K-ras codon 12 and not *TP53* mutations are predominant in advanced colorectal cancers

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Background. Colorectal cancer (CRC) is one of the most common types of cancer, affecting 3 - 5% of the global population. *K-ras* proto-oncogene and *TP53* tumour suppressor gene mutations are among the most common genetic alterations detected in advanced colorectal tumours.

Objective. To investigate the role of *K-ras* codon 12 and *TP53* exons 5 - 9 mutations in late-stage CRC patients.

Methods. Blood samples were collected from 249 CRC patients, of whom 147 presented with advanced carcinoma. *K-ras* codon 12 mutations were analysed using polymerase chain reaction-restriction fragment length polymorphism, while direct sequencing was used in screening for *TP53* exons 5 - 9 mutations.

Results. No significant changes were observed in *TP53* exons 5 - 9, except for two cases in which nucleotide replacements were observed in the non-coding regions in intron 4 (c.376-19C>T) and intron 9 (c.993+12T>C). Heterozygous mutations in *K-ras* codon 12 were observed in 79 individuals suffering from advanced CRC (53.7%). Colon and rectal tumours were equally distributed among the heterozygotes, but colon tumours were mostly present in wild-type homozygotes (84.6%). There was also a predominance of Caucasians among heterozygotes and a predominance of Asians among the wild-type homozygotes.

Conclusion. Analysis of peripheral blood samples of CRC patients suffering from advanced carcinoma has prognostic value only for *K-ras* codon 12 mutations, and not for *TP53* mutations.

Colorectal cancer (CRC) is one of the most common cancers, affecting 3 - 5% of the population, with an incidence peak in the age range 50 - 70 years. CRC is the third leading cause of cancer death in both men and women worldwide, and continues to be one of the most common fatal types of cancer. Worldwide, approximately 1.5 million individuals are diagnosed with CRC and 500 000 die from the disease annually.[1] Among Eurasian countries, Kazakhstan has the seventh-highest incidence of CRC.

CRC develops slowly over several years and progresses through cytologically distinct benign and malignant stages of growth, ranging from single-crypt lesions through adenoma to malignant carcinoma with the potential for invasion and metastasis. The majority of CRC cases are sporadic, with 20 - 30% being familial.[1]

The classic model of colorectal tumourigenesis includes several genetic changes that are required for cancer initiation and progression. The earliest genetic trigger is the inactivation of the *APC* pathway. Mutations in other tumour suppressor genes (*APC, SMAD2, SMAD 4, DCC, TP53*) and oncogenes (*K-ras*) and several other genes/pathways accompany the transition of the tumour towards malignancy and metastasis. In addition to the gene mutations, deregulated expression of oncogenes and/or tumour suppressor genes can also be mediated via epigenetic modifications such as DNA methylation and histone acetylation. Rapidly developing insights into the molecular genetics of CRC have led to the identification of predictive and prognostic biomarkers for CRC.[2]

K-ras proto-oncogene and *TP53* tumour suppressor gene mutations are among the most common genetic alterations in advanced colorectal tumours. The *Ras* genes code for a family of membrane-bound small guanosine triphosphate-bound proteins that play a key role in the transduction of extracellular mitogenic signals. Constitutively activating *Ras* mutations are present in a significant proportion of colorectal adenomas and carcinomas.[1-2] Approximately 40% of CRCs harbour *K-ras* mutations, 90% of which occur in codons 12 and 13; these mutations occur less frequently in codons 61, 63, and 146. Mutations in any of these codons lead to a constitutively activated RAS protein.[3-6] The relationship between *K-ras* mutations and the clinical/morphological parameters of CRC has been extensively investigated. Some epidemiological studies show a prevalence of *K-ras* codon 12 mutations over mutations in codon 13 in CRC tumours.[7] A study of more than 1 000 colorectal cancers showed that *K-ras* codon 12 mutations (in particular c35G>T, pG12V), but not codon 13 mutations, are associated with poor prognosis in wild-type CRCs.[3] The prognostic value of codon 12 mutations was revealed in a functional study that showed that malignant transformation of NIH3T3 cells with mutant codon 12 *K-ras* had a more aggressive phenotype when compared with mutant codon 13 *K-Ras*.[3] A recent study of Chinese CRC patients revealed that a high frequency of codon 12 *K-ras* mutations was associated with poorly differentiated tumours, liver metastasis and poor survival.[6] Although the role of *K-Ras* mutations in tumour tissues is widely accepted to be associated with poor prognosis and anti-epidermal growth factor receptor (EGFR) therapy,[4,12] the predictive value of *K-ras* mutations in blood samples remains uncertain.

