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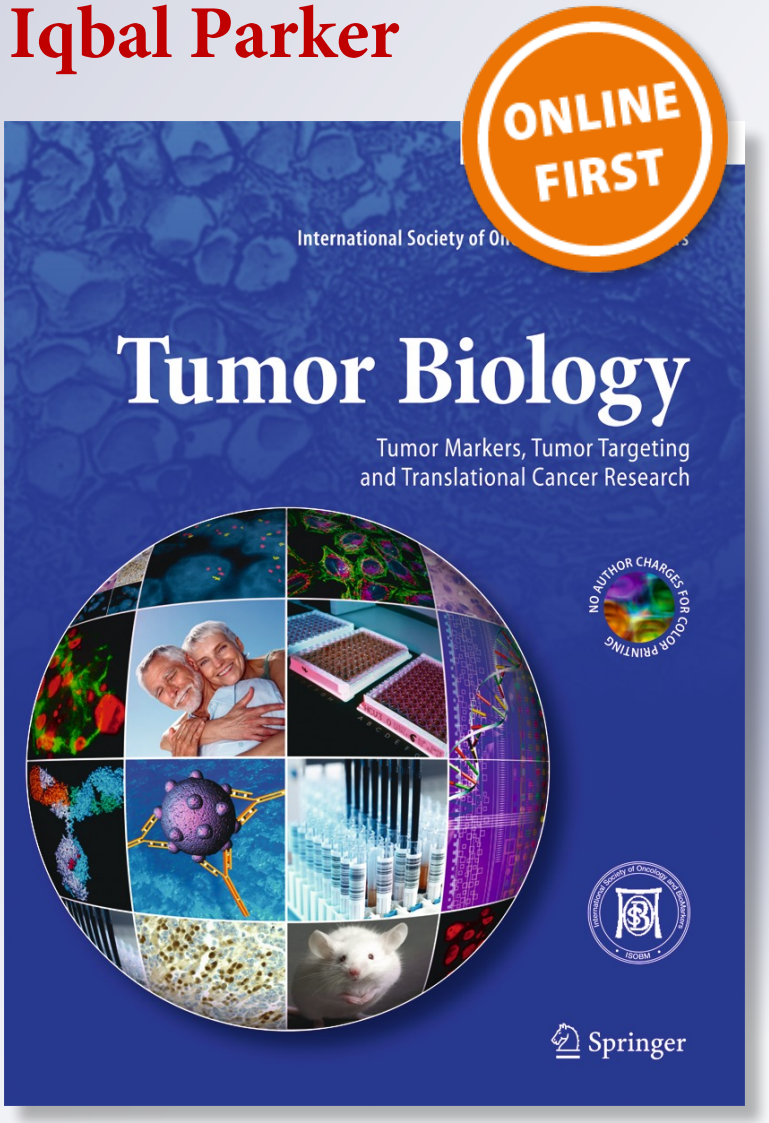

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RESEARCH ARTICLE

Association of *DCC*, *MLH1*, *GSTT1*, *GSTM1*, and *TP53* gene polymorphisms with colorectal cancer in Kazakhstan

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Abstract This study presents the first results of a molecular-genetic study of colorectal cancer (CRC) in Kazakhstan. Blood samples were collected from patients diagnosed with rectal or colon cancer (249 individuals) as well as a control cohort of healthy volunteers (245 individuals), taking into account the age, gender, ethnicity, and smoking habits of the CRC patients. Combined analysis of data obtained from individuals of either Kazakh or Russian decent showed a significant association with increased CRC risk in the following genotypes: *DCC* (32008376G/G and G/A versus A/A; OR=3.45, 95 % confidence interval (95 %CI)=1.75–6.81, χ^2=14.07, p<0.0002), *MLH1* (-93G/G versus G/A and A/A; OR=1.45, 95 %CI=1.02–2.07, χ^2=4.21, p<0.04), *TP53* (Pro72Pro; OR=3.80, 95 %CI=2.46–5.88, χ^2=61.27, p<0.0001), combination *GSTT1* deletions with heterozygotes versus normal homozygotes (OR=1.43, 95 %CI=1.00–2.04, χ^2=3.90, p<0.05), and *GSTM1* deletions (OR=1.83, 95 %CI=1.28–2.63, χ^2=11.04, p<.001). Analysis for ethnicity and smoking for each of the investigated polymorphisms showed that some genotypes can have a predictive value for susceptibility to CRC, at least those that demonstrate statistically significant ORs either for the combined mixed population of Kazakhstan or for both main ethnic groups separately (Kazakhs and Russians): *TP53* Pro72Pro homozygous (for Kazakh—OR=3.40, 95 %CI=1.63–7.06, χ^2=11.35, p<0.003; for Russian—OR=4.69, 95 %CI=2.53–8.66, χ^2=53.19, p<0.0001) and *GSTM1* deletions (for Kazakh—OR=2.30, 95 %CI=1.21–4.40, χ^2=8.42, p<0.01; for Russian—OR=1.64, 95 %CI=1.01–2.66, χ^2=7.82, p<0.02).

Keywords Single nucleotide polymorphisms · Tumor suppressor genes · DNA mismatch repair genes · GST deletions

Introduction

Colorectal cancer (colon and rectal cancer—CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women worldwide. More than 1.5 million new cases are recorded worldwide every year, and around 50 % of patients die from the disease [1]. Among Eurasian countries, Kazakhstan has the seventh highest incidence of CRC. Most cases are diagnosed at a very late stage of cancer progression (stages III–IV), when treatment is expensive and the prognosis is very poor.

