research article

Association between polymorphisms in segregation genes BUB1B and TTK and gastric cancer risk

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Background. Malignant transformation of normal gastric cells is a complex and multistep process, resulting in development of heterogeneous tumours. Susceptible genetic background, accumulation of genetic changes, and environmental factors play an important role in gastric carcinogenesis. Single nucleotide polymorphisms (SNPs) in mitotic segregation genes could be responsible for inducing the slow process of accumulation of genetic changes, leading to genome instability.

Patients and methods. We performed a case-control study of polymorphisms in mitotic kinases *TTK* rs151658 and *BUB1B* rs1031963 and rs1801376 to assess their effects on gastric cancer risk. We examined the *TTK* abundance in gastric cancer tissues using immunoblot analysis.

Results. C/G genotype of rs151658 was more frequent in patients with diffuse type of gastric cancer and G/G genotype was more common in intestinal types of gastric cancers (p = 0.049). Polymorphic genotype A/A of rs1801376 was associated with higher risk for developing diffuse type of gastric cancer in female population (p = 0.007), whereas A/A frequencies were increased in male patients with subserosa tumour cell infiltration (p = 0.009). T/T genotype of rs1031963 was associated with well differentiated tumours (p = 0.035). TT+CT genotypes of rs1031963 and GG+AG genotypes of rs1801376 were significantly associated with gastric cancer risk (dominant model; OR = 2,929, 95% CI: 1.281–6.700; p = 0.017 and dominant model; OR = 0,364, 95% CI: 0.192–0.691; p = 0.003 respectively).

Conclusions. Our results suggest that polymorphisms in mitotic kinases *TTK* and *BUB1B* may contribute to gastric tumorigenesis and risk of tumour development. Further investigations on large populations and populations of different ethnicity are needed to determine their clinical utility.

Key words: cancer susceptibility; chromosomal instability; chromosome segregation; mitotic checkpoint; serine/threonine kinase; genetic association

Introduction

Gastric cancer is one of the major contributors to cancer-related deaths worldwide with estimated 989 600 new cases and 738 000 deaths in 2008.[1,2] It is believed that complex interplay of genetic and environmental factors triggers the accumulation of numerous genetic and epigenetic alterations in cells, resulting in deregulation of normal cell functions and disruption of stomach linen homeostasis.[3-6] Individual genetic factors probably contribute to aberrant processes in the genesis of malignant phenotype. Among them, single nucleotide polymorphisms (SNPs) and other genetic variants play an important role as the main genetic elements in the aetiology of several complex diseases, including gastric cancer.[7-11] In gastric carcinogenesis this is further supported by the fact that only

a small proportion of individuals exposed to the known environmental risk factors develop adenocarcinoma.[5,10] Therefore, there is continuing interest for determining simple genetic tests for identifying individuals at high risk for the development of gastric tumours and for identifying patients with high risk for recurrence in order to ensure improved and early diagnosis as well as better survival of patients.

A majority of gastric cancer patients show chromosomal instability (CIN) resulting in aneuploidy.[4,12,13] It has been suggested that tumour cells acquire aberrant chromosome numbers and other chromosomal defects as a result of deregulation of mechanisms responsible for maintaining the chromosomal number stability, such as spindle assembly checkpoint and chromosome segregation.[14,15] However, mutations in mitotic genes are rare, due to the fact that severe defects of these genes would trigger cell death by cell-surveillance early in the development.[14-17] Studies revealed that subtle changes in mitotic segregation genes, controlling chromatids separation or regulating the progress of mitosis, could be prime candidates for inducing the slow process of accumulation of genetic changes, leading to CIN.[15,18-20] The novel hypothesis is further supported by the fact that this process is slow, and explains the late onset of sporadic epithelial cancers[21,22], as well as heterogeneous mutation load observed in different sections of tumours from individual patients.

The multidomain protein kinase BUB1B (BUB1-related kinase, known as MAD3 in yeast) plays a central role in the process of spindle assembly checkpoint (SAC), which prevents defects in the segregation of sister chromatids by delaying their separation until all chromatids have achieved correct attachments to the mitotic spindle.[23,24] BUB1B is part of the mitotic checkpoint complex (MCC), which together with BUB3, MAD2 and CDC20 inhibits the anaphase-promoting complex/cyclosome (APC/C), delaying the onset of anaphase and ensuring proper chromosome segregation.[25] The protein BUB1B has also been localized to the kinetochores and is important for stabilizing the kinetochore-microtubule interactions and chromosome alignment.[26] A dual specificity protein kinase TTK (alias MPS1) is crucial for the spindle assembly checkpoint, for chromosome biorientation on the mitotic spindle and for ensuring accurate chromosome segregation.[27,28] Inhibitor and chemical genetics studies showed that TTK activity facilitates the conformational activation of MAD2 from open to closed form (C-MAD2) capable of CDC20 binding and inhibition, thus delaying the onset of anaphase.[29] TTK is probably implicated in the recruitment of the MAD1–C-MAD2 complex to kinetochores and during mitosis its activity is continuously required to recruit O-Mad2 to the Mad1–C-Mad2 core.[30] Furthermore, TTK is required for CENP-E recruitment, whose activity is essential for metaphase chromosome alignment.[30]

In the present study we examined polymorphisms rs151658 (C>G) in *TTK* gene, rs1031963 (C>T) and rs1801376 (A>G) in *BUB1B* gene in the population of Slovenian patients with an advanced gastric cancer and their impact on gastric cancer risk. We also examined the associations of these genetic variants with clinico-histopathological features of patients.

