cm. However, the colonoscopy procedure is painful and in some cases is not recommended to the patients with heart diseases, because of possible adverse cardiopulmonary reactions that are usually related to the sedation [2] as the colonoscopy is performed under narcosis. Also, the colonoscopy meets some difficulties in case of altered topography of a colon [3]. Thus, the development of less invasive tools for screening CRC is a relevant problem of the modern oncology.

© Institute of Molecular Biology and Genetics, NAS of Ukraine, 2014

DNA methylation is a stable epigenetic mark which is associated with gene silencing in the case of promoter localization in CpG-island [4]. The gene hypermethylation frequently targets the potential tumor-suppressor genes (TSG), inactivation of which promotes the tumor development. According to Toyota *et al*., the colon cancer can be ascribed to the tumors with a high frequency of gene hypermethylation. Approximately 17 % of CpG-islands are hypermethylated in CRC [5].

The circulating cancer cells and cell-free DNA (cf DNA) are frequently detected in the patients with different types of the malignant disease. Moreover, a level of cfDNA is elevated in the cancer patients in comparison with the healthy individuals [6].

In the proposed paper DNA hypermethylation of the well-known tumor associated genes like *LRRC3B, FHIT, APC* and *HIC1* was detected by methylation-specific PCR (MSP) in the plasma and tumor samples

from the CRC patients [7–10]. Adjacent to the tumor non-malignant tissues of bowel were used as a control. Additionally, the levels of cfDNA in plasma from the CRC patients and healthy donors were mesuared by quantitative PCR (Q-PCR). All experiments were carried out with the tumors, adjacent non-malignant tissues and plasma samples of the same patients.

Material and methods. *Ethics statements*. The samples were collected in accordance with the Declaration of Helsinki and approved by the guidelines issued by the Ethic Committee of the National Cancer Institute of the Academy of Medical Sciences, Kyiv, Ukraine.

Sample collection, genomic and cell-free DNA isolation. Twenty surgically excised tissue samples of CRC were used in the present study (Table 1). All tumor samples were paired with non-malignant tissues, which were taken as the normal tissue samples. Immediately after surgery, the tissue samples were frozen in liquid nitrogen and stored at –70 °C. Neither chemotherapy nor radiotherapy was conducted for any patients prior to surgery. Each tissue sample was accompanied by a corresponding blood sample. All tissue samples were characterized histologically. Blood of 21 healthy donors was used as a control group.

Genomic DNA was purified by GenElute Mammalian Genomic DNA Miniprep Kit («Sigma-Aldrich», USA) according to the manufacturer’s recommendations. Briefly, 50 mg of tissue were homogenized in liquid nitrogen, subjected to lysis and purified with GenElute Miniprep Binding Columns. The quality and size of genomic DNA were assessed by gel electrophoresis («Sigma-Aldrich»).

Plasma from the cancer patients and the healthy donors was obtained by the multistage centrifugation of the blood in range from 1000 to 3000 *g*, using EDTA as an anticoagulant. The cell free circulating DNA from 200 μl of plasma was isolated by the Proba NK DNA («DNA Technology», Russia) isolation kit, according to the manufacturer’s instructions.

Determination of concentrations of cell free DNA. To detect the concentration of cell free circulating DNA in the plasma, the Q-PCR was used. The Q-PCR was performed with SYBR Green mixture («Thermo Scientific», USA) and primers for the *GAPDH* gene: gRef For 5'-GGCTCCCACCTTTCTCATC-3' and gRef Rev 5'-AGCGTACTCCCCACATCA-3', using the following reaction conditions: 95 °C – 4 min, then 35 cycles at 95 °C – 15 s, 60 °C – 20 s and 72 °C – 30 s. To carry out Q-PCR the CFX real-time PCR detection system («BioRad», USA) was used. To determine the concentration of DNA in the plasma the calibration curve in coordinates of genomic DNA concentrations and *Ct* was plotted. The range of genomic DNA concentration was 2.44–2440 pg. The range of concentrations was created by DNA dilution. The initial concentration of DNA for a calibration curve was determined by means of ND-2000 («Thermo Scientific»).