CRC is age related, colonic cancer more so than rectal, with an incidence peak within the range 50–70 years and being higher in men than in women. The median age at diagnosis is about 70 years in developed countries and about 60 years in developing countries. In recent years, there has been a considerable worldwide increase in CRC morbidity and a significant rejuvenation of this type of cancer. The possible reasons include a change in lifestyle such as reduced physical activity, poor nutrition, and increased smoking and alcohol consumption habits among young people.

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CRC develops slowly over several years and progresses through cytological distinct benign and malignant stages of growth ranging from single crypt lesions through adenoma to malignant carcinoma with the potential for invasion and metastasis. Adenoma, diffuse polyposis, and non-specific ulcerative colitis increase the risk of cancer development and therefore have been considered as precancerous states. Diffuse polyposis malignancy occurs in almost 100 % cases. Despite the fact that the majority of colorectal cancer cases are sporadic, about 20–30 % of patients present with familial history of CRC. Various studies have shown that 15–20 % of patients with colorectal cancer have one or more first-degree relatives suffering from the same types of cancer [1].

Colorectal carcinogenesis is characterized by the successive accumulation of mutations in genes controlling epithelial cell growth and differentiation, leading to genomic instability whereby widespread loss of DNA integrity is perpetuated. The classic model for colorectal tumorigenesis includes several genetic changes that are required for cancer initiation and progression. The earliest genetic trigger is the inactivation of the adenomatous polyposis coli pathway (*APC*). Mutations in other tumor suppressor genes (*APC*, *SMAD2*, *SMAD4*, *DCC*, *TP53*) and oncogenes (*KRAS*) and several other genes/pathways accompany transitions in the pathology of the lesions that provide the impetus for the drive toward malignancy and metastasis. In addition to these gene mutations, a deregulated expression of oncogenes and/or tumor suppressor genes can also occur following epigenetic modifications of their promoters.

DNA sequence analysis of 13,023 genes in 11 colorectal cancers revealed that the spectrum of mutated genes in CRC cases is not limited by the few genes identified to date. This study showed that individual tumors accumulate about 177 mutations in 105 genes but that on average only 11 gene mutations per tumor are required for neoplastic transformation [2].

Polymorphisms of candidate genes responsible for interaction with environmental factors related to genetic susceptibility to CRC have been extensively investigated for sporadic CRC [3–6]. Case–control studies of different populations identified strong associations between CRC and deletion polymorphisms in phase two detoxification genes such as the glutathione S-transferases [5]. Polymorphisms within the DNA repair genes have also been widely reported to be associated with CRC susceptibility, particularly for Lynch syndrome [3]. Some of these genes may contribute to CRC susceptibility in combination with other factors such as the interaction between smoking status and genes participating in detoxification [5]. Epidemiologic and mechanistic evidence suggests that folate may be involved in colorectal neoplasia since polymorphisms in genes involved in folate metabolism, such as methionine synthase and methylenetetrahydrofolate reductase, have also been associated with increased CRC risk [7]. Some polymorphisms showing a strong association with CRC and ethnicity, such as promoter polymorphisms of the COX2 gene, that leads to overexpression of COX2 have been shown to be potential risk factors for CRC in Asians but not in Europeans [8].

Rapidly growing insights into the molecular genetics of CRC and the rapidly expanding development of new technologies for genome analysis have led to the identification of predictive and prognostic biomarkers of CRC that also presents an opportunity to estimate the possibility of malignancy [4]. CRC is one of the diseases for which the preventive measures are most effective. Screening, for example, has been shown to reduce CRC incidence and mortality, but organized screening programs are still to be implemented in most countries, especially in developing countries. Primary prevention aims to identify germ-line mutations associated with a high risk of developing cancer. It is known that screening leading to early diagnosis reduces the risk of developing CRC by 56 % and the mortality by 65 %. Secondary prevention of cancer is aimed at screening the relatives of probands in order to identify families with a higher cancer burden, to provide medico-genetic counseling and regular clinical examination of high-risk patients. While this is the most successful approach to reduce the impact of hereditary cancers, this program is unfortunately still in its embryonic form in Kazakhstan, although the National Screening Program for malignant neoplasms of colon and rectum began in 2011.

In our selection of the candidate genes for analysis of CRC patients in Kazakhstan, we took into account several facts. The combination of genes involved in CRC development differs for sporadic cases and of familial syndromes, and we focused our study on polymorphisms and mutations of key genes that show association with CRC in ethnically different populations. Because the population in Kazakhstan is ethnically mixed and since this is the first case–control study for CRC in Kazakhstan, we included in our analysis some polymorphisms that show contradictory results in the Asian and Caucasian populations. The following polymorphic sites were selected for the case–control study: *DCC* G32008376A (c.985+67534A>G, rs 714) [9–11], *TP53* (Arg72Pro) (rs 1042522) [12], *hMLH1* G-93A (rs 1800734) [13–15], and deletions of *GSTM1* and *GSTT1* [5].

Materials and methods

Patient sampling

For this case–control study, blood samples were collected from 249 patients diagnosed with CRC at the Almaty Oncology Centre (Almaty, Kazakhstan) after receiving informed consent from the patients. Control bloods were collected from 245 healthy donors. The control group of healthy individuals was selected according to the age, gender, ethnicity, and

smoking habits in our CRC patient cohort. Also controls were biologically unrelated to the patients and had no known family history of malignancies. Detailed questionnaires and informed consents were obtained prior to the collection of samples. The detailed questionnaire included information on sociodemographic status, diet, occupation, tobacco/alcohol habits, previous illness, illnesses of relatives, radiation exposure, etc. The study protocol was approved by the Ethics Committee of the Asfendiyarov Kazakh National Medical University (Almaty, Kazakhstan).