Patients and methods

Research subjects

The study population (n = 284) consisted of 108 Slovenian patients with gastric cancer and 176 control subjects who at the time of peripheral blood extraction did not have cancer. Tumour and corresponding non-tumour tissues at least 7 cm away from the edge of the adenocarcinoma were collected from patients who were admitted to the Clinical Department for Abdominal Surgery at the University Medical Centre Ljubljana and Department for Pathology at the Institute of Oncology Ljubljana during the years 2000–2008. Samples were macrodissected by pathologist, frozen in liquid nitrogen and stored at -70°C. Comprehensive medical data were obtained from registries and pathologist's evaluation. The following clinico-histopathological parameters were recorded: tumour differentiation (grade), location, blood and lymphatic vessel invasion (vascular invasion, perineural invasion), occurrence of tumour cells in the lymphatic vessels (lymphatic invasion), depth of invasion (pT), lymph node involvement (pN), and presence of distant metastases (pM). The gastric cancer cases were classified into diffuse type (n = 46) and intestinal (n = 58) according to Lauren classification. The mean age ± standard deviation (SD) of patients was 66.12 ± 12.02 (range, 33–87 years), and the percentage of men was 63.0%. Cases lost to follow-up (n = 6) and those, who died within 30 days after surgery (n = 2), were excluded from survival analyses. The control population was randomly selected during the years 1999–2007 and shared the ethnic and geographic background of the gastric cancer patients. The research was ap-

proved by the National Medical Ethics Committee of the Republic of Slovenia and confidentiality of personal medical data as well as other data relating to individual identification has been assured in accordance with the Helsinki Declaration.

Genotyping

Genomic DNA from gastric tumour and non-tumour tissues was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and QuickGene™ DNA Tissue Kit S (Fujifilm Corporation, Tokyo, Japan) on QuickGene-810 DNA isolation system (Fujifilm) according to manufacturer's protocol. Genomic DNA from control population was extracted from peripheral blood samples using Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's protocol. The DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). Genotyping for polymorphism rs151658 (C>G) in *TTK* gene, and polymorphisms rs1031963 (C>T) and rs1801376 (A>G) in *BUB1B* gene was performed using TaqMan-based allele-specific polymerase chain reaction assays on the ABI Prism 7000 Sequencing Detection System apparatus (Applied Biosystems, Foster City, CA, USA) according to the procedure recommended by Applied Biosystems. The 10 μL reaction volume contained 100 ng of DNA. Assay IDs were: C_3181603_10, C_1237153_10, and C_3052718_1. In order to confirm the veracity of the results, the polymorphisms were re-genotyped by direct sequencing on a randomly selected smaller batch of samples.

Immunoblot analysis

A total of 21 paired gastric adenocarcinoma (GA) and adjacent control tissue samples were ground with a mortar and pestle in liquid nitrogen and lysed with 7 mol/L urea, 2 mol/L thiourea, 40 g/L CHAPS, with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). For every 10 mg tissue, 50 μl lysis buffer was added. After sonication on ice (3 x 10 s), the samples were incubated for 1 h on ice with occasional vortexing, and then centrifuged at 20,000×g for 1 h at 4°C. The supernatants were collected and the protein concentrations were determined using the commercial Bradford reagent (Thermo Fisher Scientific, Waltham, MA, USA) with BSA used as the standard. Immunoblot analysis was performed on 42 samples. A total of 30 μg protein per sample was loaded onto 10% gels, separated using SDS-PAGE, and transferred onto PDVF membranes (Millipore, Billerica, MA, USA), which were then blocked in 50 g/L skimmed milk 1 h. The primary antibody was used in the following dilution: anti-Mps1 (anti-TTK) antibody, 1 μg/ml (ab11108, Abcam, Cambridge, UK). Horseradish peroxidase-conjugated secondary antibody was used in the following dilution: goat anti-mouse antibody, 1:5000 (115-035-062, Jackson ImmunoResearch, Newmarket, Suffolk, UK). The proteins were revealed by chemiluminescence using LAS-4000 CCD camera (Fujifilm, Tokyo, Japan). The blots were then quantified with Multi Gauge software (Fujifilm) and the intensities were normalized to Ponceau-S-stained membranes, to allow for loading and transfer variations.