The *TP53* gene product is a nuclear phosphoprotein that is expressed at low levels in most normal tissues and plays a key role in the control of cell cycle progression, genome stability and apoptosis. *TP53* is one of the most frequently mutated genes in human cancers. Somatic mutations of *TP53* are associated with more advanced stages of the disease, with loss of *TP53* gene heterozygosity being a marker for the conversion of adenoma to carcinoma. In CRC tumours, more than 50% of mutations occur in the *TP53* gene. The most common mutations are single-base substitutions that alter protein function. However, in contrast to the activating *K-ras* gene mutations, which are concentrated in only a few positions, mutations in the *TP53* gene are scattered over a large region of the gene.[8] Most *TP53* gene mutations in CRC tumours occur in exons 5 - 9, which contain four highly conserved domains that are important in the clinical outcome of CRC and have been associated with increased malignant potential.[8,9] *TP53* mutations occur with a higher frequency in distal colon and rectal tumours and with a lower frequency in proximal, mucinous and microsatellite instability-positive CRCs. Alterations to this gene are likely to have very little or no prognostic significance in CRC patients treated with surgery alone, but may be associated with marginally worse survival for patients treated with chemotherapy. There is some evidence that different *TP53* mutations are associated with different clinical properties, including prognosis and response to therapy. *TP53* status appears to have predictive value for the survival benefit of CRC patients on 5-fluorouracil (5FU) chemotherapy.[10]

Several studies have reported combined mutations in *K-Ras* and *TP53* genes in CRC tumours,[7] but the clinical usefulness of *K-ras* and/or *TP53* gene mutations is still somewhat controversial. The data on *K-ras* and *TP53* aberrations and their relationship to patient survival and prognosis are insufficient to recommend the use of such mutations as prognostic indicators.[9] It is therefore clear that prospective studies to assess the prognostic utility of these genetic abnormalities are required. Although most reports indicate that altered *K-Ras* and *TP53* genes in tumour tissues present at advanced stages, it would be interesting to assess the status of these genes in peripheral blood samples of CRC patients, because the gene heterozygosity can have a predictive value for determining prognosis.

The Kazakhstan National Screening Program for malignant neoplasms of the colon and rectum, initiated in 2011, showed that most cases were diagnosed at a very late stage when treatment is expensive and ineffective. The prediction of malignant potential and resistance to EGFR or 5FU chemotherapy at earlier stages of CRC development before surgery may improve patient survival.

Objective

Because the *K-ras* codon 12 and *TP53* mutations are the most promising biomarkers of CRC tumour progression, the objective of this study was to assess *K-Ras* and *TP53* gene status in blood samples of patients with advanced colorectal carcinoma.

Methods
Sampling

Blood samples were collected from 249 CRC patients at Almaty Oncology Centre, Almaty, Kazakhstan. Informed consent and detailed demographic information were obtained. Histological testing showed that 147 of these patients (59.0%) had presented with advanced CRC, and they were chosen as subjects for this study. The study protocol was approved by the Ethics Committee of the Asfendiyarov Kazakh National Medical University.

DNA isolation

Genomic DNA was isolated from peripheral blood leucocytes of the 147 patients with advanced CRC (stages III and IV) using the standard phenol-chloroform method with modifications in the composition of the lysis buffer (0.2M sodium acetate and 1% sodium dodecyl sulphate, pH 8.0) and precipitated in ice-cold ethanol.[9] The quantity and quality of the DNA samples were evaluated spectrophotometrically using an Eppendorf BioPhotometer (Eppendorf, Germany), and they were stored at –20°C until required.

Detection of *K-ras* mutations in codon 12

All known nucleotide mutations in codon 12 were screened by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). One hundred nanograms of total DNA was amplified in a 20 μL reaction mixture containing 10 pM of each specific primer (sense-5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′, anti-sense-5′-TCAAAGAATGGTCCTGGACC-3′), 10 mM of each deoxynucleotide triphosphate (dNTP), 1 mM MgCl2, 0.5 KCl, 0.1% gelatin and 1 U Taq-polymerase (Sigma-Aldrich, USA). Denaturation was performed at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, at 54°C for 40 seconds and at 72°C for 1 minute, with a final elongation step at 72°C for 7 minutes.[11] Five microlitres of each PCR product (157 bp) was digested with 1 U MvaI (BstN1, ThermoScientific, USA) at 60°C for 3 hours. Digestion products were analysed on 1.4% agarose gels. The normal *K-ras* allele is indicated by the presence of a 114-bp fragment as opposed to the 143-bp fragment in the mutant *K-ras* allele. Heterozygotes display both the 143- and 114-bp fragments.

