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SEARCH FOR POTENTIAL GASTRIC CANCER MARKERS USING miRNA DATABASES AND GENE EXPRESSION ANALYSIS

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***Aim*: The aim of this study was to identify genes that are differentially expressed in gastric tumors and to analyze the association of their expression level with tumor clinicopathologic features. *Methods*: In the present research, we used bioinformatic-driven search to identify miRNA that are down-regulated in gastric tumors and to find their potential targets. Then, the expression levels of some of the target mRNAs were investigated using reverse transcription polymerase chain reaction (RT-PCR) analysis. *Results*: As a result of the bioinformatics analysis, fifteen genes were found to be potentially differentially expressed between the tumors and normal gastric tissue. Five of them were chosen for the further analysis (*WNT4*, *FGF12*, *EFEMP1*, *CTGF*, and *HSPG2*) due to their important role in cell proliferation and differentiation. Expression levels of these genes were evaluated in our collection of frozen tissue samples of gastric tumor and paired normal stomach epithelia. Increased *FGF12* expression was observed in diffuse type of gastric cancer while *WNT4* mRNA was found to be down-regulated in intestinal type of gastric cancer. Besides, *CTGF* gene overexpression was revealed in diffuse type of stomach cancer in comparison with that in intestinal type. Up-regulation of *CTGF* was also associated with lymph node metastasis. *Conclusions*: The findings show its expedient to perform further investigations in order to clarify diagnostic and prognostic value of *CTGF*, *FGF12,* and *WNT4*'s in stomach cancer as well as the role of these genes in carcinogenesis.**

***Key Words*: gastric cancer, molecular markers, bioinformatic search, gene expression, RT-PCR.**

Gastric cancer is one of the most common cancers worldwide and one of the leading cause for cancer-related deaths. Incidence rate of gastric cancer in Russia was 17.2/100 000 in 2009 ranking second among men and third among women. Gastric cancer is also characterized with high one-year mortality rates (53.2%), and low overall 5-year relative survival regardless of gender (20.2–21.1%.) [1]. Overall prognosis of gastric cancer is generally poor due to late manifestation of the disease. On the other hand, the decline in cancer mortality trends is related not only to improvement of treatment methods but also to early manifestation and accurate prognosis of given disease for treatment optimization. If gastric cancer is detected at an early stage, the 5-year survival rate is approximately 69.3–75.5%. In contrast, the 5-year survival rate of patients with advanced-stage of the tumor is only 5.1–6.4% [2].

In the early stages of gastric cancer, most patients are asymptomatic, making it difficult to control the malignancy rate through early diagnosis and motivational therapy. Although there are several diagnostic tools for detecting the clinically divergent conditions of gastric carcinomas, such as computed tomography, scanning, endoscopic ultrasound (EUS), magnetic resonance imaging (MRI), and positron emission tomography (PET) scans, they are no effective in early diagnosis and adequate prognosis of gastric cancer. There are practically no reliable methods for early gastric cancer diagnosis based on molecular markers detection. The most promising variants of biological molecules that are already used for that purpose are CA-19.9 (cancer antigen 19.9), CA 72-4, and CEA (carcinoembryonic antigen). All these markers have a moderate prognostic value for approximately one third of patients and also have significant cross-reactivity and thus don't effective enough for early diagnosis [3]. In the past few years some evidences have been obtained illustrating potential diagnostic value of a number of secreted proteins such as NF2, NEK6, INHBA, CDH17, and PDCD6. However further investigations are needed to confirm their clinical value [4, 5].

Adenocarcinoma accounts for over 95% of all malignant gastric neoplasms, and generally the term gastric cancer refers to adenocarcinoma of the stomach. They can be classified based on histopathological features. The most commonly used classifications of gastric cancer are the World Health Organization (WHO) [6] and the Laurén classification. Laurén classification describes two main histological types, diffuse and intestinal, having different clinicopathological characteristics that affect disease prognosis and outcome [7, 8]. Although most of the genetic alterations that have been reported are observed in both intestinal and diffuse types of tumors, it is considered that molecular pathogenesis of diffuse and intestinal types of gastric cancer differs significantly [9]. It is thought that knowledge of these

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Abbreviations used: cDNA – complementary DNA; CTGF – connective tissue growth factor; EFEMP1 – epidermal growth factor-containing fibulin-like extracellular matrix protein 1; FGF12 – fibroblast growth factor 12; HSPG2 – heparan sulfate proteoglican 2; miRNA – microRNA; RT-PCR – reverse transcription polymerase chain reaction; WNT4 – wingless-type MMTV integration site family, member 4.

subtypes of gastric carcinomas due to identification and investigation of their genetic differences may become the key step to make the diagnosis and treatment of gastric cancer more effective.

