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HUMAN GENETICS

Heterogeneity in Colorectal Primary Tumor and Synchronous Liver Metastases

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Abstract—The expression profile of the *ZEB1*, *ZEB2*, *VIM*, *CDH1*, *SFRP2*, *FOXQ1*, *TNC*, *MACC1*, *PLS3*, *CFTR*, *FLNA*, *MUC2*, *TFF3*, and *RARRES3* genes, as well as the mutational status of the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes, were investigated in 40 patients with colorectal cancer and liver metastases. A comparative analysis of changes in gene expression in primary tumor cells and liver metastases was performed. Statistically significant differences were found between the expression levels of the *ZEB2* ($p = 0.004$), *VIM* ($p < 0.001$), *FLNA* ($p = 0.04$), and *MUC2* ($p < 0.001$) genes. It was demonstrated that the overall frequency of mutations of the *KRAS* gene was 18/40 (45%) and the *PIK3CA* gene was 9/40 (23%). Mutations in the *NRAS* and *BRAF* genes were not found. The concordance between the primary tumor and metastases in the liver by mutation status was 100%.

Keywords: colorectal cancer, liver metastases, intratumor heterogeneity, gene mutations, gene expression, epithelial-mesenchymal transition

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INTRODUCTION

One of the main causes of growth, progression, metastasis, and resistance to antitumor therapy is a high intratumor heterogeneity [1]. Molecular genetic changes revealed in colorectal cancer (CRC) made it possible to identify several subtypes of tumors [2]. All subtypes are associated with a change in expression and the mutational status of the genes. The following genes were identified as potential markers: *ZEB1*, *ZEB2*, *VIM*, *CDH1*, *SFRP2*, *FOXQ1*, *TNC*, *MACC1*, *PLS3*, *CFTR*, *FLNA*, *MUC2*, *TFF3*, *RARRES3*, *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*. Most often, synchronous metastases of CRC occur in the form of liver metastases (25%) and peritoneal carcinomatosis (4.8–15%) [3]. It is interesting what cells or subpopulations of tumor cells are prone to metastasis and how much the tumor and its metastases are heterogeneous. In order to answer this question, we studied the expression and mutational status of genes in primary CRC tumors and their liver metastases.

MATERIALS AND METHODS

The analysis of the expression of the *ZEB1*, *ZEB2*, *VIM*, *CDH1*, *SFRP2*, *FOXQ1*, *TNC*, *MACC1*, *PLS3*, *CFTR*, *FLNA*, *MUC2*, *TFF3*, and *RARRES3* genes and mutations in the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes was performed in a primary tumor of the large intestine and synchronous liver metastases (SLM). The material for the study was obtained from 40 patients who underwent a one-stage removal of the colon tumor and SLM at the State Scientific Centre of Coloproctology in the period from January 1, 2013, to December 31, 2016. Chemo- and/or radiation therapy served as a criterion for excluding patients from the study. A comparative analysis was performed among the following samples: histologically unchanged colon mucosa, primary tumor tissue, and SLM tissue. All the samples were morphologically verified. Demographic and clinical morphological data of patients are given in Table 1.

The tumor material and SLM were immediately placed in a lysis solution (buffer GA, Tiangen) and frozen at –70°C until further analysis.

Measurement of the level of expression of the investigated genes was carried out on a StepOnePlus System (Applied Biosystems, United States) using oligonucleotide primers designed by the specialists at the Laboratory Genetics Department of the State Scientific Centre of Coloproctology using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). Twenty-five microliters of the reaction mixture included 0.25 μM of each original oligonucleotide primer, 200 μM of each nucleoside triphosphate, 1 unit of *Taq* polymerase, PCR buffer (500 mM Tris and 500 mM KCl, pH 8.74), 2.5 μL of $MgCl_2$ (25 mM), intercalating dye