Table 1
Concentrations of cfDNA in plasma samples of CRC patients and healthy donors

N, CRC patients	cfDNA concentration for CRC patients, ng/ml	N, healthy donors	cfDNA concentration for healthy donors, ng/ml
100	14.0	1	6.1
102	4.5	2	7.8
103	8.6	3	5.3
104	7.1	4	7.9
108	10.3	5	10.0
109	11.9	6	5.4
110	25.5	7	6.8
116	3.2	8	11.0
117	2.7	9	5.3
118	12.6	10	6.7
119	10.7	11	2.7
120	23.8	12	3.2
121	12.1	13	6.3
122	69.9	14	4.8
124	15.1	15	3.9
126	18.2	16	6.0
127	21.1	17	18.8
129	36.5	18	8.2
130	10.8	19	8.3
131	32.8	–	–
132	252.7	–	–

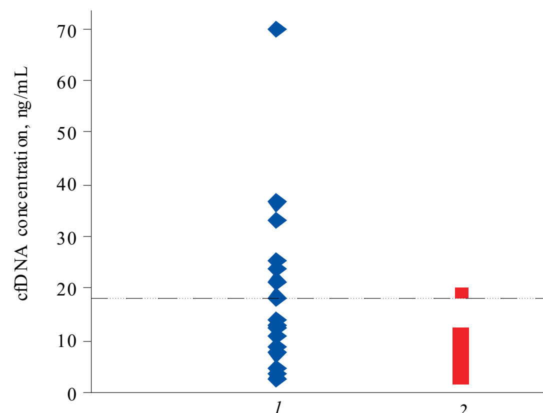
DNA bisulfite conversion and determination of methylated DNA fragments. The bisulfite treatment of ge-

nomic DNA was performed, using the EZ DNA Methylation kit («ZYMO Research», USA), according to the manufacturer's protocol. For the bisulfite treatment of the plasma DNA 44 µl of solution of the dissolved DNA sediments with co-precipitator were used. To protect the plasma DNA during the reaction, 450 ng of Lambda DNA were added per a reaction sample of bisulfite treatment.The bisulfite treated DNA was dissolved in 20 µl of Elution buffer.

To determine the methylation status of the *LRRC3B*, *APC*, *FHIT*, and *HIC1* genes in tissue samples, the methylation-specific PCR (MSP) was used. MSP was performed using primers that were described previously [11–14]. Amplification of the bisulfite converted sequence of the *COL2A1* gene was used as a control of the DNA input [14]. The PCR mixture of MSP reactions contained 10 × PCR Dream buffer («Thermo Scientific»), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 0.3 µM primers, 50 ng of modified DNA and 1 U of DreamTaq polymerase («Thermo Scientific»). Amplification was performed for 40 cycles (30 s at 95 °C, 30 s at 63 °Cand 30 s at 72 °C), initiated with DNA denaturation at 95 °C for 4 min. The final extension was at 72 °C for 5 min. Amplified products were detected by electrophoresis in 12 % polyacrylamide gel with subsequent ethidium bromide staining.

To determine methylation status of the *APC*, *FHIT*, *LRRC3B*, and *HIC1* genes in the cell free circulating DNA, the real-time MSP (RT-MSP) was used. RT-MSP was performed with the above-mentioned primer set for MSP. Each sample of the amplification reaction contained 1 × SYBR Green mixture («Thermo Scientific»), 0.4 µM of each primer and 4µl of bisulfite treated DNA. The conditions of RT-MSP were 95 °C for 10 min, for 50 cycles; 95 °C for 15 s, 62–65 °C for 20 s and 72 °C for 30 s. It was considered the positive methylation status of the sample if *Ct* of reaction was < 45 cycles. The quality of amplified products was checked by electrophoresis in 2.5 % agarose gel and melting curve analysis with the CFX real-time PCR detection system («BioRad»). To verify RT-MSP data, the MSP sequencing assay was performed.