DNA isolation

Genomic DNA was isolated from peripheral blood leukocytes using the standard phenol–chloroform method with modifications in the composition of the lysis buffer: 0.2 M sodium acetate and 1 % sodium dodecyl sulfate, pH 8.0 [16]. The DNA was dissolved in distilled water and the quantity and quality of the dissolved DNA samples were evaluated by spectrophotometric analysis (Eppendorf BioPhotometer plus). The dissolved DNA samples were stored at −20 °C until further use.

Genotyping by site-specific PCR amplification

The genotyping of *GSTM1* and *GSTT1* deletion polymorphisms was carried out by multiplex PCR amplification [17]. The PCR-RFLP assay was used for the genotyping of *DCC* g.32008376A>G [18, 19], *MLH1* -93G>A [20], and *TP53* Arg72Pro [21] single nucleotide polymorphisms. About 20–100 ng of target DNA was amplified in a total volume of 20 μl of PCR mixture using the "Mastercycler" (Eppendorf, Germany). PCR reactions contained 15 pM of each specific primer, 10 mM of each dNTP, 2 μl of 10× PCR buffer (10 mM KCl, 100 mM Tris HCl, pH 9.0) and 0.5 U of Taq polymerase (Sigma-Aldrich, USA). The samples were analyzed on 1.4 % agarose gels using a Lambda/Hind III DNA marker (Sigma-Aldrich, USA) for the sizing of the amplified DNA fragment lengths. The PCR products were digested at 37 °C for 8–16 h with 1–3 U of the corresponding restriction enzymes (Thermo Scientific, USA) and analyzed on 2–3 % agarose MetaPhor (Lonza, Switzerland) gels. All genotyping was performed in duplicate for all samples and the genotypes were scored by different individuals, and thus it is unlikely that these results are due to genotyping error. The PCR details and relevant information are provided in Table 1.

Statistical analysis

Student's *t* test was used to compare the distribution of variables between case and control cohorts. A *p* value of less than 0.05 was considered significant. The allele frequencies were calculated in accordance with standard Hardy–Weinberg

Table 1 The site-specific PCR amplification protocols

Genes	Primers for PCR (5′→3′)	PCR conditions	Length of amplified fragments (bp)	Restriction enzyme	Restricted product length and corresponding genotype
GSTM1	f-GAACTCCCTGAAAAGCTAAAGC, r-GTTGGGCTCAAATATACGGTGG	Initiation: 94 °C, 5 min; 35 cycles of 94 °C for 2 min, 59 °C for 1 min, 72 °C for 1 min; final elongation: 72 °C, 10 min [17]	215	not used	–
GSTT1	f-TTCCTTACTGGTCCTCACATCTC, r-TCACCGGATCATGGCCAGCA		480	not used	–
β-Globin (internal control)	f-CCACTTCATCCACGTTCACC, r-GAAGAGCCTAGGACAGGTAC		268	not used	–
DCC g.32008376A>G rs 714	f-TGCACCATGCTGAAGATTGT, r-AGTACAACACAAGGTATGTG	Initiation: 95 °C, 5 min; 35 cycles of 94 °C for 40 s, 56 °C for 40 s, 72 °C for 40 s; final elongation: 72 °C, 7 min [18, 19]	396	*Msp*I (*Hpa*II), 10× Tango buffer	AA—396 bp; GG—256, 140 bp; GA—396, 256, and 140 bp
MLH1 -93G>A rs 1800734	f-CCGAGCTCCTAAAAACGAAC, r-CTGGCCGCTGGATAACTTC	Initiation: 95 °C, 5 min; 36 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; final elongation: 72 °C, 10 min [20]	387	*Pvu*II, 10× Tango buffer	AA—387 bp; GG—207, 180 bp; GA—387, 207, and 180 bp
TP53 Arg72Pro rs 1042522	f-TGAGGACCTGGTCCTCTGAC, r-AGAGGAATCCCAAAGTTCCA	Initiation: 94 °C, 2 min; 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s; final elongation: 72 °C, 5 min [21]	412	*Bsh* 1236 (*Acc*II), 10× R buffer	Arg/Arg—252 and 160 bp; Arg/Pro—412, 252, and 160 bp; Pro/Pro—412 bp

equilibrium. To estimate the relative risk of CRC development, we used multivariate analysis and logistic regression for odds ratio (OR) and the 95 % confidence intervals (95 %CI) calculation using the general model (analysis of each genotype separately), the dominant model (normal homozygotes versus combination of heterozygotes with polymorphic homozygotes), and the recessive model (combination of normal homozygotes with heterozygotes versus polymorphic homozygotes). We did separate analyses for main ethnic groups (Kazakh and Russian). A p value of <0.05 was considered statistically significant.

All statistical analyses of the data were performed using GraphPad InStat™ Software (V. 2.04. Ralf Stahlman, Purdue University) and "Case–control Study Estimating Calculator" from Gene Expert company ("GosNII Genetika" State Scientific Centre of Russian Federation, http://gen-exp.ru/calculator_or.php).

Results

Characteristic of the study population

The case–control study comprised 249 CRC patients and 245 healthy individuals with no history of any cancer. Among the CRC patients, there were 28 patients (11.2 %) with early cancer development (28–50 yrs), including 16 patients (6.4 %) with family history of CRC. Adenocarcinoma was the predominant tumor type among these patients, and 55.82 % of the cases were well and moderately differentiated adenocarcinoma. All tumors were staged using TNM criteria: stage I—16 cases (6.4 %), stage II—86 cases (34.5 %), stage III—108 cases (43.4 %), and stage IV—39 cases (15.7 %).

When selecting the control group of healthy individuals, every attempt was made to match them with the cancer patients according to the basic population characteristics (Table 2).