Table 1. Clinical morphological characteristics of the patients

Data		Value
Age		53.5 ± 11
Sex	Female	21 (53%)
	Male	19 (48%)
CEA*		13.4 (3; 48)
CA 19-9**		14.5 (3; 36)
Localization:		
rectum		22 (55%)
colon		18 (45%)
Median of tumor size of large intestine, cm (quartile)		5 (4; 7)
T2		1 (2%)
T3		23 (58%)
T4		16 (40%)
N0		6 (15%)
N+		34 (85%)
Amount of lymph nodes with SLM		
<5		26 (67%)
5 and more		13 (33%)
MDA***		32 (80%)
LGA****		5 (13%)
AM*****		3 (7%)
Perivasal, perineural invasion		33 (83%)
Median number of SLM (quartiles)		3 (1; 5)
solitary		13 (32%)
single		15 (38%)
multiple		12 (30%)
Median size of SLM, cm (quartiles)		3 (2; 6)
<5		27 (68%)
5 or more		13 (32%)

* Cancer embryonic anigen.
** Carbon antigen 19-9 (tumor marker).
*** Moderately differentiated adenocarcinoma.
**** Low-grade adenocarcinoma.
***** Adenocarcinoma mucosa.

EvaGreen, and deionized water. The change in gene expression by ±2 times was considered significant. RNA was isolated using the RNAprep Pure Kit for Tissue (Tiangen, China) according to the manufacturer's protocol. The RNA quality was evaluated on a Nanophotometer P300 (Implen, Germany). RNA with a concentration of at least 10 ng/μL and parameters A260/230 of 1.8–2.1 was used. The reverse transcription reaction was performed using Random6 hexaprimers (Syntol, Russia), Thermo Scientific RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania), and Thermo Scientific RiboLock RNase Inhibitor (Thermo Scientific, Lithuania) on the Veriti System (Applied Biosystems, United States). The concentration of cDNA was 250–450 ng/μL (LID10), at which the change in gene expression was linear. Measurement of the expression level of genes was carried out in threefold repetition. The *GAPDH* and *ACTB* genes were used as reference. The data obtained was analyzed in StepOnePlus Software v. 2.2.2 and according to the work of Pfaffl [4].

To determine the mutational status, the fragments of the *KRAS* (RefSeq_NM_004985) (exons 2–4), *NRAS* (RefSeq_NM_002524) (exons 2–4), *BRAF* (RefSeq_NM_004333) (exon 15), and *PIK3CA* (RefSeq_NM_006218) (exons 9 and 20) genes were amplified during the polymerase chain reaction (PCR) on a programmable thermocycler TP4-PCR-01-Tertsik (DNA-Technology, Russia) using oligonucleotide primers designed by the specialists at the Department of Laboratory Genetics of the State Scientific Centre of Coloproctology using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). Composition of the reaction mixture: 0.1–1.0 μg of genomic DNA; 0.25 μM of each original oligoprimer; 200 μM of each nucleoside triphosphate; 1 unit of *Taq* polymerase; PCR buffer (500 mM Tris and 500 mM KCl, pH 8.74), 2.5 μL $MgCl_2$ (25 mM); deionized water; 30 μL of mineral oil. Mutations in the genes were determined on an ABI PRISM 3500 automatic sequencer (Applied Biosystems, United States). For mutation interpretation, software from Applied Biosystems was used.

Determination of the status of microsatellite instability (MSI) was carried out by fragment analysis on the ABI PRISM 3500 System (8 capillaries; Applied Biosystems, United States) using five mononucleotide markers (NR21, NR24, NR27, BAT25, and BAT26) described in [5, 6].

The data were analyzed using Statistica 12.0.

RESULTS

The gene expression levels in the primary tumor and synchronous metastases were obtained for 40 patients with CRC. The median levels of gene expression and the results of the comparative analysis are shown in Fig. 1.

Expression of the *ZEB2*, *VIM*, *FLNA*, and *MUC2* genes in metastases was statistically significantly different from the data for the tumor (Table 2). Against the reduced expression of the *ZEB2*, *VIM*, and *MUC2* genes in the primary tumor, in metastasis, in comparison to the samples of histologically unchanged mucous membrane of the large intestine, the expression of the *ZEB2* gene did not change; the expression of the *VIM* and *MUC2* genes increased and decreased, respectively. In contrast to the aforementioned three genes, there is a further increase in the level of expression of the *FLNA* gene in SLM compared with an increased expression of this gene in the primary tumor.