Although the most cases of gastric cancer are seemed to be sporadic two hereditary syndromes have been characterized — hereditary diffuse gastric cancer (HDGC) and gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) [10]. HDGC syndrome is often attributed to disorders in the work of E-cadherin gene (*CDH1*, cadherin 1, type 1, the major regulator of cell adhesion) as a consequence of the gene mutations or promoter hypermethylation. Sporadic malignant diffuse gastric neoplasms, in turn, are associated with functional disorders of following genes: *RUNX3, CDH1 FGFR2/KSAM,* and *CDKN2A* [11–13].

GAPPS is a unique gastric polyposis syndrome with a significant risk of gastric adenocarcinoma with areas of dysplasia or intestinal type of gastric cancer. Mutations in *APC, MUTYH, CDH1, SMAD4, BMPR1A, STK11,* and *PTEN* genes were studied in several families by sequencing of the genes exons and their deletions and duplications assays but causal genetic defect remains unidentified [10]. Typical genetic alterations of sporadic intestinal gastric carcinomas are inactivation of *HMLH1* gene and mutation of *CDX2* (caudal type homeobox transcription factor 2) as well as *ps2, RARB, HER-2/neu,* etc. CDX2 is a transcription factor which is involved in epithelium differentiation of colon, pancreas and gallbladder. It interacts with such tumor suppressors as APC, E-cadherin as well as with bcl-2 (transcription regulator of such colon epithelium differentiation factors genes as *MUC2, MUC5AC, MUC6,* and *CDX2*). The loss of *CDX2* expression in the case of intestinal type of gastric cancer may be the marker of tumor progression [11–13].

In order to find novel molecular markers, transcription and proteomic approaches are used. The major goal of transcriptional investigations is to identify miRNAs mostly expressed in tumors but not in normal tissues. It is reached through the analysis of databases containing microarray gene expression data for thousands of genes, and through the use of commercial microarrays for estimating gene expression level in own collection of normal and tumor tissue specimens [14, 15].

The study of proteins expression level by means of comparative analysis of the proteome of normal and tumor tissue with 2D electrophoresis followed by the identification of differently expressed proteins with mass spectrometry is also productive enough. The confirmation of differences in gene expression is held with the use of RT-PCR and WB-analysis of tumor and paired normal tissue samples in that case [4, 15–17].

The aim of this study was to reveal the potential biological markers of different histological types of gastric cancer by means of the bioinformatics-driven search for cancer-related miRNA and comparative gene expression analysis of their targets. The expression analysis was performed in paired specimens of normal and tumor tissue. During the bioinformatics search with miRNA expression databases, not only transcriptional level of candidate genes but also their miRNA regulation was estimated. All these greatly improve the reliability of the method and restrict the number of identified genes taken for further analysis.

MATERIALS AND METHODS

Clinical specimens. This study was approved by the Institutional Ethics Committee of Tomsk Cancer Research Institute. Informed consents were obtained from all patients prior to analysis. Gastric adenocarcinoma and paired normal tissue samples were obtained from 37 patients who underwent surgical resections at the Department of Abdominal and Thoracic Oncology of Tomsk Cancer Research Institute of Siberian Branch of Russian Academy of Science (Tomsk, Russia). Surgical pathologic staging was assigned according to the TNM Classification [18]. A summary of clinicopathologic information for all patients is shown in Table 1.

Table 1. Clinicopathologic features of all patients (n=37)

Clinicopathological parameter		N (%)
Depth of invasion	T1	3 (8.11)
	T2	15 (40.54)
	T3	10 (27.03)
	T4	9 (24.32)
Nodal status	N0	14 (37.83)
	N1	16 (43.24)
	N2	6 (16.22)
	N3	1 (2.70)
Metastasis	M0	33 (91.7)
	M1	3 (8.3)
Age	⩽ 50	8 (21.62)
	> 50	29 (78.38)
Histological type	diffuse	16 (43.24)
	intestnal	21 (56.76)
Stage	I	10 (27.78)
	II	7 (19.44)
	III	11 (30.56)
	IV	8 (22.22)
Gender	male	21 (55.56)
	female	16 (44.44)
Degree of differentiation	G1	1 (3.1)
	G2	14 (43.75)
	G3	17 (53.15)

Notes: TNM classification: T – the extent of the primary tumor; N – the absence or presence and extent of regional lymph node metastasis; M – the absence or presence of distant metastasis.