Sequencing of exons 5 - 9 of the *TP53* gene

Direct sequencing was used to screen for mutations in exons 5 - 9 of *TP53*.[9] The PCR was performed in a final volume of 20 μL containing 50 ng template DNA, 1 × PCR buffer, 0.2 mM of each dNTP, 1.5 mM MgCl2, 0.4 μM of each primer (Table 1) and 0.5 U of FastStart Taq DNA polymerase using the Veriti Dx 96-well Thermal Cycler (Applied Biosystems, USA). PCR products were visualised by electrophoresis on 1.5% agarose gels.

PCR products were purified using Nucleofast 96-well PCR plates (Macherey-Nagel, Germany). Sequencing reactions were performed

Table 1. The PCR amplification primers and parameters used for direct sequencing of *TP53* exons 5 - 9			
Amplified regions of *TP53* gene	Primer pairs (5′→3′)	PCR conditions	Length of PCR products
Exons 5 - 6	f-TGTTCACTTGTGCCCTGACT r-TTAACCCCTCCTCCCAGAGA	1 cycle of 2 min at 94°C, 20 cycles (94°C for 30 s, 63°C for 45 s with –5°C per cycle, 72°C for 1 min), followed by 30 cycles (94°C for 30 s, 60°C for 45 s, 72°C for 1 min), and a final cycle of 10 min at 72°C	467 bp
Exon 7	f-CTTGCCACAGGTCTCCCCAA r-AGGGGTCAGAGGCAAGCAGA		237 bp
Exons 8 - 9	f-TTGGGAGTAGATGGAGCCT r-AGTGTTAGACTGGAAACTTT		445 bp

using specific primers and the BigDye Terminator v3.1 sequencing kit (Applied Biosystems) and an ABI 3730xl Genetic Analyser (Applied Biosystems). The cycle sequencing reactions contained 50 - 100 nmol of purified PCR product, 2 µl of BigDye Terminator, 1 µL of Sequencing Buffer and 3.2 pmol of primer in a total volume of 20 µL. Cycle sequencing was performed using 55 cycles at 96°C for 5 minutes, 60°C for 5 minutes and 96°C for 5 minutes. After cycle sequencing, sodium dodecyl sulphate was added and the samples were purified on Sephadex columns using a Tecan EVO150 robotic workstation (Agilent Technologies, USA). The purified sequencing products were dried, suspended in Hi-Di (Life Technologies, USA) and denatured at 95°C for 2 minutes before sequencing electrophoresis. Electrophoresis was performed on an ABI 3730xl using a 50 cm capillary array and POP7 (Applied Biosystems).

DNA sequencing data were analysed using ChromasPro version 1.7.4 software, confirmed by reverse sequencing and compared with the Leiden Open Variation and Universal Mutation databases to search for mutations.

Statistical analysis

Student's *t*-test was used to compare the distribution of variables between groups, with a *p*-value of <0.05 considered significant.

Results
### Characteristics of the study population

Adenocarcinoma was the predominant tumour type among the 249 CRC patients first diagnosed with CRC in the period 2012 - 2014, and 55.8% of the tumours were well or moderately differentiated. Table 2 summarises the data on tumour localisation and stage, showing that rectal tumours predominated at 47.7% of all sites.

For the analysis of *K-ras* codon 12 mutations and *TP53* exons 5 - 9 mutations as potential markers of cancer progression, the advanced carcinoma cases were chosen after histological verification. A total of 147 patients (59.0%) had advanced CRC (stages III and IV by TNM criteria). In this cohort there were 73 women (49.7%) and 74 men (50.3%), and the ethnic distribution was as follows: Kazakhs *n*=39 (26.5%), Russians *n*=86 (58.5%), other Asians (Turks, Uzbeks, Tatars, Uighurs, Koreans, Dungans) *n*=17 (11.6%), and other Caucasians (Armenians, Ukrainians, Belarusians, Germans) *n*=5 (3.4%). There were 50 cases of rectal cancer

Table 2. Localisation and stage of the tumours in the CRC cohort used in this study