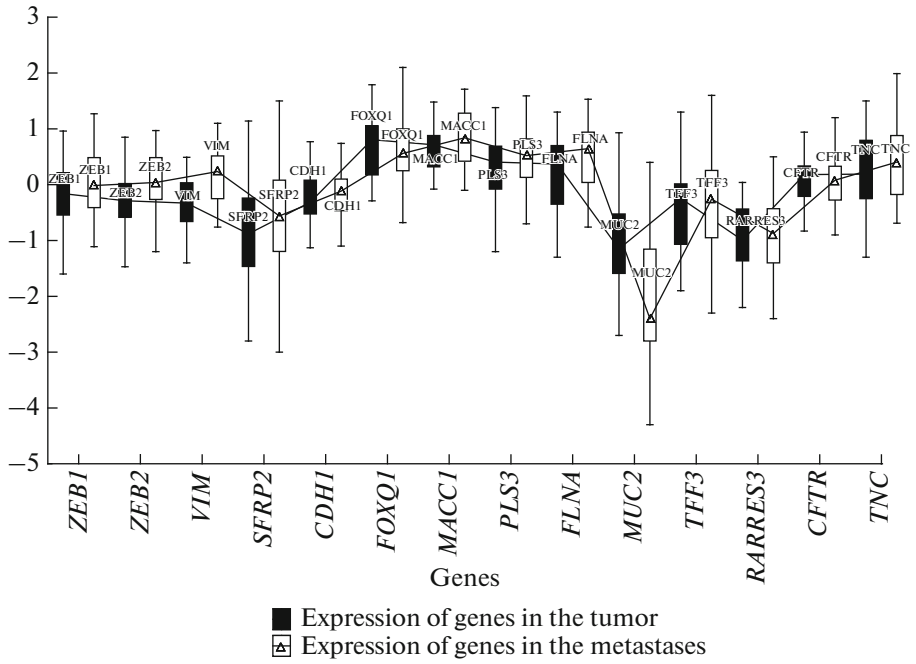

Fig. 1. Comparison of the expression of the genes in the primary tumor and synchronous liver metastases (SLM).

In 10 genes (*ZEB1*, *SFRP2*, *CDH1*, *FOXQ1*, *MACC1*, *PLS3*, *TFF3*, *RARRES3*, *CFTR*, and *TNC*) of 14, the expression levels in the primary tumor and SLM were changed to a lesser extent (Table 2). Of these 10 genes, increased expression was observed among genes *FOXQ1*, *MACC1*, and *PLS3*; reduced expression was observed among *SFRP2*, *CDH1*, and *RARRES3*; and unchanged expression was noted among *ZEB1*, *CFTR*, *TNC*, and *TFF3*.

To determine the dependence of the expression level of genes in the SLM in respect of the primary tumor, a correlation analysis was performed (Table 3). In nine genes (*ZEB1*, *ZEB2*, *CDH1*, *SFRP2*, *TNC*, *MACC1*, *PLS3*, *FLNA*, and *RARRES3*), the tendency to increase the expression in the SLM was determined. Separately, there is a strong relationship between the expression of the *PLS3* gene in metastasis and in the primary tumor ($r = 0.75$). Expression of the *FOXQ1*, *MUC2*, *TFF3*, and *CFTR* genes was suppressed more strongly in SLM relative to the primary tumor. It is worth noting that the decrease in the expression of the *MUC2* and *TFF3* genes in SLM occurs even against the suppressed expression of these genes in the primary tumor. Despite a statistically significant increase in the expression of the *VIM* gene in the SLM (Table 2), the relationship between the expression of this gene in the SLM and primary tumor samples was not revealed (Table 3).

Somatic mutations of the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes and the status of MSI were determined in the primary tumor and SLM. The total frequency of mutations of the *KRAS* gene was 18/40 (45%) and the *PIK3CA* gene was 9/40 (23%) (Table 4). Mutations in the *NRAS* and *BRAF* genes were not found. The status of all primary tumors and SLM by microsatellite markers was stable (MSS). The concordance between primary tumor and SLM concerning mutational and microsatellite status was 100%.