There were no significant differences in the distribution of age, gender, and ethnicity between the cases and controls. The percentage of non-smokers in cancer patients and healthy individuals did not differ significantly, but there was a small difference between smokers in case and control cohorts (t_{st}= 2.255, p=0.024) because there are ex-smokers in both cohorts.

Case–control study for sporadic colorectal cancer

The case–control study was conducted to investigate any association between the studied polymorphisms and CRC risk. Genotyping of the candidate genes (*DCC* g.32008376A>G, *hMLH1* -93G>A, *TP53* Arg72Pro, deletions of *GSTM1* and *GSTT1*) was performed for both case

Table 2 The characteristics of the colorectal cancer case and control cohorts

Cohort (persons)	Years of birth (average age)	Sex, persons (%)		Ethnicity, persons (%)				Smoking habit, persons (%)		
		Males	Females	Kazakh	Other Asians	Russian	Other Europeans	Smokers	Non-smokers	Ex-smokers
Case (249)	1924–1983 (65.14±9.04)	116 (46.59)	133 (53.41)	70 (28.11)	31 (12.45)	134 (53.82)	14 (5.62)	29 (11.65)	189 (75.91)	31 (12.45)
Control (245)	1924–1984 (64.22±9.12)	106 (43.27)	139 (56.73)	90 (36.73)	16 (6.53)	135 (55.10)	4 (1.63)	51 (20.82)	175 (71.43)	19 (7.76)
t_{st}	0.072	0.549	0.497	1.642	1.845	0.194	1.495	2.255	0.578	1.512
p value	0.944	0.582	0.617	0.101	0.064	0.849	0.133	0.024	0.562	0.131

and control cohorts, and the frequencies of the allele variants are shown in Table 3. All the genotyping results are all in Hardy–Weinberg equilibrium. The goodness-of-fit χ^2 test values for *DCC* g.32008376A>G, *hMLH1* -93G>A, *TP53* Arg72Pro, *GSTT1*, and *GSTM1* genotype distribution in cancer-free controls are 0.87 (p=0.35), 0.16 (p=0.69), 0.61 (p=0.43), 69.21 (p<0.001), and 60.85 (p<0.001), respectively.

Statistical analyses of association between a genetic polymorphism and development of CRC were determined by evaluating the data using the general, dominant, and recessive models for each type of polymorphism. Table 3 shows the adjusted association of candidate gene polymorphisms that were calculated using the general model of inheritance for each genotype separately.

Separate analyses were conducted for each of the main ethnic groups represented in the inhabitants of Almaty (Kazakh and Russian) with reference to smoking habits. In view of the small number of current smokers in our population, we have combined smokers with ex-smokers. According to the general model (Table 3), there is a significant risk of CRC associated with the *DCC 32008376*G/G (OR=1.23) and G/A (OR=1.22) genotypes. This was confirmed using the recessive model where G/G and G/A versus A/A (OR=3.45, 95 %CI=1.75–6.81, χ^2=14.07, p=0.0002), while the A/A genotype had a protective effect (OR=0.29, 95 %CI=0.15–0.57, χ^2=14.07, p=0.0002). Kazakhs also showed this risk association (for the G/G genotype—OR=1.46, 95 %CI=0.78–2.74, and the G/A genotype—OR=1.21, 95 %CI=0.64–2.28, while the AA genotype—OR=0.25, 95 %CI=0.08–0.77) was also protective. But, in Russians, only the GA genotype demonstrates the increased CRC risk (OR=1.29, 95 %CI=0.80–2.08, χ^2=6.90, p=0.03); there was no association of the GG genotype with CRC risk (OR=1.08, 95 %CI=0.67–1.74, χ^2=6.90, p=0.03), but the protective effect of AA genotype was indicated (OR=0.25, 95 %CI=0.08–0.76, χ^2=6.90, p=0.03). The high CRC risk correlates with smoking in G allele carriers (combination of G/G and G/A genotypes versus A/A—OR=3.28, 95 %CI=0.86–12.52, χ^2=3.29, p=0.07) (Table S1).

For the *MLH1* -93 polymorphism, the risk of CRC associated with the G/G genotype (OR=1.45, Table 3). The dominant model, where G/G versus G/A and A/A, confirmed this finding (OR=1.45, 95 %CI=1.02–2.07, χ^2=4.21, p=0.04). The general model of inheritance (Table 3) shows that the protective effect is expressed for G/A genotype (OR=0.68) but not for the A/A genotype (OR=1.02). Separate analysis of main ethnic groups revealed that -93G/G genotype strongly associated with increased CRC risk in Kazakhs (OR=2.67, 95 %CI=1.35–5.30, χ^2=10.61, p=0.005) but not in Russians (OR=1.02, 95 %CI=0.63–1.64, χ^2=0.01, p=1). The -93 A/A genotype demonstrated a marginally significant association of CRC risk with smoking (for AA genotype—OR=1.99, 95 %CI=0.72–5.51; for G/G genotype—OR=0.71, 95 %CI=0.35–1.44; for G/A genotype—OR=1.00, 95 %CI=0.50–2.01; for all genotypes—χ^2=2.08, p=0.35).