Localisation of tumours	Patients *n* (%)	TNM stage, *n* (%)			
		I	II	III	IV
Rectum	115 (47.7)	8 (3.2)	44 (17.7)	48 (19.3)	15 (6.0)
Colon	48 (19.3)	3 (1.2)	14 (5.6)	21 (8.4)	10 (4.0)
Cecum	18 (7.5)	0 (0)	7 (2.8)	7 (2.8)	4 (1.6)
Sigmoid	57 (22.9)	5 (2.0)	17 (6.8)	28 (11.2)	7 (2.8)
Rectosigmoid	11 (4.6)	0 (0)	4 (1.6)	4 (1.6)	3 (1.2)
Total	249 (100.0)	16 (6.4)	86 (34.5)	108 (43.4)	39 (15.7)
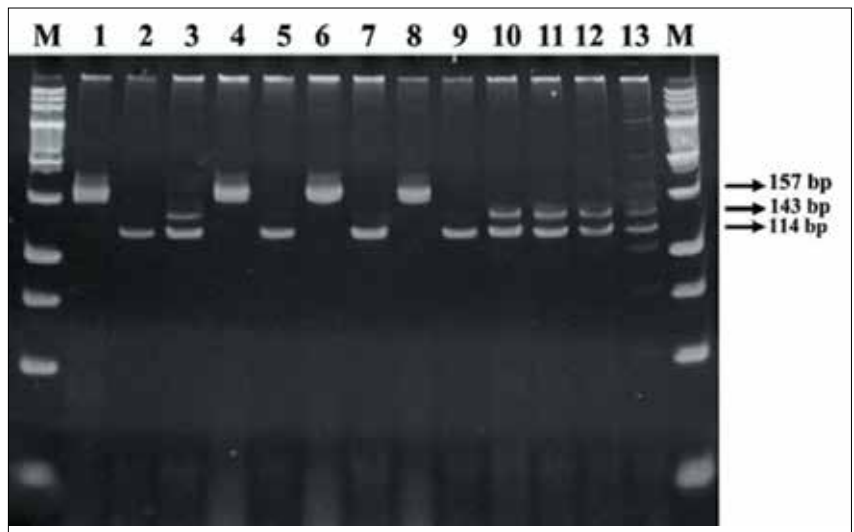
Fig. 1. Genotyping of the K-ras *codon 12 mutations. Blood DNA was isolated and subjected to PCR amplification, digestion with the restriction MvaI (BstN1) and separation of the digestion products on 3% agarose gels as described under 'Methods'. The 114-bp fragment indicates the normal* K-ras *while the 143-bp fragment indicates the mutant* K-ras *codon 12 allele. Lane M is a 100-bp DNA ladder, while lanes 1, 4, 6 and 8 are the undigested 157-bp PCR products; lanes 2, 5 and 9 show the normal homozygous individuals represented by the 114-bp band, while lanes 3, 7, 10, 11, 12 and 13 show the heterozygous individuals. The gel shows only a sample of 13 of the 147 patients with advanced carcinoma investigated in this study.*

(34.0%) and 97 of colon cancer (66.0%), with 126 cases of stage III cancer (85.7%) and 21 of stage IV cancer (14.3%). Among patients suffering from advanced CRC there were 5 smokers (3.4%), 14 ex-smokers (9.5%), and 128 non-smokers (87.1%).

K-ras mutations in codon 12 in patients with advanced CRC

The 147 selected CRC patients with advanced carcinoma were screened for *K-ras* codon 12 mutations (Gly12Asp (GGT→GAT), Gly12Ala (GGT→GCT), Gly12Val (GGT→GTT), Gly12Ser (GGT→AGT), Gly12Arg (GGT→CGT), Gly12Cys (GGT→TGT)).

The genotyping data for the *K-ras* codon 12 mutations (Fig. 1) revealed 79 heterozygous individuals (53.7%), while 68 (46.3%) of the patients were homozygous wild type. Some differences were observed

Table 3. Differences in the stage of CRC in individuals with homozygous and heterozygous *K-ras* codon 12 genotypes

Genotype	TNM stage	
	III	IV
Mutant heterozygous, *n* (%)	53 (67.1)	26 (32.9)
Normal homozygous, *n* (%)	58 (85.3)	10 (14.7)
Odds ratio	1.25	1.76
p-value	>0.05	>0.05

in the stage of cancer progression between individuals who were mutant heterozygous and normal homozygous for *K-ras* codon 12 (Table 3). The percentage of patients who had progressed to stage IV was higher among the mutant allele carriers than

among the normal homozygotes (32.9% v. 14.7%).