Statistical analysis. Statistical analysis was performed using STATISTICA 7.0 program («StatSoft Inc», USA). If the p-value was < 0.05 the results were considered statistically significant. The nonparametric Mann-Whitney U Test was used to calculate the difference between concentration of plasma cfDNA in CRC patients and healthy donors.



CfDNA concentration in plasma of 20 CRC patients (*1*) and 19 healthy donors (*2*) was detected by Q-PCR. The upper cut-off value of the free-circulating DNA concentration of healthy donors is depicted as a dashed line at 17.7 ng/ml level of plasma. To prevent merger of data points one CRC (N 132) concentration was not included here, it equals 252,7 ng/ml

Results and discussion. *The levels of cell free circulating DNA in the plasma from CRC patients are higher than in healthy donors*. Using Q-PCR, the level of cell free circulating DNA was determined in the plasma of CRC patients and healthy donors (Table 1, Figure). It was shown that the mean value of concentration of plasma cfDNA was significantly higher in CRC patients, compared with the healthy donors ($p < 0.01$). Thus, the mean value (MV) of concentration of cell-free circulating DNA in the plasma from CRC patients was 29.45 ± 12.24 ng/ml (MV ± St. Error), whereas it was 7.07 ± 0.82 (MV ± St. Error) in healthy donors. In order to generate the upper cut-off value of the cell free circulating DNA concentration in plasma we used the highest permissible concentration in the healthy donors. It was defined as a mean value concentration of cfDNA of healthy donors and three standard deviations. So, the upper cut-off value of the free-circulating DNA concentration was 17.7 ng/ml of the plasma in healthy donors (Figure).

Therefore, there were 8 out of 20 samples of CRC which fell into criteria as samples with an abnormally increased DNA level.

Hypermethylation of tumor suppressor genes in the cancer tissues and plasma samples from CRC patients. *APC*, *FHIT*, *LRRC3B* and *HIC1* are the colon cancer as-

Table 2
Total data of cfDNA concentration and methylation of APC, FHIT and LRRC3B genes from plasma of CRC patients.

	Tissue				Plasma				
N/Genes	*LRRC3B*	*APC*	*FHIT*	Totally	*LRRC3B*	*APC*	*FHIT*	cfDNA	Totally
100	M	M	M	+	ND	M	ND	L	A
102	U	M	U	+	ND	M	ND	L	A
103	U	U	U	–	ND	ND	ND	L	NC
104	M	U	M	+	M	ND	ND	L	A
108	M	U	U	+	ND	ND	ND	L	NC
109	M	U	U	+	ND	ND	ND	L	NC
110	M	M	M	+	ND	ND	M	H	A
116	M	M	U	+	ND	ND	ND	L	NC
117	M	M	M	+	ND	ND	ND	L	NC
118	M	M	M	+	ND	M	ND	L	A
119	U	M	M	+	ND	M	M	L	A
120	U	U	M	+	ND	ND	ND	H	A
121	M	U	M	+	ND	ND	M	L	A
122	M	M	M	+	ND	ND	ND	H	A
126	U	U	M	+	ND	ND	ND	H	A
127	M	M	M	+	ND	ND	ND	H	A
129	M	U	M	+	M	ND	M	H	A
130	U	U	U	–	ND	M	ND	L	A
131	U	U	M	+	ND	ND	ND	H	A
132	M	M	M	+	M	M	ND	H	A
Frequency	65 % (13/20)	50 % (10/20)	70 % (14/20)	90 % (18/20)	15 % (3/20)	30 % (6/20)	20 % (4/20)	40 % (8/20)	75 % (15/20)

N o t e . M – methylated DNA was detected; U – unmethylated DNA was detected; H – cfDNA concentration is higher than upper cut-off value; L – cfDNA concentration is lower than upper cut-off value; ND – methylated DNA was not detected; «+» – methylation was detected in one or more genes for the tissue; «–» – no methylation was detected for at least one genes for the tissue; A – abnormal plasma; NC – no changes have been detected in the plasma.

sociated genes. Their alterations due to the promoter CpG-island methylation were described previously.