Statistical and bioinformatic analyses

Statistical evaluation of the genotyping data was carried out using the χ^2 or Fischer's exact tests to compare the groups regarding genotype frequencies. Hardy-Weinberg (HW) equilibrium was calculated with an online program (http://www.genes.org.uk/software/hardy-weinberg.shtml).[31] Survival was assessed by the Kaplan-Meier method and differences between groups were evaluated using the log-rank test. Multivariate survival analyses were further performed using the Cox proportional-hazards regression model. In the Cox multivariate analyses, forced entry procedure was used to determine the predictor variables. Only the variables that resulted in p-values < 0.05 in the Kaplan-Meier test were entered into the Cox proportional hazard model for the determination of independent prognostic factors for gastric cancer. The postoperative period was measured from the date of surgery to the date of the last follow-up or death. Statistical software used for calculations was IBM® SPSS® Statistics Version 20. For all statistical tests, a probability level (p-value) of less than 0.05 was considered significant.

To assess the statistical significance of altered protein abundance in the immunoblotting (as the tumour vs. non-tumour paired samples), non-parametric Wilcoxon signed-rank test was used. The tests were double-sided and the values with $p < 0.05$ with a confidence level of 95% were considered to be statistically significant. To assess the correlation of the altered protein abundance from the immunoblotting with the histopathological parameters, repeated measures ANOVA was used. The values with $p < 0.05$ were considered to be statistically significant. Bonferroni post-tests were used to deter-

TABLE 1. Clinicopathological characteristics of patients with gastric cancer

Parameter	Number of patients (%)
Age (years ± standard deviation) (n = 108)	66.12 ± 12.02
Gender (n = 105)	
Male	68 (63.0)
Age (years ± standard deviation) (n = 66)	65.07 ± 12.03
Female	40 (37.0)
Age (years ± standard deviation) (n = 39)	67.90 ± 11.94
Lauren's classification (n = 104)	
Intestinal	58 (55.8)
Diffuse	46 (44.2)
Location (n = 101)	
Upper	40 (39.6)
Lower	34 (33.7)
Mixed	27 (26.7)
Grade/differentiation (n = 105)	
Well	9 (8.6)
Moderate	24 (22.9)
Poor	72 (68.6)
Vascular invasion (n = 80)	
Present	27 (33.8)
Not present	53 (66.3)
Perineural invasion (n = 95)	
Present	44 (46.3)
Not present	51 (53.7)
Lymphatic invasion (60)	
Present	53 (88.3)
Not present	7 (11.7)
pN (n = 105)	
0	24 (22.9)
1–2	15 (14.3)
3–6	20 (19.0)
> 7	46 (43.8)
pT (n = 105)	
Muscularis propria	6 (3.7)
Subserosa	50 (42.6)
Serosa	49 (36.1)

pN = number of positive regional lymph nodes; pT = tumour invasion

TABLE 2. Multivariate survival analysis of clinic-pathological variables in gastric cancer patients

Variable	B*	SE (B)	OR (95% CI)	P
pN	0.670	0.127	1.954 (1.525–2.504)	0.000
Lauren's classification	0.591	0.246	1.807 (1.116–2.925)	0.016

*Predicted change in the hazard for a unit increase in the predictor.
CI = confidence interval; OR = odds ratio; pN = number of positive regional lymph nodes; SE = standard error

mine where the differences were significant. All of the analyses were performed using Microsoft Office Excel 2007 (Microsoft Corporation, Washington, USA) and GraphPad Prism 5 (GraphPad Software, Inc., California, USA).

In order to functionally evaluate intronic polymorphisms we identified their effect in the context of polymorphic biological sequences on protein binding motifs. We used web-based software PROMO, which is part of the ALGGENE web-server.[32,33] The search for putative binding sites was performed using the following parameters: human species, all motifs, and all factors. The data for comparisons of genotype frequencies in European populations of examined SNPs in this study was extracted from the 1000 Genomes Project data platform using a specific version of the Ensembl browser (http://browser.1000genomes.org).[34]

Results

Patients' survival is associated with certain clinico-pathological features

The clinical information and demographic characteristics of selected patients with gastric cancer in this study are summarized in Table 1. At the end of a period of up to 11 years of follow-up, a total of 69 patients out of 100 have died.

The overall 5-year survival was 33.5%. No statistically significant association between tested genetic variations and survival was observed ($p > 0.05$). Univariate survival analysis showed that only Lauren's classification and lymph node involvement (pN) were significant prognostic factors. Diffuse type predicted shorter 5-year survival (log-rank test, $\chi^2 = 5.516$, $p = 0.019$) with overall mean estimate of survival for patients with intestinal type 64.67 months ± 7.74 (SE) (CI = 49.50–79.84) and 39.73 months ± 6.83 (SE) (CI = 26.75–53.11) for patients with diffuse type of gastric cancer. Regarding the parameter pN, patients with 7 or more positive lymph nodes had shorter survival time of 21.93 months ± 4.30 (SE) with CI = 13.5030–36 (log-rank test, $\chi^2 = 34.169$, $p = 0.000$). Multivariate analysis was performed for the same set of patients with complete clinical data sets. Cox regression model included both significant variables, pN and tumour classification. The enter method showed significant improvement ($p < 0.05$) if both parameters were entered into the model (Table 2).