The heterozygous genotype was present in 50% of patients with rectal tumours and in 50% of those with colon tumours. Most of the normal homozygotes were diagnosed with colon tumours (84.6%), and only 15.4% with rectal tumours. These differences were statistically significant (for colon tumours odds ratio (OR) 2.7; *p*<0.01 and for rectal tumours OR 2.84; *p*<0.01).

The prevalence of Caucasians among mutant allele carriers (70% Russians and other Caucasians), and the prevalence of Asians among normal homozygotes (53.9% Kazakhs and other Asians) were observed as significant (for Caucasians OR 2.09; *p*<0.05 and for Asians OR 2.32; *p*<0.05).

The percentage of smokers and ex-smokers among heterozygotes was 13.4%, while among normal homozygotes there were only ex-smokers (11.5%). Distribution by gender was similar between heterozygotes (40 males and 39 females) and normal homozygotes (34 males and 34 females).

Analysis of *TP53* mutations

No *TP53* exon 5 - 9 mutations were observed by direct sequencing, as shown in Fig. 2. However, three cases of nucleotide replacements were noticed in intron 9 (c.993+12T>C) (Fig. 2, A), and another two cases in intron 4 (c.376-19C>T, Fig. 2, B), all in the heterozygous state. Table 4 summarises the DNA sequencing data in individuals with *TP53* mutations. Two different types of mutations were detected in five patients from among the 147 patients with advanced carcinoma.

Discussion

It is well known that mutations in the tumour suppressor gene *TP53* and *K-ras* oncogene are not responsible for initiation of the development of CRC, but are critical for tumour progression and metastasis.[1-2,8-9,12]

The most common *TP53* gene mutations are single-base substitutions that alter protein function. While loss-of-function mutations are generally inherited or early events, some of the oncogenic mutations that confer gain-of-function properties are late-stage events, mostly exon 5 - 9 mutations, that play an important role in the clinical outcome of CRC.[1,9]

Our study on peripheral blood samples of 147 patients with advanced CRC did not show any significant changes in *TP53*

exon 5 - 9 mutations, except for two novel heterozygous nucleotide replacements in the non-coding regions of the gene. One change in intron 4 (c.376-19C>T) was observed in two patients, and another nucleotide substitution was detected in intron 9 (c.993+12T>C) in three patients. No information on the significance of these mutations detected in CRC patients from Kazakhstan is available in the literature. It should also be noted that the *TP53* mutations were examined in the peripheral blood DNA of the patients and not in the tumours; these are therefore inherited mutations. However, it cannot be ruled out that additional genetic events may occur along the way. Although intronic mutations do not alter the amino acid sequence of a gene product, they can influence the regulation of gene activity by altering the binding of regulatory proteins or miRNA, leading, for example, to aberrant splicing (IARC TP53 Database).

The most common *K-ras* codon 12 mutations result in the substitution of glycine for valine, thus changing the spatial conformation of RAS, leading to a constitutively activated form where the RAS signalling cascade to other participants occurs regardless of EGFR status. This explains why the predictive value of *K-ras* mutations in determining whether or not patients will respond to anti-EGFR therapy is of much interest.[13,14]

Screening of all known *K-ras* codon 12 mutations (Gly12Asp, Gly12Ala, Gly12Val, Gly12Ser, Gly12Arg, Gly12Cys) in the blood DNA of 147 CRC patients with advanced CRC showed heterozygous mutations in more than 50% of the patients (79/147). Our data are in agreement with the published data showing *K-ras* codon 12 mutation frequencies ranging from 30% to 60% in tissue samples from rectal and colon tumours.[10,13-14]