Thus, hypermethylation of *APC*, *FHIT*, *LRRC3B* and *HIC1* was detected in 45, 37, 77 and 42 % of CRC samples correspondingly [15–18]. Therefore, the MSP-based detection of the methylated fragments of these genes was used for further development of screening panel for CRC. In the current research we have found that the *APC*, *FHIT*, and *LRRC3B* genes were hypermethylated in 50 % (10/20), 70 % (14/20) and 65 % (13/20) of tumor samples, correspondingly. Altogether, the hypermethylation of at least one of the selected genes was detected in 90 % (18/20) of samples.

Using MSP and the subsequent melting curve analysis, the methylated fragments of *APC*, *FHIT* and *LRRC3B* genes were detected in 30 % (6/20), 20 % (4/20) and 15 % (3/20) of the plasma of CRC patients, respectively. The hypermethylation of at least one selected gene was found in 50 % (10/20) of the plasma samples from CRC patients.

No amplification was found in reactions with MSP primers and bisulfite treated Lambda DNA which did not include the human DNA. No methylated fragments of the selected genes were identified in plasma of healthy donors.

Therefore, the hypermethylated fragments of *APC*, *FHIT*, and *LRRC3B* genes in the plasma were detected in 50 % (5/10), 31 % (4/13) and 23 % (3/13) of tumors which were positive for hypermethylated fragments of the abovementioned genes, respectively. To verify specificity of MSP, the PCR products of the *APC* and *LRRC3B* genes were sequenced after amplification with the primers for methylated DNA.

To study specificity of the gene panel, hypermethylation of the *APC*, *FHIT* and *LRRC3B* genes was studied in the plasma of healthy donors. Moreover, the methylation of *APC*, *FHIT* and *LRRC3B* in plasma was detected in 92 % (12/13) of the samples with pre-detected methylation of these genes in tumor tissue. Initially we had shown the same tendency in hypermethylation of the *APC*, *FHIT* and *LRRC3B* genes in the plasma of CRC patients [19].

Additionally, we have registered a high frequency of the *HIC1* hypermethylation in the plasma of CRC patients – 80 % (8/10). Similar study of the healthy donor's plasma has revealed the hypermethylation of *HIC1* at the similar level – 80 % (12/15). No difference in the *HIC1* and *COL2A1* hypermethylation was observed in CRC patients and healthy donors: mean values of Ct_{CRC} 32.7 – *HIC1* and 28.6 – *COL2A1* and $Ct_{\mathrm{healthy\ donors}}$ 35.0 – *HIC1* and 30.5 – *COL2A1*.

This indicates a low selectivity of the *HIC1* hypermethylation in CRC patients. Therefore, we concluded that the *HIC1* hypermethylation is not a valuable marker for the prediction of CRC.

We proposed that a two-stage verification must be applied for CRC screening. These stages include the measurement of the cell-free circulating DNA and the following detection of the methylated fragments of *APC*, *FHIT*, and *LRRC3B* genes in the CRC patient plasma. This allows us to achieve a sensitivity of the panel in CRC detection up to 75 % (Table 2).

However, our research has not resulted in the sensitivity of 100 % for the CRC registration that is essential for the prevention of this disease. We hope that a higher sensitivity might be achieved by further extension of the gene panel for the identification of methylated DNA in the plasma of CRC patients.

Conclusions. In the present work we have characterized hypermethylation of the *APC*, *FHIT*, *LRRC3B*, and *HIC1* genes in the patients with CRC in comparison with the healthy donors. We have found that hypermethylation of the *APC*, *FHIT*, and *LRRC3B* gene fragments in the plasma fully corresponds to hypermethylation of these genes in the tumors.

We have proposed and tested the novel approach for CRC screening, based on the detection of cell-free DNA and methylated fragments of the well-known tumor associated genes, such as *APC*, *FHIT*, *LRRC3B*, and *HIC1* in the blood plasma. With such approach 75 % of sensitivity could be achieved. The sensitivity for CRC detection might be increased by the analysis of additional tumor-associated genes.