SNPs in *TTK* and *BUB1B* are associated with type, grade, and location of gastric cancer

Associations between clinicopathological parameters and genotypes of SNPs are presented in Table 3. Statistical analysis revealed a weakly significant association for rs151658 genotypes C/G and risk of developing diffuse type of gastric cancer, and genotype G/G and risk of developing intestinal type of

cancer (p = 0.049). Similar results were obtained for both male and female populations of patients (p = 0.047 and p = 0.024, respectively). Genotype A/A of rs1801376 polymorphism was significantly associated with higher risk of developing the diffuse type of gastric cancer in total and female populations of patients (p = 0.007). Interestingly, A/G genotype was under-represented in populations with diffuse type of gastric cancer. Genotype A/A of this polymorphism was also associated with the invasion of tumour cells into subserosa layer of stomach in male population (p = 0.009). A/A genotype was also associated with tumour location, namely, A/A frequencies were increased in patients with tumours disseminated across the whole stomach (p = 0.035).

Genotype T/T of rs1031963 was associated with well differentiated tumours in total population (p = 0.035); however, when we stratified it into female and male populations, we observed a significant association of this genotype with moderately differentiated tumours in the female population (p = 0.004). Clinico-pathological features lymph node involvement (pN), depth of invasion (pT), vascular invasion, perineural invasion and lymphatic invasion did not show significant associations with investigated polymorphisms.

SNPs in BUB1B are associated with gastric cancer risk

The analyses of genotype frequencies in selected SNPs between cases and controls are shown in Tables 4 and 5. The frequencies of all genotypes in cases and control groups were in Hardy-Weinberg equilibrium.

The tested polymorphisms did not show significant differences between gastric cancer patients and control group. In contrast, when we stratified the population for gender, we found significant association between *BUB1B* rs1801376 genotypes and higher risk for developing gastric tumours (p = 0.029). Similarly, dominant model combining genotypes A/G and G/G showed comparable results (p = 0.010; p [Yates correction] = 0.017). Furthermore, tests for association showed analogous results and confirmed significantly higher frequency of G allele in female population of patients with gastric cancer (0.41 *vs.* 0.28 in control group). We also observed allele frequency difference in male patient population for *BUB1B* rs1031963. The dominant model, combining genotypes TT+CT versus CC, showed that patients with C/C homozygous allele had significantly higher risk for developing gastric cancer.

TABLE 3. Comparison of clinic-pathological features and genotypes *TTK* rs151658, *BUB1B* rs1031963, and *BUB1B* rs1801376 in patients with gastric cancer

Parameter		Subject	Variant/Genotype			P
			***TTK* rs151658**			
			GG	CG	CC	
Lauren's classification	Intestinal	Total	17	21	20	**0.049**
	Diffuse		5	25	16	χ^2=6.033
	Intestinal	Male	9	10	14	**0.047**
	Diffuse		5	20	8	χ^2=6.113
	Intestinal	Female	8	11	6	**0.024**
	Diffuse		0	5	8	F=7.499
			***BUB1B* rs1801376**			
			AA	AG	GG	
	Intestinal	Total	18	33	7	**0.007**
	Diffuse		28	13	5	χ^2=9.951
	Intestinal	Male	15	14	4	0.472
	Diffuse		20	9	4	F=1.836
	Intestinal	Female	3	19	3	**0.007**
	Diffuse		8	4	1	F=9.688
			***BUB1B* rs1031963**			
			CC	CT	TT	
Tumour differentiation	Well	Total	1	4	4	**0.035**
	Moderate		7	9	7	F=9.642
	Poor		23	39	7	
	Well	Male	0	2	3	0.139
	Moderate		6	7	2	F=6.439
	Poor		19	20	6	
	Well	Female	1	2	1	**0.004**
	Moderate		1	2	5	F=12.549
	Poor		19	20	6	
			***BUB1B* rs1801376**			
			AA	AG	GG	
pT	Muscularis propria	Total	1	4	1	0.232
	Subserosa		27	18	5	F=5.250
	Serosa		18	25	6	
	Muscularis propria	Male	0	3	1	**0.009**
	Subserosa		22	6	2	F=11.832
	Serosa		13	14	5	
	Muscularis propria	Female	1	1	0	0.816
	Subserosa		5	12	3	F=1.967
	Serosa		5	11	1	
			***BUB1B* rs1801376**			
			AA	AG	GG	
Tumour location	Upper	Total	14	23	3	**0.035**
	Lower		13	15	6	F=10.104
	Whole		18	6	3	