Our data detected a significant association between CRC and polymorphism of codon 72 of the *TP53* gene. The general model of inheritance (Table 3) revealed that the Pro/Pro genotype was associated with an increased risk of CRC (OR=3.80). According to the recessive model of OR calculation (Table S2), the combination of Pro/Pro and Pro/Arg genotypes is also associated with CRC susceptibility (OR=4.29, 95 %CI=2.77–6.63, χ^2=46.23, p<0.001), while the Arg/Arg genotype had a strongly protective effect (OR=0.23, 95 %CI=0.15–0.36, χ^2=46.23, p<0.001). In Kazakh, the CRC risk was associated only with the Pro/Pro genotype (for Pro/Pro genotype—OR=3.40, 95 %CI=1.63–7.06; for Pro/Arg genotype—OR=0.57, 95 %CI=0.30–1.07; for Arg/Arg genotype—OR=0.57, 95 %CI=0.26–1.21; for all genotypes—χ^2=11.35, p=0.003). In the Russians, the Pro allele carriers exhibit significantly increased CRC risk (for the Pro/Pro genotype—OR=4.69, 95 %CI=2.53–8.66, and the Pro/Arg genotype—OR=1.55, 95 %CI=0.96–2.53, while it was protective for the Arg/Arg genotype—OR=0.13, 95 %CI=0.07–0.25; for all genotypes—χ^2=53.19, p<0.001). The recessive model confirmed this finding (for the combination of Pro/Pro and Pro/Arg versus Arg/Arg—OR=7.49, 95 %CI=4.02–13.94, χ^2=46.25, p<0.001). In correlation with smoking, the increased CRC risk was significantly increased for the Pro/Pro genotype (OR=5.51, 95 %CI=2.15–14.08, χ^2=23.26, p<0.0001) and Pro/Arg genotype (OR=1.29, 95 %CI=0.64–2.59, χ^2=23.26, p<0.0001). Also the strong protective effect of Arg/Arg genotype was defined in smokers (OR=0.17, 95 %CI=0.07–0.41, χ^2=23.26, p<0.0001; Table S3).

No CRC risk was associated with homozygous *GSTT1* deletions (OR=0.92, Table 3), but the heterozygous genotype correlated with CRC susceptibility (OR=2.01, Table 3). The recessive model of inheritance (−/− versus the combination of +/+and +/− genotypes) did not show any correlations with CRC development, but the dominant model demonstrated an increased CRC risk for the combination of +/− and −/− genotypes (OR=1.43, 95 %CI=1.00–2.04, χ^2=3.90, p=0.05). The presence of the functional allele variants of *GSTT1* in the homozygous state showed a strong protective effect (OR=0.70, 95 %CI=0.49–1.00, χ^2=9.37, p=0.009) which was confirmed by the dominant model of OR calculation. There were no significant differences between Kazakhs (for the +/+ genotype—OR=0.71, 95 %CI=0.37–1.36; for the +/− genotype—OR=1.92, 95 %CI=0.94–3.93; or for the −/− genotype—OR=0.83, 95 %CI=0.44–1.57; for all genotypes—χ^2=3.30, p=0.19) and Russians (for the +/+ genotype—OR=0.85, 95 %CI=0.52–1.37; for the +/− genotype OR=1.96, 95 %CI=1.01–3.83; for the −/− genotype—OR=0.78, 95 %CI=0.45–1.33; for all genotypes—χ^2=4.18, p=0.12).

Table 3 Association between genetic polymorphism and development of colorectal cancer

Type of polymorphism	Genotype	CRC patients (%)	Controls (%)	OR	95 %CI	*p* value	Population group
DCC 32008376	G/G	120 (48.19)	104 (42.98)	1.23	0.86–1.76	0.0009	All ethnic groups
		36 (50.70)	36 (41.38)	1.46	0.78–2.74	0.04	Kazakh
		62 (46.27)	60 (44.44)	1.08	0.67–1.74	0.03	Russian
	G/A	117 (46.99)	102 (42.15)	1.22	0.85–1.74	0.0009	All ethnic groups
		31 (43.66)	34 (39.08)	1.21	0.64–2.28	0.04	Kazakh
		68 (50.75)	31 (43.66)	1.29	0.80–2.08	0.03	Russian
	A/A	12 (4.82)	36 (14.88)	0.29	0.15–0.57	0.0009	All ethnic groups
		4 (5.63)	17 (19.54)	0.25	0.08–0.77	0.04	Kazakh
		4 (2.99)	15 (11.11)	0.25	0.08–0.76	0.03	Russian
MLH1(*-93*)	G/G	126 (50.60)	101 (41.39)	1.45	1.02–2.07	0.09	All ethnic groups
		31 (43.66)	20 (22.47)	2.67	1.35–5.30	0.005	Kazakh
		72 (53.73)	72 (53.33)	1.02	0.63–1.64	1	Russian
	G/A	94 (37.75)	115 (47.13)	0.68	0.48–0.97	0.09	All ethnic groups
		27 (38.03)	56 (62.92)	0.36	0.19–0.69	0.005	Kazakh
		51 (38.06)	52 (38.52)	0.98	0.60–1.60	1	Russian
	A/A	29 (11.65)	28 (11.48)	1.02	0.59–1.77	0.09	All ethnic groups
		13 (18.31)	13 (14.61)	1.31	0.56–3.04	0.005	Kazakh
		11 (8.21)	11 (8.15)	1.01	0.42–2.41	1	Russian
TP53 72	Pro/Pro	99 (39.92)	36 (14.88)	3.80	2.46–5.88	<0.001	All ethnic groups
		29 (41.43)	15 (17.24)	3.40	1.63–7.06	0.003	Kazakh
		54 (40.30)	17 (12.59)	4.69	2.53–8.66	<0.001	Russian
	Pro/Arg	113 (45.56)	104 (42.98)	1.11	0.78–1.59	<0.001	All ethnic groups
		28 (40.00)	47 (54.02)	0.57	0.30–1.07	0.003	Kazakh
		64 (47.76)	50 (37.04)	1.55	0.96–2.53	<0.001	Russian
	Arg/Arg	36 (14.52)	102 (42.15)	0.23	0.15–0.36	<0.001	All ethnic groups
		13 (18.57)	25 (28.74)	0.57	0.26–1.21	0.003	Kazakh
		16 (11.94)	68 (50.37)	0.13	0.07–0.25	<0.001	Russian
GSTT1	+/+	110 (44.18)	130 (53.06)	0.70	0.49–1.00	0.009	All ethnic groups
		22 (30.99)	35 (38.89)	0.71	0.37–1.36	0.19	Kazakh
		73 (54.48)	79 (58.52)	0.85	0.52–1.37	0.12	Russian
	+/−	61 (24.50)	34 (13.88)	2.01	1.27–3.20	0.009	All ethnic groups
		23 (32.39)	18 (20.00)	1.92	0.94–3.93	0.19	Kazakh
		28 (20.90)	16 (11.85)	1.96	1.01–3.83	0.12	Russian
	−/−	78 (31.33)	81 (33.06)	0.92	0.63–1.35	0.009	All ethnic groups
		26 (36.62)	37 (41.11)	0.83	0.44–1.57	0.19	Kazakh
		33 (24.63)	40 (29.63)	0.78	0.45–1.33	0.12	Russian
GSTM1	+/+	90 (36.14)	118 (48.16)	0.61	0.43–0.87	0.004	All ethnic groups
		26 (36.62)	37 (40.66)	0.84	0.45–1.60	0.01	Kazakh
		47 (35.07)	70 (51.85)	0.50	0.31–0.82	0.02	Russian
	+/−	34 (13.65)	40 (16.33)	0.81	34 (13.65)	0.004	All ethnic groups
		10 (14.08)	27 (29.67)	0.39	0.17–0.87	0.01	Kazakh
		18 (13.43)	12 (8.89)	1.59	0.73–3.45	0.02	Russian
	−/−	125 (50.20)	87 (35.51)	1.83	1.28–2.63	0.004	All ethnic groups
		35 (49.30)	27 (29.67)	2.30	1.21–4.40	0.01	Kazakh
		69 (51.49)	53 (39.26)	1.64	1.01–2.66	0.02	Russian