Table 2. Expression levels of the genes in primary tumor and SLM

Genes	Median expression, log10		*p*
	primary tumor, $n = 40$	SLM, $n = 40$	
ZEB1	−0.16	−0.02	0.11
ZEB2	−0.29	0.04	0.004
VIM	−0.33	0.24	<0.001
SFRP2	−0.88	−0.56	0.44
CDH1	−0.32	−0.12	0.54
FOXQ1	0.81	0.56	0.76
MACC1	0.72	0.84	0.08
PLS3	0.41	0.52	0.25
FLNA	0.37	0.65	0.04
MUC2	−1.15	−2.4	<0.001
TFF3	−0.25	−0.26	0.74
RARRES3	−1.0	−0.88	0.87
CFTR	0.18	0.08	0.6
TNC	0.19	0.41	0.09

Table 3. Correlation of expression of genes between primary tumor and SLM

Genes	*r*	*p*
ZEB1	0.38	0.016
ZEB2	0.34	0.033
VIM	0.09	0.577
SFRP2	0.48	0.002
CDH1	0.62	0.0001
FOXQ1	0.42	0.007
MACC1	0.63	0.0001
PLS3	0.75*	0.0001
FLNA	0.63	0.0001
MUC2	0.42	0.007
TFF3	0.67	0.0001
RARRES3	0.62	0.0001
CFTR	0.58	0.0001
TNC	0.33	0.037

Table 4. Mutations of the *KRAS* and *PIK3CA* genes in the primary tumor and SLM

Gene	Tumor	SLM
KRAS	18 (45%)	18 (45%)
PIK3CA	9 (23%)	9 (23%)
KRAS + *PIK3CA*	4 (10%)	4 (10%)

DISCUSSION

In 1976, P.C. Nowell hypothesized that most tumors arise from a single cell of origin, and tumor progression is the result of acquired genetic changes within the original clone [7]. Later, in 1988, Fearon and Vogelstein [8] proposed a model for the development of colorectal cancer. According to this model, the development and progression of the tumor begins at the adenoma stage by the gradual accumulation of genetic changes in it. At the first stage, a mutation in the *APC* gene occurs in the colon mucosa cells, which leads to the activation of the Wnt/β-catenin pathway involved in cell differentiation and proliferation. As a result, an adenoma is formed. At the next stage, the *KRAS* acquires a mutation. Mutations in this proto-oncogene lead to activation of the RAS/BRAF/MAPK pathway, as a result of which the adenoma begins to progress, changing in size. Moreover, the active growth of the adenoma induced by the *KRAS* mutation leads to its polyclonality and the formation of cancer stem cells (CSC), which are distinguished by the absence of a mutation in the *APC* gene [9]. It is believed that the *KRAS* gene is a key driver in the formation of CRC. According to the results of our study, the frequency of mutations in the *KRAS* gene in CRC was 45%, which indicates the presence of intratumoral heterogeneity; i.e., during the invasion, cells with different molecular characteristics can bud from the tumor: with or without mutation in the *KRAS* gene or other molecular-genetic changes. The presence of tumor heterogeneity is also confirmed by the combined mutations of the *KRAS* and *PIK3CA* genes detected both in the tumor and in its metastases (Table 4). The total concordance of the mutational status of the *KRAS* and *PIK3CA* genes between the primary tumor and metastasis may indicate that the latter is formed from predominant tumor subclones, and other subclones may exist in a small amount and activate under certain conditions [10].

Thus, liver metastases are less heterogeneous than the primary tumor. Sadanandam et al. [11] identified marker genes whose expression changes are associated with different tumor subtypes developing from different cells of the Lieberkun crypt. A classification is proposed that includes six subtypes. The stem-like subtype is characterized by a high level of expression of the *SFRP2* gene. The inflammatory subtype has a high level of expression of the *RARRES* gene. The transit-amplifying (TA) subtype, which in turn is divided into cetuximab-resistant (CR-TA) and cetuximab-sensitive (CS-TA), is characterized by a change in the expression of the *CFTR* and *FLNA* genes. Moreover, the level of *CFTR* expression was increased in both the cetuximab-resistant and cetuximab-sensitive subtypes and *FLNA* was increased only in the cetuximab-resistant subtype. The goblet-like subtype has a high level of expression of the *MUC2* and *TFF3* genes. In the enterocyte subtype, the biomarkers are the same as in the goblet-like one. However, it has a lower level of expression of the *TFF3* gene.