RNA isolation and cDNA synthesis. Tissue samples were obtained from patients during operation, kept in "RNAlater" solution ("Ambion", USA) at temperature of +4°C overnight and then stored at –80°C. These samples were homogenized with Sartorius Mikro Dismembrator U ("Eppendorf", Germany) at 7300 rpm under cooling with liquid nitrogen. The RNA was extracted from the tissue samples using the RNeasy mini kit plus DNase I digestion (Qiagen, Gmbh, Hilden; Cat no: 74106) as per the manufacturer's instructions. RNA was reverse transcribed into cDNA using the RevertAid kit with random hexanucleotide primers ("Fermentas", Lithuania) following the manufacturer's instructions.

RNA quality determination. RNA concentration and quality were measured with a NanoDrop-2000 spectrophotometer ("Thermo Scientific", USA). The concentration of RNA ranged from 80 to 250 ng/μl. The optical density ratios at 260/280 and 260/230 to ex-

amine RNA quality were in the range of 1.95–2.05 and 1.90–2.31, respectively. RNA integrity was assessed by visualization of the 28S and 18S ribosomal RNA in 1.5% agarose gels followed by 0.02% ethidium bromide staining. RNA was stored at –80°C for further use. In order to estimate genomic DNA contamination of extracted RNA no template control (NTC) was performed. The control RT reactions contained all components including template RNA, except for the reverse transcriptase.

Quantitative real time PCR. Validation of the gene expression was done using comparative Cq method of quantitative real time RT-PCR. ACTB was chosen for normalizing the data [8]. Each reaction was performed in a total volume of 15 µl.

qRT-PCR was performed in triplicate reactions in a volume of 15 µl containing 250 IM dNTPs ("Sibenzyme", Russia), 300 µM forward and reverse primers, 200 µM probe, 2.5 mM Mgastric cancerl2, 19 SE buffer (67 mM Tris–HCl pH 8.8 at 25 °C, 16.6 mM (NH4)2SO4, 0.01% Tween-20), 2.5U Hot Start Taq polymerase ("Sibenzyme", Russia), and 50 ng of template cDNA. Samples were heated for 10 min at 95 °C, followed by 40 cycles of amplification for 10 s at 95 °C and 27 s at 60 °C.

Primer and probes were designed using Vector NTI 11.5 and NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2).

Table 2. Sequence of the primers and probes used in the study

Gene	GenBank Accession		Sequence	Design
ACTB (actin beta)	NM_001101.3 75 bp	F R Probe	5'-gagaagatgacccagatcatgtt-3' 5'-atagcacagcctggatagcaa-3' FAM 5'-agaccttcaacaccccagccat-3'BHQ1	**OrD**
CTGF (connective tissue growth factor)	NM_001901.2 78 bp	F R Probe	5'-gtgtgcaccgccaaagat-3' 5'-gctgctctggaaggactctc-3' FAM 5'-tgctccctgcatcttcggtgg-3'BHQ1	**OrD**
EFEMP1 (EGF containing fibulin-like extracellular matrix protein 1)	NM_001039348.2 102 bp	F R Probe	5'- agtcacaggacaccgaagaa-3' 5'-gtcacattcatcaatatctttgca-3' FAM 5'-tgcactgacggatatgagtggga-3'BHQ1	**OrD**
FGF12 (fibroblast growth factor 12)	NM_004113.5 77 bp	F R Probe	5'-cgaaaacagcgactacactct-3' 5'-tagccttcactccttggatgg-3' FAM 5'-ccgtgggcctgcgtgtagt-3'BHQ1	**OrD**
HSPG2 perlecan (heparan sulfate proteoglycan 2)	NM_005529.5 137 bp	F R Probe	5'-cctgccaggactgtgcc-3' 5'-gttgtgctggcattgcga-3' FAM 5'-cagacctgtgccacccagagact-3'BHQ1	**OrD**
WNT-4 (wingless-type MMTV integration site family, member 4)	NM_030761.4 76 bp	F R Probe	5'-cgagcaactggctgtacct-3' 5'-ttgagtttctcgcacgtctc-3' FAM 5'-cgtcggtggggagcatctca-3'BHQ1	**OrD**

Notes: M number according to NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore); F – forward primer; R – reverse primer; OrD – Original design.