Analysis for smoking and *GSTT1* polymorphism did not reveal any association with CRC risk.

However the "null" (−/−) genotype of another glutathione S-transferase gene *GSTM1* demonstrated a strong correlation

with CRC susceptibility (OR=1.83, Table 3) while the homozygous state of the functional allele and heterozygotes showed resistance to CRC development (for +/+ genotype—OR=0.61, for +/− genotype—OR=0.81, Table 3) in the general population. These findings were also confirmed by the dominant (+/+ versus the combination of +/− and −/− genotypes) and recessive models of OR calculation (Table S4). The strong protective effect was expressed for the combination of +/+and +/− genotypes (OR=0.55, 95 %CI=0.38–0.78, χ^2=10.88, p=0.001). The strong association of *GSTM1* null genotype were determined in Kazakhs (for the +/+ genotype the OR=0.84, 95 %CI=0.45–1.60, for the +/− genotype the OR=0.39, 95 %CI=0.17–0.87, while for the −/− genotype the OR=2.30, 95 %CI=1.21–4.40; for all genotypes—χ^2=8.42, p=0.01). The Russian carriers of the *GSTM1* deletion alleles, however, have an increased risk of CRC development for both the heterozygous state (OR=1.59, 95 %CI=0.73–3.45) and the homozygous deletion (OR=1.64, 95 %CI=1.01–2.66; for all genotypes—χ^2=7.82, p=0.02). The CRC risk of *GSTM1* null genotype in smokers was considerably higher (for the +/+ genotype—OR=0.42, 95 %CI=0.20–0.87; for the +/− genotype—OR=0.36, 95 %CI=0.12–1.08; for the −/− genotype—OR=3.77, 95 %CI=1.82–7.81; for all genotypes—χ^2=13.51, p=0.001).

Discussion

In this study, we investigated sporadic CRC in patients representing the various population groups in Kazakhstan. The choice of candidate genes was based on well-known associations of a limited number of genes implicated in CRC etiology and pathogenesis [5, 9–15]. Numerous molecular epidemiological studies have been devoted to the determination of biomarkers for sporadic and familial CRCs. Moreover, more than 15 % of sporadic CRCs develop through fundamentally different pathways of molecular events, and differences in population genetics are crucial in this process. Therefore, our study focused on polymorphisms and mutations of key genes, for which the association with CRC in ethnically different population groups have been shown.

Epidemiological studies require a careful selection of the control group to be used in the research. This cohort should correspond to a case cohort on many parameters in order to ensure a reliable association between genetic polymorphisms and risk of disease, especially so in the cases of small sample sizes or rare allele frequency. The cohorts used in this study represent inhabitants of one geographic zone (the city of Almaty) in Kazakhstan. To minimize the effects of ethnicity, age, sex, and smoking influence on the susceptibility to CRC, we used the same parameters for the healthy control groups (Table 2). It should be noted that both case and control populations are mixed by ethnicity, and therefore represent several different ethnic groups in Eurasia. However, Russian (approximately 55 %) and Kazakh (approximately 30 %) represent the majority of both cohorts.

Because this represents the first genetic polymorphism study on the population groups in Kazakhstan, we compared the frequency of allele variants in the control cohort with data presented in the National Center for Biotechnology Information (NCBI) SNP database and the literature [11, 19, 22–25]. Table 4 presents mainly data on NCBI SNP database, but the necessary data on the glutathione S-transferase deletion polymorphisms [22–24] and some allele frequencies of *DCC* G32008376A were also included [11, 19, 25].