In order to determine what cells or subpopulations of cells are more often metastasized to the liver, we performed a comparative analysis of the expression of the *SFRP2*, *RARRES*, *CFTR*, *FLNA*, *MUC2*, and *TFF3* genes, proposed by the authors of [11]; *FOXQ1*, *MACC1*, *PLS3*, and *TNC*, associated with a high metastatic potential [12–15]; and *ZEB1*, *ZEB2*, *VIM*, and *CDH1*, associated with the epithelial-mesenchymal transition (EMT) [16], between the samples of primary tumors and metastases. In our study, for 10 of the 14 genes, the expression values in tumors and SLMs coincide. However, these values are multidirectional. An increased level of expression is observed among the *FOXQ1*, *MACC1*, and *PLS3* genes. Reduced expression is observed among *CDH1*, *SFRP2*, and *RARRES3*. Unchanged expression is seen among *ZEB1*, *CFTR*, *TNC*, and *TFF3*. As already mentioned, high expression of the *FOXQ1*, *MACC1*, and *PLS3* genes is associated with unfavorable prognosis, high metastatic potential, and rapid growth of tumor and metastases. The low level of expression of the *SFRP2* and *RARRES3* genes indicates the absence or very low concentration of stem-like and inflammatory cells in the tumor and SLM. Reduced expression of the *CDH1* gene indicates a violation of intercellular adhesion

[17]. In addition, low expression of *CDH1* is associated with EMT. The EMT program plays an important role in the formation and differentiation of various organs and tissues during embryonic development. As a pathological process, EMT triggers a tumor progression, forming the ability of cells to migrate, invade the adjacent stroma, and reach the bloodstream. In the development of EMT in cancer cells, the transcription profile of a significant number of genes changes—some transcription factors (ZEB1/2, SNAIL1/2, VIM, etc.) and mesenchymal markers are overexpressed, and epithelial phenotype markers (CDH1) are suppressed [16]. In our study, the expression of the *ZEB1*, *ZEB2*, and *VIM* genes in the primary tumor is low, but in metastases, the expression of *ZEB2* and *VIM* is increased, and *ZEB1* tends to increase (Table 2). Expression of markers of the goblet-like subtype (*MUC2* and *TFF3*) is reduced both in the primary tumor and in metastasis. Interestingly, the expression of the *MUC2* gene is 2 times lower in metastasis relative to the primary tumor ($p = 0.001$), which may indicate that the metastasis contains a very small amount of mucin-producing cells, and a low expression level of this gene can be a marker of liver metastases. The expression of the *CFTR* and *FLNA* genes in the primary tumor is increased, which indicates the presence of a subpopulation of cells with a cetuximab-resistant subtype. In metastases, the level of *CFTR* expression is lower relative to the primary tumor and does not differ from that in normal mucosa. Expression of *FLNA*, conversely, in metastases is significantly increased relative to the primary tumor.

Summarizing the results, we should emphasize that statistically significant differences between the primary tumor and metastasis have been revealed by expression levels of the following genes: *ZEB2* ($p = 0.004$), *VIM* ($p < 0.001$), *FLNA* ($p = 0.04$), and *MUC2* ($p < 0.001$). In conclusion, it should also be noted that the high level of concordance of the primary tumor and metastasis for the mutation status, in particular, for the *KRAS* gene, confirms the fact that the material of the primary tumor is sufficient to select the drug treatment of CRC. Further investigation of the heterogeneity of primary and secondary tumor lesions can potentially influence the choice of drug therapy and determine the prognosis of the disease.

The work was carried out within the framework of the registered prospective study "Effects of Gene expression pattern and RAS/BRAF Mutations on the Course of Colorectal Cancer" ISRCTN18093312 (https://doi.org/10.1186/ISRCTN18093312).