pT = tumour invasion

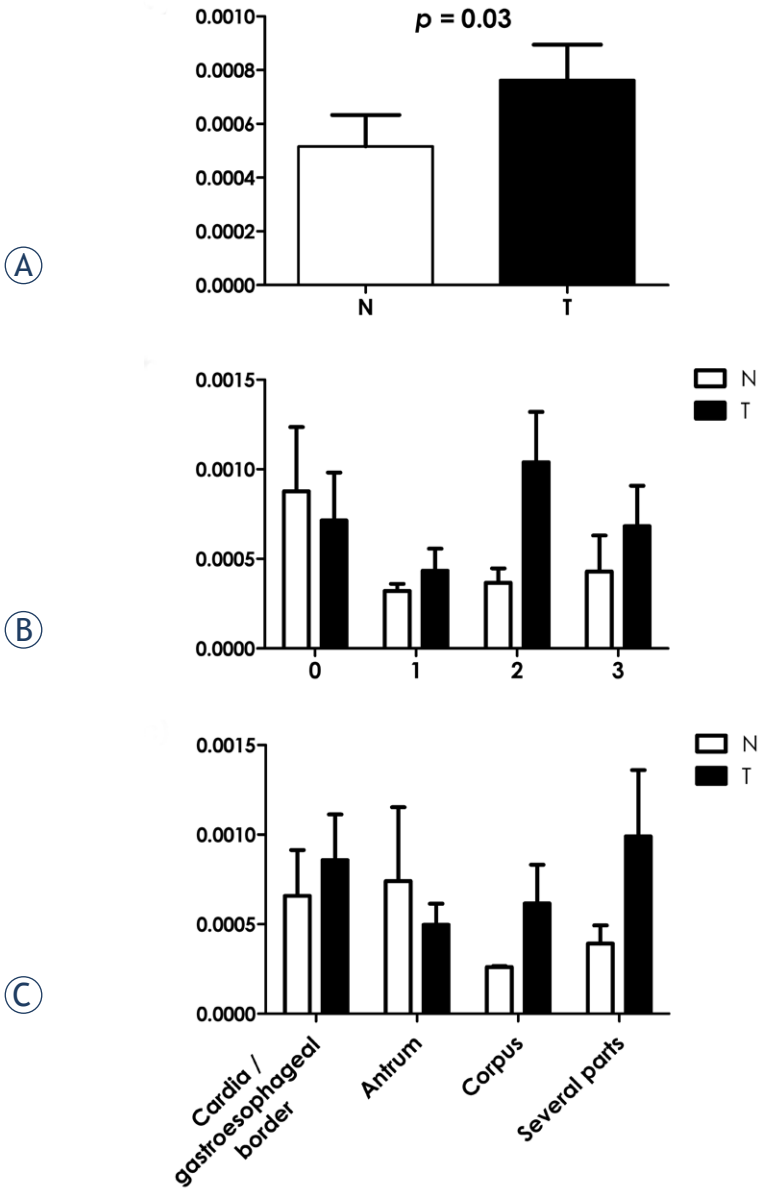

FIGURE 1. Immunoblotting of TTK. (A) Densitometry quantification analysis for the relative band densities from the protein abundance immunoblotting for the indicated protein in the non-tumour (N) and tumour (T) gastric tissues. The p value given (Wilcoxon signed-rank test) indicates the significance of the difference between the non-tumour (N) and tumour (T) gastric tissue samples. (B, C) Densitometry quantification analysis for the relative band densities from the protein abundance immunoblotting for TTK in the non-tumour (N) and tumour (T) gastric tissues samples according to lymph node involvement (pN) and location of the tumours.

Distribution and genotype frequencies of SNPs in *TKK* and *BUB1B* between European and Slovenian populations

Comparisons of the SNPs' genotype frequencies between our test groups and European populations are presented in Figure 3. Genotype frequencies of rs151658, rs1031963 and rs1801376 in our groups of populations showed significant differences from European population (Table 6). The frequency of rs151658 C/C genotype was higher than expected in the Slovenian population of patients compared to total European population ($p = 0.015$). Similarly, we observed more rs1031963 C/C genotypes in the male population of Slovenian patients with gastric cancer ($p = 0.042$) compared with total European population and European population stratified for males. The rs1801376 A/G genotype was higher and A/A genotype was under-represented in female population of patients with gastric cancer compared to the total European population ($p = 0.034$) and female European population ($p = 0.014$).

TTK abundance is altered in tumour tissues of gastric cancer patients

Immunobloting data on individual samples (Figure 1A) demonstrated statistical significance for the increased abundance of TTK ($p = 0.03$) in the tumour tissues. No statistically significant correlation of TTK abundance with clinical histopathological parameters or rs151658 genotypes was observed. However, some trends were observed (Figures 1B and C) for lymph node involvement (pN) and antral tumour location: TTK abundance was higher in normal tissues compared to tumour tissues when no regional nodes were invaded with tumour cells (pN = 0) and when the tumours were located at the bottom of the stomach (antrum).

Prediction of binding motifs showed that polymorphic sites in *TTK* and *BUB1B* bind different transcription factors

To determine if different intronic polymorphisms could affect binding of transcription factors, we performed *in silico* analysis of conserved human motifs using polymorphic sequences as templates (Figure 2). We identified distinct recognition sites for different proteins for both *TTK* rs151658 and *BUB1B* rs1031963. TFII-I, c-Myb, NFI/CTF, and HNF-4 alpha binding motifs were recognized if rs151658 polymorphic site contained C allele. In contrast, if G allele was present, GR, TFII-I, NCFI/CTF, and HNF-4 alpha were identified. Comparison of rs1031963 alleles showed that if allele T was present, several binding motifs were predicted, whereas in the case of allele C, there were no recognizable binding patterns.