The distribution of the *GSTM1* deletions are similar among the Asian (0.490–0.540) and European (0.420–0.540) populations while the frequency of *GSTM1* deletions in healthy residents of Almaty city (0.437) was lower than that of the Asian populations and similar to that of the European population. The frequency of *GSTT1* deletions in our study is defined between the known Asian and European populations. The frequency of the rare alleles, 72Pro of *TP53* (0.364) and -93A of *hMLH1* (0.350), corresponded to the populations from Europe and is lower than that of the Asians. The frequency of 32008376A allele of *DCC* gene in Almaty residents was closer to the two population groups in India [19, 25] but lower than that of the European groups represented in the NCBI SNP database. One of the possible explanations for the differences in allele frequencies in our population groups with known populations from Europe and Asia is the mixed ethnic composition of residents in Almaty city (Table 4). Also it should be noted that most of the studied populations from Asia, represented in NCBI SNP database and other sources (Chinese, Japanese, Malaysian etc.), were distinct from the Kazakh population.

The identification of associations between candidate gene polymorphisms and CRC is not surprising. The tumor suppressor gene *TP53* regulates the cell cycle, apoptosis, and genome stability and is one of the most frequently mutated genes in human cancers. The most common mutations are single base substitutions that alter protein function with some of the mutations being oncogenic and conferring gain-of-function properties.

The *TP53* Arg72Pro polymorphism can play a dual role in cancer development [12, 26]. On the one side, the protein product of the 72Arg allele is more effective in inducing apoptosis [27], resulting in an extension of the G1 phase of the cell cycle in which DNA repair processes are very active [28]. Also, it has been shown that the E6 oncoprotein of HPV-16 and HPV-18 can interact with p53 protein to induce its degradation. Furthermore, the 72Arg allele is associated with faster degradation than 72 Pro [29]. Both the Arg and Pro alleles have been shown to be associated with a high risk of malignancy. In a meta-analysis of more than 300 published

articles [26], the Arg72Pro polymorphism of *TP53* was associated with different patterns of cancer susceptibility according to cancer site, ethnicity, allele frequency, and histological type of tumor. Our previous study on the Kazakhstan population demonstrated a strong association of *TP53* 72Pro allele with susceptibility to esophageal cancer and the 72Arg allele with cervical cancer development [16].

The role of the *TP53* Arg72Pro polymorphism in CRC susceptibility has been examined in several studies [12, 26, 30], with an overall controversial outcome. A number of studies have found an association of CRC risk with the Pro72 allele in ethnically different populations [31–36]. However, one meta-analysis [30] that includes information on 3603 CRC cases and 5524 controls indicates that the *TP53* codon 72 polymorphism is not associated with CRC risk. Twenty studies (total 7184 CRC cases and 9332 controls) were included in another meta-analysis [12], and the association of CRC or adenoma risk with 72Pro has been shown in studies in Korea, India, China, Czech Republic, Greece, Italy, Spain, Caucasian, and Afro-American, while the 72Arg in three studies in Germany, Italy, and Argentina did not observe any association. This suggests that ethnicity and the type of tumor may modulate the penetrance of *TP53* Arg72Pro in CRC susceptibility. In our combined study of the ethnically mixed population from Kazakhstan with a prevalence of Russian (54 %), Kazakh (28 %), other Asians (12 %), and other Europeans (6 %), the CRC risk for the Pro/Pro genotype was increased (OR=3.80). In the Russian population of Almaty, the risk was much higher (the Pro/Pro OR=4.69, the Pro/Arg OR=1.55, while the combination of Pro/Pro and Pro/Arg versus Arg/Arg was OR=7.49). In the Kazakh population, however, the CRC risk is associated only with the Pro/Pro genotype (OR=3.40) which also demonstrated a strong association with smoking. CRC risk for smokers combined with ex-smokers was significantly higher (OR=5.51) than that for non-smokers (OR=3.37).

Glutathione S-transferases (GSTs), a multigene family of the phase II metabolizing enzymes are active in the detoxification of a wide variety of potentially toxic and carcinogenic substances by conjugating them to glutathione. Deletions of GST genes are associated with susceptibility to many cancer types. While several studies have shown that deletions of *GSTM1*, rather than *GSTT1*, are associated with CRC susceptibility in the Caucasian [37, 38], Japanese [39], and mixed American populations [40, 41] and an even stronger association with smoking [40, 41], other studies did not find any association [42, 43]. Moreover, a case–control study of a population from Scotland did not show the interaction between the *GSTM1* or *GSTT1* polymorphisms and smoking, meat intake, and green leafy vegetable consumption [43]. A meta-analysis of 20 studies [44] determined that *GSTM1* status has no effect on the risk of developing colon cancer. Other meta-analyses [5] that included 44 studies for *GSTM1* (11,998 CRC cases, 17,552 controls) and 34 studies for *GSTT1* (8596 cases, 13,589 controls) showed that *GSTM1* null allele carriers exhibited an increased CRC risk in the Caucasian population, and no significant association was detected for Chinese subjects (pooled OR=1.025). Similarly, while the *GSTT1* null allele carriers exhibited an increased CRC risk in the Caucasian populations (pooled OR=1.312), the association in Chinese subjects was not significant (pooled OR=1.068). The results of our study did not show any significant CRC risk of the *GSTT1* null genotype (OR=0.92), but defined the strong association of *GSTM1* null genotype (OR=1.83 for ethnically mixed population), which is higher in Russians (OR=2.30) than in Kazakhs (OR=1.64), and also significantly correlates with smoking (OR=3.77).