Bioinformatics analysis. Bioinformatics analysis using databases and analysis of literature sources was performed for detection of miRNA with significant down-regulation in tumor tissue. To identify the most probable targeted mRNAs the original algorithm of TargetScan (http://www.targetscan.org.) and SAGE (http://cgap.nci.nih.gov/SAGE) data analysis was performed [19]. The search for up-regulated tumor-associated mRNAs was carried out with the usage of following informational resources: Oncomine (https://www.oncomine.com/), dbEST (http://www.ncbi.nlm.nih.gov/dbEST/), SAGE and Chen sample collection [20].

Statistical analysis. Mann — Whitney U-test was used to assay the significance of the differences obtained. The median and range for the expression value of each gene in each group were calculated using Statistica 8.0 software (ver. 6.1, serial number 1203d, StatSoft, Inc.). A single gene was defined as overexpressed when more than twofold expression increase was observed during the analysis in a tumor tissue sample versus its paired normal. The significance of these differences was evaluated using the Chi-square test. Two-tailed test and Yates' chi-squared test were used for obtaining the *p* value in the case of small groups (less than 5) being analyzed.

RESULTS AND DISCUSSION

miRNAs are small single-stranded regulatory RNAs capable of interfering with mRNAs and thus are important element of post-transcriptional gene silencing mechanism. There are approximately 700 miRNAs in humans and up to one third of human total mRNA are potential miRNA targets. It is believed that different miRNAs can be involved in carcinogenesis playing oncogenic as well as tumor suppressor role [21]. Determination of miRNA expression pattern can be used for identification of potential protein tumor markers [22]. For it to be effective a number of significantly down-regulated miRNA as well their targets should be found in tumor. Here one faced with two important circumstances. The first of them is a possibility that a single mRNA could be targeted by several different miRNA providing different regulating influence on the gene expression. The second is the requirement to consider the targeted both mRNA transcription profile and its translation activity in tumor. These problems were solved using our approach of the double selection method testing the transcriptional as well as translational levels of genes under study [18]. The influence of both up and down-regulated miRNA in tumors was also estimated.

At the first stage of bioinformatics-driven search, approximately one hundred of literature sources were analyzed. As a result, 90 miRNA were found to be differentially expressed in tumor compared to normal tissue according to literature data. 50 of them were found to be significantly down-regulated in tu-

mor, whereas the others were up-regulated. Then, mRNA targets prediction with the use of TargetScan (http://www.targetscan.org.) database as well as the choice of more perspective mRNA-miRNA pairs were carried out. During the procedure each of these pairs were assigned to the ε number (score in TargetScan) showing the accuracy of the prediction as well as the miRNA influence on its targeted mRNA translation. As a result a list was obtained containing miRNA capable to regulate 15 998 genes of the whole human genome. Then 935 of genes coding secreted proteins were chosen for further work. 100 of them were predicted as the most perspective using the value of scoring-function S (that reflects the probability for protein translation to be increased). Genes with the highest S were considered to be the most promising.

$S = \Sigma\, \varepsilon_i^- - 2\, \Sigma\, \varepsilon_i^+$

ε_i^+ and ε_i^- are the ε value from TargetScan database for up- and down-regulated miRNAs in tumors respectively. 2 — coefficient reflecting the strictness of used criteria.

To identify the number of clones in clone libraries of normal and tumor tissue, GeneHub GEPIS web resource of dbEST (Gene Expression Profiling In Silico, http://research-public.gene.com/Research/genentech/genehub-gepis/index.html.) was used. The expected changes in the transcriptional level of identified genes were estimated with Oncomine database (www.oncomine.org) based on the microarray investigations.

Identification of proteins that could enter the bloodstream using Babelomics web-service (http://babelomics.bioinfo.cipf.es/) was the next stage of our research. The additional check was held with the GeneCards database (http://genecards.org). The further screening for biological markers was carried out with Oncomine, dbEST (http://www.ncbi.nlm.nih.gov/dbEST/) and SAGE (http://cgap.nci.nih.gov/SAGE) databases. The genes under investigation were selected for further analysis when the information about their overexpression (two-fold increase or higher) in gastric tumors was received at least from two of mentioned databases. This search resulted in sixteen gene list as final candidates because the increase of both their transcriptional and translational levels in tumors was probable. To validate differential gene expression in stomach cancer specimens versus normal tissue with RT-PCR analysis we selected five genes (*WNT4*, *FGF12*, *EFEMP1*, *CTGF*, and *HSPG2*) that are considered to contribute to carcinogenesis regulating cell proliferation, differentiation, division, migration, apoptosis, gene expression and regeneration.