Discussion

In this study, we investigated the effects of selected polymorphisms in mitotic kinases *TTK* and *BUB1B* and risk of developing gastric cancer in Slovenian population. We also determined the associations between tested polymorphisms and clinic-pathological features of patients. The results provide evidence that *TTK* rs151658, *BUB1B* rs1031963, and rs1801376 could potentially serve as prognostic biomarkers for determining tumour differentiation and invasion. Furthermore, rs1801376 G allele could be used as one of determinants for gastric cancer screening in female population and CC genotype in rs1031963 could be used for selection of male population at higher risk for developing gastric cancer.

TTK gene harbours more than 600 different one-nucleotide polymorphisms (data obtained from GeneCards, http://www.genecards.org/). We investigated intronic polymorphism, because this gene has many alternative transcripts, and intronic one-nucleotide variants could have an effect on splicing and/or ubiquitination.[35,36] SNP rs151658 lies between exons 12 and 13. In its vicinity there are binding sites for TFII-I, c-Myb, NFI/CTF, and HNF-4 alpha transcription factors, if C allele is present and binding sites for GR, TFII-I, NFI/CTF, and HNF-4 alpha, if G allele is present

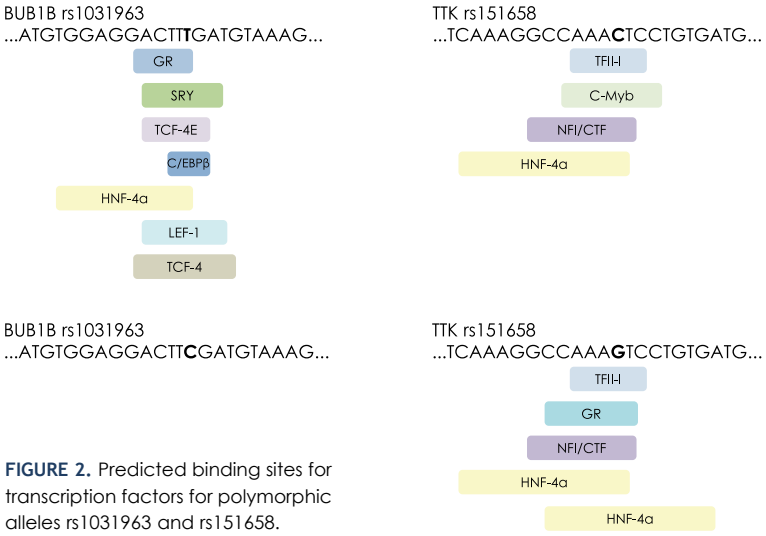

FIGURE 2. Predicted binding sites for transcription factors for polymorphic alleles rs1031963 and rs151658.

TABLE 4. Distribution of genotype frequencies of *TTK* rs151658, *BUB1B* rs1031963, and *BUB1B* rs1801376 between gastric cancer patients and control subjects

Variants	Genotype	Cases (n)	Controls (n)	P	HWE (cases)	HWE (controls)
TTK rs151658	GG	24	55	χ^2=3,628 0.163	χ^2=1.08 0.299	χ^2=2.87 0.090
	CG	48	76			
	C/C	36	44			
BUB1B rs1031963	CC	32	48	χ^2=1.059 0.589	χ^2=0.345 0.557	χ^2=0.035 0.852
	CT	54	89			
	TT	18	39			
BUB1B rs1801376	AA	47	89	χ^2=2.291 0.318	χ^2=0.021 0.885	χ^2=0.005 0.941
	AG	49	69			
	GG	12	13			
Male population	AA	36	49	χ^2=1.186 0.553	χ^2=1.530 0.216	χ^2=0.040 0.841
	AG	24	39			
	GG	8	7			
Female population	AA	11	40	F=6.955 **0.029**	χ^2=3.352 0.067	χ^2=0.013 0.909
	AG	25	30			
	GG	4	6			

F = Fisher statistics; HWE = Hardy-Weinberg Equilibrium; χ^2 = chi-square statistics

TABLE 5. Odds ratios for *TTK* rs151658, *BUB1B* rs1031963, and *BUB1B* rs1801376 between the cases and controls and their effect on gastric cancer risk