Funding. This work was supported by a grant from the Ukrainian Academy of Sciences (41/12).

Порівняльний аналіз епігенетичних маркерів у плазмі крові пацієнтів, хворих на рак товстого кишечника

О. Г. Кондратов, К. А. Некрасов, Л. В. Лотоцька,
Г. В. Панасенко, Л. А. Столяр, Ю. В. Лапська,
О. О. Колесник, І. Б. Щепотін, А. В. Риндич, В. І. Кашуба

Резюме

Мета. *Розробка менш інвазивних методик для скринінгу злоякісних пухлин товстого кишечника (CRC).* ***Методи***. *Використано кількісну ПЛР і метил-специфічну ПЛР.* ***Результати***. *Показано, що середнє значення концентрацій вільно циркулюючої ДНК у плазмі крові є статистично достовірно вищим у пацієнтів з CRC порівняно зі здоровими донорами (p < 0,01). Встановлено гіперметилювання генів APC, FHIT, LRRC3B і HIC1 у пухлинах та плазмі хворих на CRC.* ***Висновки***. *Нами запропоновано і перевірено новітній підхід для скринінгу CRC, який базується на визначенні позаклітинної ДНК і метильованих фрагментів ДНК у плазмі.*

Ключові слова: злоякісні пухлини товстого кишечника, вільно циркулююча ДНК, метилювання ДНК, APC, FHIT, LRRC3B, HIC1.

Сравнительный анализ эпигенетических маркеров в плазме крови пациентов, больных раком толстого кишечника

А. Г. Кондратов, К. А. Некрасов, Л. В. Лотоцкая,
Г. В. Панасенко, Л. А. Столяр, Ю. В. Лапская,
О. О. Колесник, И. Б. Щепотин, А. В. Рындич, В. И. Кашуба

Резюме

Цель. *Разработка менее инвазивных методик для скрининга злокачественных опухолей толстого кишечника (CRC).* ***Методы***. *Использована количественная ПЦР и метил-специфическая ПЦР.* ***Результаты***. *Показано, что среднее значение свободно циркулирующей ДНК в плазме статистически достоверно выше у паци-*

ентов с CRC по сравнению со здоровыми донорами (p < 0,01). Установлено гиперметилирование генов APC, FHIT, LRRC3B и HIC1в опухолях и плазме пациентов с CRC. ***Выводы***. *Нами предложен и проверен новейший подход для скрининга CRC, базирующийся на определении внеклеточной ДНК и метилированных фрагментов ДНК в плазме.*

Ключевые слова: злокачественные опухоли толстого кишечника, свободно циркулирующая ДНК, метилированная ДНК, APC, FHIT, LRRC3B, HIC1.