It is important that some of these genes are functionally linked to each other through the key transcriptional cancer-related factor, TGFβ (transforming growth factor beta) and regulatory glycoprotein HSPG (heparan-sulfate proteoglycan), that are responsible for epithelial-mesenchymal transition (EMT), as a principal step for tumor dissemination.

The expression analysis of *WNT4, FGF12, EFEMP1, CTGF*, and *HSPG2* genes was done using quantitative RT-PCR in the own collection of frozen tumor and paired normal samples collected from patients treated in Tomsk Cancer Institute.

No significant differences were found in the expression levels of genes to be tested when the analysis was done for the total group of patients. There was also no significant association between *WNT4*, *FGF12, EFEMP1*, *CTGF*, and *HSPG2* gene expression level and tumor differentiation status, stage, metastasis, as well as age and gender of patients ($p > 0.05$, data not shown). However, the individual analysis showed more than two-fold *FGF12* expression increase in tumor tissue versus normal gastric mucosa among 42.5% of patients ($p = 0.01$, Yates' chi-squared test, Table 3).

Table 3. *WNT4, HSPG2, CTGF, EFEMP1* and *FGF12* gene expression alterations in gastric tumor tissue and paired normal specimens

Genes	Increased expression n (%)	Decreased expression n (%)	Equal expression n (%)
WNT4	8 (20)	13 (32,5)	19 (47,5)
HSPG2	5 (12,5)	6 (15)	29 (72,5)
CTGF	12 (30)	7 (17,5)	21 (52,5)
EFEMP1	10 (25)	11 (27,5)	19 (47,5)
FGF12	17 (42,5)	11 (27,5)	12 (30)

Increased expression of *CTGF* gene in the tumor compared with normal tissue was observed in patients with metastases in regional lymph nodes (0.0625 (0.0510; 0.1090) versus 0.0463 (0.0250; 0.0883), $p = 0.042$, median (range), Mann — Whitney U-test). No differences in *CTGF* gene expression were found in patients with local tumor growth.

When patients were stratified by tumor histological type, the increased *FGF12* expression was found in diffuse type of stomach cancer in comparison with normal tissue (p=0.049, Mann — Whitney U-test, Table 4), the increase in *FGF12* gene expression level in tumors was observed in 62,5% of patients, while there was no differential *FGF12* expression in intestinal type of tumors. These findings suggest the *FGF12* involving in pathogenesis of diffuse type of gastric cancer.

The significant down-regulation of *WNT4* gene was found in intestinal type of tumors versus normal mucosa (Table 5). Two-fold expression decrease was observed in 47.6% of patients, while gene up-regulation was noted only in one person (4.8%).

The *CTGF* gene expression was significantly higher in patients with diffuse type of stomach cancer than in patients with intestinal type ($p = 0.012$; Tables 4, 5), Mann — Whitney U-test).

Thus, the analysis of gene expression revealed a significant difference in only three genes (*FGF12, WNT4,* and *CTGF)* between the tumor of different histological types and normal tissue. *FGF12* gene showed differential expression in diffuse type of cancer samples for more than 60% of patients. All members of FGF family are known to play an important role in embryogenesis, morphogenesis and regeneration as well as cell mitosis, survival and proliferation [23]. Although the specific function of *FGF12* is still to be studied, evidence exists that it contains clusters of basic resi-

dues that have been demonstrated to act as a nuclear localization signal. When transfected into mammalian cells, FGF12 was accumulated in the nucleus, but was not secreted [24]. On the other hand, it participates in tissue regulation through internalization into cells interacting with its cell-penetrating domen (CPP) [24]. There are although indirect evidences of FGF12 involvement in angiogenesis [25] as well as inhibition of apoptosis suggesting its potential oncogenic role [26]. However, participation of FGF12 in diffuse type of gastric cancer pathogenesis suggested in our research requires clarification.

Table 4. *WNT4, HSPG2, CTGF, EFEMP1* and *FGF12* gene expression level in diffuse type of gastric tumors and paired normal mucosa

Genes	Diffuse gastric cancer Me (Q25–75)	Normal tissue Me (Q25–75)	p
WNT4	0.0520 (0.0135; 0.0762)	0.0314 (0.0131; 0.0639)	0.373
HSPG2	0.1091 (0.0715; 0.2693)	0.1317 (0.0931; 0.1872)	0.756
CTGF	0.0932 (0.0586; 0.1492)	0.0744 (0.0364; 0.1333)	0.33 (0.012*)
EFEMP1	0.0556 (0.0403; 0.1057)	0.0415 (0.0309; 0.0747)	0.093
FGF12	0.0015 (0.0010; 0.0024)	0.0009 (0.0002; 0.0019)	0.042

Notes: Me – median; Q25–75 – range; *p* – Mann – Whitney U-test; **p*-value obtained for the groups "diffuse" and "intestinal" (Table 5).