Genotype model	Cases (n)/Control group (n)	OR (95% CI)*	P	P_Y
***BUB1B* rs1031963**				
Dominant TT+CT vs. CC	72/120 vs. 32/48	0.900 (0.527-1.536)	χ^2=0.149 0.699	χ^2=0.062 0.803
Recessive TT vs. CT+CC	18/36 vs. 86/137	0.797 (0.426-1.491)	χ^2=0.507 0.476	χ^2=0.309 0.578
Heterozygous CT vs. CC	54/89 vs. 32/48	0.910 (0.520-1.594)	χ^2=0.108 0.742	χ^2=0.035 0.853
Male population				
Dominant TT+CT vs. CC	41/101 vs. 26/26	0.364 (0.192-0.691)	χ^2=9.848 **0.002**	χ^2=8.834 **0.003**
Recessive TT vs. CT+CC	11/26 vs. 56/101	0.763 (0.351-1.659)	χ^2=0.467 0.494	χ^2=0.241 0.623
Heterozygous CT vs. CC	30/75 vs. 26/26	0.400 (0.201-0.797)	χ^2=6.959 **0.008**	χ^2=6.057 **0.014**
Female population				
Dominant TT+CT vs. CC	31/57 vs. 6/22	1.994 (0.731-5.437)	χ^2=1.862 0.172	χ^2=1.281 0.258
Recessive TT vs. CT+CC	7/13 vs. 30/66	1.185 (0.429-3.269)	χ^2=0.107 0.743	χ^2=0.004 0.949
Heterozygous CT vs. CC	24/44 vs. 6/22	2.000 (0.714-5.606)	χ^2=1.775 0.183	χ^2=1.188 0.276
***BUB1B* rs1801376**				
Dominant GG+AG vs. AA	61/82 vs. 47/89	1.409 (0.868-2.287)	χ^2=1.927 0.165	χ^2=1.601 0.206
Recessive GG vs. AA+AG	12/13 vs. 96/158	1.519 (0.666-3.465)	χ^2=0.999 0.318	χ^2=0.615 0.433
Heterozygous AG vs. AA	49/69 vs. 47/89	1.345 (0.808-2.237)	χ^2=1.304 0.253	χ^2=1.025 0.311
Male population				
Dominant GG+AG vs. AA	32/46 vs. 36/49	0.947 (0.508-1.766)	χ^2=0.029 0.864	χ^2=0.0002 0.990
Recessive GG vs. AA+AG	8/7 vs. 60/88	1.676 (0.577-4.868)	χ^2=0.917 0.338	χ^2=0.466 0.495
Heterozygous AG vs. AA	24/39 vs. 36/49	0.838 (0.430-1.630)	χ^2=0.272 0.602	χ^2=0.124 0.725
Female population				
Dominant GG+AG vs. AA	29/36 vs. 11/40	2.929 (1.281-6.700)	χ^2=6.719 **0.010**	χ^2=5.737 **0.017**
Recessive GG vs. AA+AG	4/6 vs. 36/70	1.296 (0.344-4.889)	χ^2=0.147 0.701	χ^2=0.001 0.971
Heterozygous AG vs. AA	25/30 vs. 11/40	3.030 (1.292-7.108)	χ^2=6.732 **0.009**	χ^2=5.709 **0.017**

* p value with Yates correction
χ^2 = chi-square statistics; OR = odds ratio; CI = confidence interval

(PROMO, ALGGEN server) (Figure 2).[32,33] This indicated that different polymorphic alleles bind different proteins, which could in turn affect splicing or gene expression. Studies, performed on breast cancer patients, confirmed the significant association of this polymorphism with cancer risk[15]; however, we did not find any studies regarding the effect of rs151658 on gastric cancer risk. In our study, we identified the association of G/G genotype with intestinal type of gastric cancer, while C/G genotype was significantly increased in cases with diffuse type of gastric cancer. Interestingly, comparison of genotype distribution for rs151658 between Slovenian patients with gastric cancer and European population showed that C/C genotype was over-represented in patients with gastric cancer. The significance of this finding is not clear and further analyses are needed on larger cohorts of patients in order to determine its usefulness in clinical setting.

To assess if the above mentioned genotypes perhaps had an effect on TTK protein levels, immunoblotting was performed. While the results regarding the effect of genotypes on protein abundance remain inconclusive, it should be noted that polymorphisms usually exert low-penetrance effects, which could more profoundly affect the pathogenesis of gastric cancer in early stages; however, when the disease progresses, the mutation load and aberrant expression of other genes mask their effects. We did, however, confirm higher abundance of TTK in tumour tissues, which is in accordance with several other studies and points out the deregulation of cell cycle homeostasis, higher proliferative trend of tumour cells and weakened spindle assembly checkpoint leading to increased genome instability and aneuploidy.[37,38] Furthermore, this study showed a trend of increased TTK abundance associated with the spread of cancer cells to regional lymph nodes indicating a possible link between TTK levels and metastatic potential of malignant gastric cells.