REFERENCES

1. *Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM*. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010; **127**(12):2893–917.
2. *Rex DK, Bond JH, Winawer S, Levin TR, Burt RW, Johnson DA, Kirk LM, Litlin S, Lieberman DA, Waye JD, Church J, Marshall JB, Riddell RH; U.S. Multi-Society Task Force on Colorectal Cancer*. Quality in the technical performance of colonoscopy and the continuous quality improvement process for colonoscopy: recommendations of the U.S. Multi-Society Task Force on Colorectal Cancer. *Am J Gastroenterol*. 2002; **97**(6):1296–308.
3. *Louis MA, Nandipati K, Astorga R, Mandava A, Rousseau CP, Mandava N*. Correlation between preoperative endoscopic and intraoperative findings in localizing colorectal lesions. *World J Surg*. 2010; **34**(7):1587–91.
4. *Bird A.* DNA methylation patterns and epigenetic memory. *Genes Dev*. 2002; **16**(1):6–21.
5. *Esteller M.* CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene*. 2002; **21**(35):5427–40.
6. *Kohler C, Barekati Z, Radpour R, Zhong XY.* Cell-free DNA in the circulation as a potential cancer biomarker. *Anticancer Res*. 2011; **31**(8):2623–8.
7. *Kim M, Kim JH, Jang HR, Kim HM, Lee CW, Noh SM, Song KS, Cho JS, Jeong HY, Hahn Y, Yeom YI, Yoo HS, Kim YS.* LRRC3B, encoding a leucine-rich repeat-containing protein, is a putative tumor suppressor gene in gastriccancer. *Cancer Res*. 2008; **68**(17):7147–55.
8. *Pichiorri F, Palumbo T, Suh SS, Okamura H, Trapasso F, Ishii H, Huebner K, Croce CM.* Fhit tumor suppressor: guardian of the preneoplastic genome. *Future Oncol*. 2008; **4**(6):815–24.
9. *Dumitrescu RG.* Epigenetic markers of early tumor development. *Methods Mol Biol*. 2012; **863**:3–14.
10. *Dehennaut V, Leprince D.* Implication of HIC1 (Hypermethylated In Cancer 1) in the DNA damage response. *Bull Cancer*. 2009; **96**(11):E66–72.
11. *Kondratov AG, Stoliar LA, Kvasha SM, Gordiyuk VV, Zgonnyk YM, Gerashchenko AV, Vozianov AF, Rynditch AV, Zabarovsky ER, Kashuba VI.* Methylation pattern of the putative tumor-suppressor gene LRRC3B promoter in clear cell renal cell carcinomas. *Mol Med Rep*. 2012; **5**(2):509–12.
12. *Li B, Wang B, Niu LJ, Jiang L, Qiu CC.* Hypermethylation of multiple tumor-related genes associated with DNMT3b up-regulation served as a biomarker for early diagnosis of esophageal squamous cell carcinoma. *Epigenetics*. 2011; **6**(3):307–16.
13. *Kvasha S, Gordiyuk V, Kondratov A, Ugryn D, Zgonnyk YM, Rynditch AV, Vozianov AF.* Hypermethylation of the 5'CpG island of the FHIT gene in clear cell renal carcinomas. *Cancer Lett*. 2008; **265**(2):250–7.
14. *Kristensen LS, Raynor MP, Candiloro I, Dobrovic A.* Methylation profiling of normal individuals reveals mosaic promoter methylation of cancer-associated genes. *Oncotarget*. 2012; **3**(4): 450–61.
15. *Segditsas S, Sieber OM, Rowan A, Setien F, Neale K, Phillips RK, Ward R, Esteller M, Tomlinson IP.* Promoter hypermethylation leads to decreased APC mRNA expression in familial polyposis and sporadic colorectal tumours, but does not substitute for truncating mutations. *Exp Mol Pathol*. 2008; **85**(3):201–6.
16. *Sinha R, Hussain S, Mehrotra R, Kumar RS, Kumar K, Pande P, Doval DC, Basir SF, Bharadwaj M. Kras* gene mutation and RASSF1A, FHIT and MGMT gene promoter hypermethylation: indicators of tumor staging and metastasis in adenocarcinomatous sporadic colorectal cancer in Indian population. *PLoS One*. 2013; **8**(4):e60142.
17. *Tian XQ, Zhang Y, Sun D, Zhao S, Xiong H, Fang J.* Epigenetic silencing of LRRC3B in colorectal cancer. *Scand J Gastroenterol*. 2009; **44**(1):79–84.
18. *Abouzeid HE, Kassem AM, Abdel Wahab AH, El-mezayen HA, Sharad H, Abdel Rahman S.* Promoter hypermethylation of RAS SF1A, MGMT, and HIC-1 genes in benign and malignant colorectal tumors. *Tumour Biol*. 2011; **32**(5):845–52.
19. *Skrypkina IYa, Kondratov OG, Tsyba LO, Panasenko GV, Nikolaienko OV, Romanenko AM, Kolesnyk OO, Morderer DYe, Nekrasov KA, Kashuba VI, Vozianov SO, Shchepotin IB, Rynditch AV.* Detection of cell-free DNA and gene-specific methylation in blood plasma of patients with renal and colon cancer. *Science and Innovation*. 2012; **8**(6):60–6.

Received 28.08.13