DNA mismatch repair (MMR) system maintain genomic integrity during DNA replication and the loss of normal MMR function leads to a mutator phenotype that is characterized by microsatellite instability (MSI) that has been detected in 10–15 % of colorectal cancers. The majority of CRC displaying MSI is caused by somatic mutation and hypermethylation of the *MLH1* CpG island promoter region, whereas a smaller portion is caused by Lynch syndrome, a familial cancer predisposition syndrome caused by autosomal dominant inheritance of defective MMR genes [1, 4, 6, 45]. The human MutL homolog 1 (*hMLH1*) gene is one of the major genes in the MMR pathway, and it plays an important role not only in recognition and repair of mismatched DNA base pairs but also in other vital cellular processes including cell cycle arrest, oxidative stress, and apoptosis [45]. *MLH1* -93G>A (rs1800734) is a single nucleotide polymorphism located in the promoter region which regulates the activity of the promoter and the rate of gene transcription [46]. Many studies have evaluated the relationship of *MLH1* -93G>A polymorphism with the risk of CRC [13–15, 47–50]. A large-scale case–control study [13] in Canada revealed the association of -93 A/A genotype with an increased risk of CRC in the Newfoundland population without MSI (OR=2.22). This genotype was highly associated with an MSI–CRC risk for the Newfoundland population (OR=8.88) while the MSI–CRC risk for Ontario has an OR=3.23. A similar association has been observed in the USA [49], in the United Kingdom [14], and in Malaysia [15]. However, in the Mexican population [50], there was a significantly reduced risk of CRC for the -93 A/A genotype. In meta-analysis including 7508 CRC cases and 7185 controls from 7 studies [45], however, no association of *MLH1* -93G>A polymorphism with a CRC risk was observed. Our results show that -93 G/G genotype strongly associated with increased CRC risk in Kazakhs (OR=2.67) but not in Russians (OR=1.02). The OR for the ethnically mixed

Table 4 The comparison of allele frequencies of healthy people of Kazakhstan population with earlier studied populations

Polymorphism	Allele	The frequency of allele		
		Control cohort (Kazakhstan population)	Integrated data from different sources	
			Asian populations	European populations
GSTM1	+	0.563	0.460–0.510 [22–24]	0.460–0.580 [22–24]
	−	0.437	0.490–0.540 [22–24]	0.420–0.540 [22–24]
GSTT1	+	0.600	0.460–0.520 [22–24]	0.610–0.840 [22–24]
	−	0.400	0.480–0.540 [22–24]	0.160–0.385 [22–24]
TP53 Arg72Pro (G215C)	Arg (G)	0.636	0.489–0.614	0.500–1.000
	Pro (C)	0.364	0.386–0.511	0.000–0.500
hMLH1 -93G>A	G	0.650	0.410–0.615	0.500–1.000
	A	0.350	0.385–0.590	0.200–1.000
DCC g.32008376A>G	G	0.640	0.623 [19], 0.644 [25]	0.500–1.000
	A	0.360	0.356 [25], 0.377 [19]	0.500–1.000; 0.283 [11]

population from Almaty (Kazakhstan) was 1.45. No significant association of CRC risk was detected for the -93 A/A genotype in smokers combined with ex-smokers (OR=1.99, p=0.35). Similar to our results, the -93 G/G genotype is also highly associated with tobacco-related oral squamous cell carcinoma in Asian Indians (OR=6.73) [51]. Also Korean women with breast cancer show an increased risk with the -93 G/G genotype (OR=1.33) [52]. So, ethnicity appears to be a crucial covariate, suggesting that the *MLH1* -93G>A polymorphism has a different penetrance according to ethnicity, cancer type, and smoking habit.

Deleted in colorectal cancer (*DCC*) gene encodes the netrin 1 receptor, a transmembrane protein that is a member of the immunoglobulin superfamily of cell adhesion molecules. Loss of heterozygosity (LOH) and MSI in the chromosome 18q21 region that contains the *DCC* have been observed for many cancers in digestive organs [53]. Some studies have demonstrated that LOH are related to poor differentiation, metastasis, and poor prognosis in CRC [54–56]. In some case–control studies, polymorphisms in *DCC* genes have been linked to the modulation of risk for colorectal [11, 57, 58] and gallbladder cancer [19, 25, 59]. Thus, polymorphism at codon 201 of the *DCC* gene is associated with carcinoma type, stage of disease, and prognosis [57]. For another SNP (rs2298606), the CC genotype was associated with the tumors located in the left colon [58]. Investigations on the role of the *DCC* g.32008376A>G (rs714) polymorphism closely associated with LOH in CRC started only recently [18]. A study on the Romanian population [25] showed that G allele is associated with protection for CRC (OR=0.34), while the AA genotype (OR=2.97) and A allele (OR=2.87) are associated with an increased risk for CRC. The statistically significant association of the AA genotype was detected in the North Indian population for gallbladder [19], esophageal, and gastric cancers [25]. Our results defined the statistically significant association of increased CRC risk with G allele carriers in Almaty (for G/G genotype—OR=1.23; for G/A genotype—OR=1.22), while the AA genotype demonstrates a strongly protective effect (OR=0.29). Separate analysis of the main ethnic groups defined that only Kazakhs showed this association (for G/G genotype—OR=1.46, for G/A genotype—OR=1.21, for AA genotype—OR=0.25). In the Russians, however, only the GA genotype demonstrated an increased CRC risk (OR=1.29) while there was no association of the GG genotype with CRC risk (OR=1.08). Smoking was associated with an elevated CRC risk in the G allele carriers (combination G/G and G/A genotypes versus A/A—OR=3.28). We propose that this SNP of *DCC* also exhibits population/ethnic-specific patterns, but this suggestion needs a further investigation.

The results of this case–control study for sporadic cases of CRC show that some polymorphisms can have predictive value for susceptibility to CRC, at least those that demonstrate statistically reliable ORs for the mixed population from Kazakhstan and for both main ethnic groups (Kazakhs and Russians). There are two important genotypes: the *TP53* homozygous Pro72Pro (mixed population OR=3.80, p<0.0001) and the *GSTM1* deletions (mixed population—OR=1.83, p=0.001).

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Conflicts of interest None

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