Table 5. *WNT4, HSPG2, CTGF, EFEMP1* and *FGF12* gene expression level in intestinal type of gastric tumors and paired normal mucosa

Genes	Intestinal gastric cancer Me (Q25–75)	Normal tissue Me (Q25–75)	p
WNT4	0.0182 (0.0069; 0.0557)	0.0505 (0.0236; 0.1109)	0.048
HSPG2	0.1141 (0.0741; 0.1935)	0.1496 (0.0879; 0.2032)	0.291
CTGF	0.0482 (0.0274; 0.0636)	0.0581 (0.0296; 0.0831)	0.402
EFEMP1	0.0338 (0.0168; 0.0826)	0.0458 (0.0247; 0.1080)	0.130
FGF12	0.0007 (0.0004; 0.0020)	0.0009 (0.0006; 0.0014)	0.705

Notes: Me – median; Q25–75 – range; *p* – Mann – Whitney U-test.

CTGF gene was found to be overexpressed in diffuse type of tumor versus intestinal one as well as in tumors with lymph node metastasis that is thought to point its contribution to cancer progression. *CTGF* was discovered in 90-s as growth factor secreted by human endothelial cells [27]. It takes part in regulation of cell proliferation, migration, survival, adhesion as well as angiogenesis and therefore could be involved in carcinogenesis [28, 29]. Indeed a functional relationship between *CTGF* and *TGFβ* as well as their involvement into EMT, which is considered as one of the major mechanisms providing tumor metastatic potential have been reported earlier. The process of intracellular signal transduction from receptors into the nucleus downstream of TGF superfamily ligands is rather conserved and acts through SMAD proteins activation [30]. TGFβ as the key player in EMT regulates *CTGF* expression by means of gastric cancer-rich response element in the *CTGF* promoter and thereby Smad1-dependent increased promoter activity [31]. The *CTGF* gene product inhibits E-cadherin expression that is similar to the major way of TGFβ-dependent EMT [32]. It is also known that E-cadherin gene down-regulation is one of the molecular mechanisms of hereditary diffuse histotype of gastric cancer development. Thus it can be assumed that data received about increased *CTGF* gene expression reflect its role in gastric cancer diffuse type pathogenesis.

There is a lot of information about *CTGF* involvement in pathogenesis of different malignant tumors (breast cancer, pancreatic cancer, ovarian cancer, glioblastomas etc.) [33]. According to some authors *CTGF* high mRNA level has prognostic value in stomach cancer and associated with metastasis and low survival rates that are in agreement with our finding [34]. In addition it is thought to contribute to tumor drug resistance [33].

As for *WNT4* gene expression level, we observed differential expression (namely its decrease in intestinal gastric tumors versus normal tissue with more than two-fold reduction) in half of the patients. *WNT4* is a member of gene family encoding secreted signal proteins regulated cell differentiation that supposes its participation in carcinogenesis. Normally WNT4 regulates breast stem cells proliferation in response to progesterone and also plays an important role in embryogenesis [35].

WNT genes up-regulation was found in gastric cancer, leukemia and some other malignances [4, 35, 36]. As for *WNT* gene family contribution to tumor pathogenesis WNT/beta-catenin pathway is thought to be crucial. Beta-catenin regulates expression of such oncogenes as c-MYC and cyclin D1 and participates in cell adhesion through interaction with cadherin. Activation of this pathway by *H. pylori* infection is critical event prior malignancy. WNT proteins promote beta-catenin gene expression contributing to malignancy although this event could be related not only to *WNT* genes activation but with up-regulation of WNT receptors genes and *RAC1* gene [37, 38]

In order to clarify the fact of *WNT* down regulation in intestinal type of gastric cancer in our study, we analyzed whether *WNT4* gene expression alteration is associated with *H. pylori* infection in gastric cancer patients but did not observed any association. It remains an open question what could be the possible reason of *WNT4* down-regulation in intestinal type of gastric tumors.

The data obtained suggest the importance of further investigations in order to make clear the role of *WNT4*, *FGF12,* and *CTGF* genes in pathogenesis of different types of gastric cancer as well as their diagnostic and prognostic value.

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