We could not find any significant differences regarding gender, age or smoking habit between patients carrying the *K-ras* codon 12 mutation and the wild-type homozygotes. The percentage of patients with stage IV cancer carrying the mutant allele was higher than among the normal homozygotes (32.9% v. 14.7%), but this was not statistically significant. Tumours of the rectum and colon were equally distributed among heterozygous patients; however, the prevalence of colon tumours (84.6%) in normal homozygous patients was statistically significant. The relationship between *K-ras* mutations and the clinical/morphological parameters of CRC has been extensively investigated.[5] There is some evidence that *K-ras* codon 12 mutations are significantly
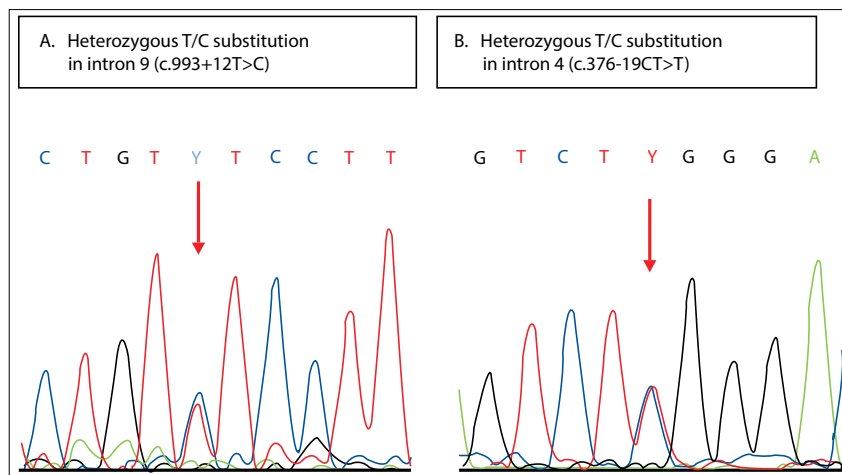

Fig. 2. DNA sequence analysis of the TP53 *gene. Blood DNA was isolated and subjected to PCR amplification using the primers defined in Table 1, and the PCR products were subjected to direct sequence analysis. A: Nucleotide change observed in intron 9 in three patients; B: Nucleotide change observed in intron 4 in two patients.*

Table 4. Nucleotide changes detected by direct PCR sequencing *TP53* exons 5 - 9 detected in five of the 147 CRC patients investigated

Gene	Exon/intron	Nucleotide substitution	Affected patients
TP53	9i	c.993+12T>C, rs1800899	One female (born 1967), rectal, stage III T3NxMo One male (born1937), rectal, stage IV T3NxM1 One male (born1937), colon, stage IV T3NoM1
TP53	4i	c.376-19C>T	One female (born 1983), rectal, stage III T4NxMo One female (born1952), colon, stage IV T4NxM1

associated with poor survival at all stages of cancer progression. Estimated event-free survival correlates with poor tumour differentiation status, pericolic fat invasion and metastasis.[5] CRCs with *K-ras* mutations are associated with distinctive morphological features. *K-ras* codon 12 mutations are more frequently observed in carcinomas of the proximal-distal axis of the colorectum, mucinous differentiation, and contiguous polyps. Our findings are in agreement with these findings.

Some ethnic differences were observed in the Kazakhstan patients: there was a higher prevalence of mutant allele carriers among the Caucasians (Russians and others) and a higher prevalence of normal homozygotes among the Asians (Kazakhs and others). It is well established that ethnic differences and lifestyle have a strong influence on the frequency of *K-ras* mutations.[15] *K-ras* codon 12 mutation is a good biomarker in stage IV CRC, predicting lack of benefit from the anti-EGFR targeted antibodies cetuximab (Erbitux) and panitumab (Vectibix).[4,15] It is possible that the findings on *K-ras* codon 12 carriers in our population will help in the choice of adjuvant therapy.

Several studies have detected compound mutations in the *K-ras* and *TP53* genes in CRC.[4,15] A Tunisian CRC study reported the detection of a *K-ras* somatic mutation in 31.5% of patients, with 81.2% having a single mutation at codon 12 and 23% having a single mutation at codon 13; 43.75% of the patients harboured combined *K-ras* and *TP53* mutations, with 71.42% of them showing *TP53* over-expression.[7] In our study two of the five patients (a woman born in 1967, rectal carcinoma, stage III T3NxMo, and a man born in 1937, rectal carcinoma, stage IV T3NxM1) with *TP53* intron mutations were also heterozygous for the *K-ras* codon 12 mutation. In both cases the mutation was in intron 9 of *TP53* (c.993+12T>C). Because the percentage of *TP53* mutations among advanced CRC patients from Kazakhstan is very low (3.4%) and nothing is known about the pathological significance of these intronic mutations, there is no evidence for an association with poor prognosis. However, our observation of the prevalence of inherited *K-ras* codon 12 mutation carriers in advanced CRC patients (53.7%), together with the literature data, supports the idea that these mutations may be a predictive marker of cancer progression and anti-EGFR therapy.

Conclusion
The study demonstrates that the analysis of peripheral blood samples of patients suffering from advanced CRC may have prognostic value only for *K-ras* codon 12 mutations, and not for *TP53* mutations.

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