REFERENCES

1. Alizadeh, A.A., Aranda, V., Bardelli, A., et al., Toward understanding and exploiting tumor heterogeneity, *Nat. Med.*, 2015, vol. 21, no. 8, pp. 846—853. doi 10.1038/nm.3915
2. Guinney, J., Dienstmann, R., Wang, X., et al., The consensus molecular subtypes of colorectal cancer, *Nat. Med.*, 2015, vol. 21, no. 11, pp. 1350—1356. doi 10.1038/nm.3967
3. Sushkov, O.I. and Achkasov, S.I., Peritoneal carcinomatosis from colon cancer: approaches to treatment (literature review), *Koloproktologiya*, 2016, vol. 58, no. 4, pp. 69—79.
4. Pffafl, M.W., A new mathematical model for relative quantification in real-time RT–PCR, *Nucleic Acids Res.*, 2001, vol. 29, no. 9, pp. 2002—2007.
5. Tsukanov, A.S., Pospekhova, N.I., Shubin, V.P., et al., Differential diagnosis of Lynch syndrome from other forms of nonpolyposis colorectal cancer among Russian patients, *Ross. Zh. Gastroenterol., Gepatol., Koloproktol.*, 2014, vol. 24, no. 2, pp. 78—84.
6. Paklina, O.V., Setdikova, G.R., Daabul', A.S., et al., Role of microsatellite instability in ampullar carcinoma, *Farmateka*, 2016, vol. 8, pp. 80—84.
7. Nowell, P.C., The clonal evolution of tumor cell populations, *Science*, 1976, vol. 194, pp. 23—28.
8. Fearon, E.R. and Vogelstein, B.A., Genetic model for colorectal tumorigenesis, *Cell*, 1990, vol. 61, pp. 759—767.
9. Moon, B.S., Jeong, W.J., Park, J., et al., Role of oncogenic K-Ras in cancer stem cell activation by aberrant Wnt/β-catenin signaling, *J. Natl. Cancer Inst.*, 2014, vol. 106, no. 2. djt373. doi 10.1093/jnci/djt373
10. Diaz, L.A., Williams, R.T., Wu, J., et al., The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers, *Nature*, 2012, vol. 486, no. 7404, pp. 537—540. doi 10.1038/nature11219
11. Sadanandam, A., Lyssiotis, C.A., Homicsko, K., et al., A colorectal cancer classification system that associates cellular phenotype and responses to therapy, *Nat. Med.*, 2013, vol. 19, no. 5, pp. 619—625. doi 10.1038/nm.3175
12. Li, Y., Zhang, Y., Yao, Z., et al., Forkhead box Q1: a key player in the pathogenesis of tumors (review), *Int. J. Oncol.*, 2016, vol. 49, no. 1, pp. 51—58. doi 10.3892/ijo.2016.3517
13. Stein, U., Walther, W., Arlt, F., et al., MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis, *Nat. Med.*, 2009, vol. 15, no. 1, pp. 59—67. doi 10.1038/nm.1889
14. Kujawski, R., Przybyłowska-Sygut, K., Mik, M., et al., Expression of the *PLS3* gene in circulating cells in patients with colorectal cancer, *Pol. Przegl. Chir.*, 2015, vol. 87, no. 2, pp. 59—64. doi 10.1515/pjs-2015-0020
15. Mettouchi, A., Cabon, F., Montreau, N., et al., The c-Jun-induced transformation process involves complex regulation of tenascin-C expression, *Mol. Cell Biol.*, 1997, vol. 17, no. 3, pp. 202—209. PMID: 9154819
16. Shelygin, Y.A., Pospekhova, N.I., Shubin, V.P., et al., Epithelial-mesenchymal transition and somatic alteration in colorectal cancer with and without peritoneal carcinomatosis, *Biomed. Res. Int.*, 2014, vol. 2014, article ID 629496. doi 10.1155/2014/629496
17. Takeichi, M., Cadherin cell adhesion receptors as a morphogenetic regulator, *Science*, 1991, vol. 251, pp. 1451—1455. PMID: 2006419

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