Homozygous mutations of critical spindle-assembly *BUB1B* are extremely rare and associated with the diseases such as mosaic variegated aneuploidy syndrome 1 (biallelic mutations) and premature chromatid separation trait, which are both characterized by aneuploidy and chromosomal instability.[39] *BUB1B* overexpression has been found in gastric cancers, although the results are often conflicting. In one study, the overexpression of *BUB1B* was associated with tumour proliferation[40], however, Enjoji *et al*. observed that patients with higher expression of *BUB1B* had improved relapse-free survival.[41] Furthermore, Ando *et al*. found that high expression of *BUB1B* correlated with invasion, lymph node metastasis, liver metastasis, and poor prognosis.[14] Bohers *et al*. confirmed that the function of *BUB1B* is dosage-dependent by gradual reduction of *BUB1B* expression by shRNA in cell lines.[42] In their experiment, residual levels of BUB1B protein below 50% of the normal level indicated premature chromatid separation and ane-

TABLE 6. Comparison of *TTK* rs151658, *BUB1B* rs1031963, and *BUB1B* rs1801376 genotypes between the European population and examined groups of Slovenian population

Population	N	Genotype counts	P
***TTK* rs151658**			
EUR	503	97 (C|C) / 246 (C|G) / 160 (G|G)	$\chi^2 = 8.391$; P = 0.015 [a] $\chi^2 = 11.143$; P = 0.004 [b] NS [c]
SI (total)[a]	283	80 (C|C) / 124 (C|G) / 79 (G|G)	
SI (cases)[b]	108	36 (C|C) / 48 (C|G) / 24 (G|G)	
SI (controls)[c]	175	44 (C|C) / 76 (C|G) / 55 (G|G)	
***BUB1B* rs1031963**			
EUR	503	125 (C|C) / 259 (C|T) / 119 (T|T)	NS [d] NS [e] $\chi^2 = 5.715$; P = 0.057 [f]
EUR - male	240	56 (C|C) / 124 (C|T) / 60 (T|T)	- [c] - [d] $\chi^2 = 6.348$; P = 0.042 [f]
SI (total)[d]	280	80 (C|C) / 143 (C|T) / 57 (T|T)	
SI (cases - female)[e]	36	6 (C|C) / 23 (C|T) / 7 (T|T)	
SI (cases - male)[f]	65	25 (C|C) / 29 (C|T) / 11 (T|T)	
***BUB1B* rs1801376**			
EUR	503	240 (A|A) / 217 (A|G) / 46 (G|G)	NS [g] F = 6.569; P = 0.034 [h] NS [i]
EUR - female	263	135 (A|A) / 109 (A|G) / 19 (G|G)	- [g] F = 8.277; P = 0.014 [h] - [i]
SI (total)[g]	279	136 (A|A) / 118 (A|G) / 25 (G|G)	
SI (cases - female)[h]	40	11 (A|A) / 25 (A|G) / 4 (G|G)	
SI (cases - male)[i]	68	36 (A|A) / 24 (A|G) / 8 (G|G)	

EUR = European population; F = Fisher statistics; SI (cases) = gastric cancer patients; SI (controls) = control population; SI (total) = combined populations of patients and controls; χ^2 = chi-square statistics; superscript letters indicate comparisons between European population and Slovenian populations

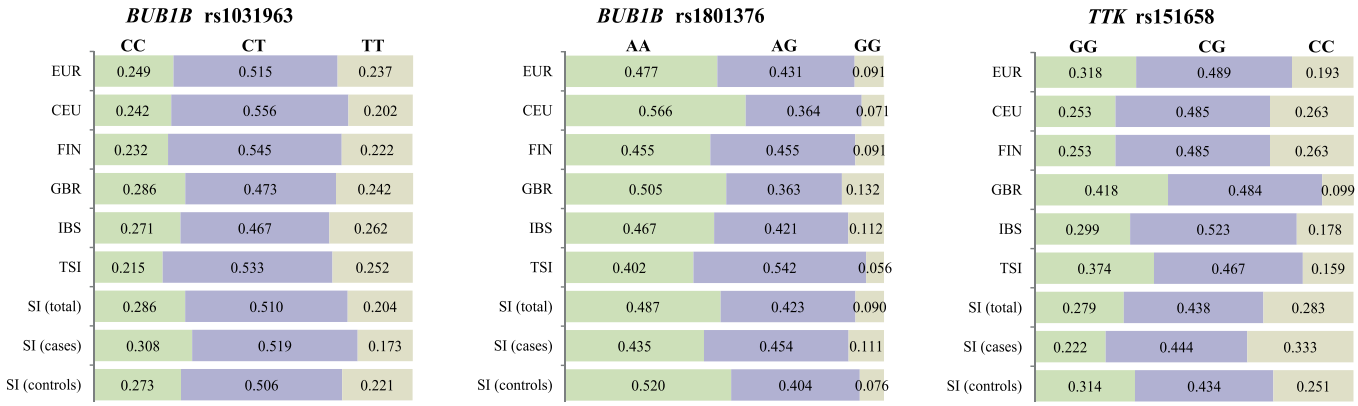

FIGURE 3. Distribution of genotype frequencies of polymorphisms rs151658, rs1031963, and rs1801376 between European populations and Slovenian population.

CEU = Utah Residents (CEPH) with Northern and Western European Ancestry; EUR = European population; FIN = Finnish in Finland; GBR = British in England and Scotland; IBS = Iberian Population in Spain; SI (cases) = gastric cancer patients; SI (controls) = control population; SI (total) = combined populations of patients with gastric cancer and healthy controls; TSI = Tuscany in Italy

uploidy. These conflicting effects of BUB1B could be mediated by different polymorphisms, present in the nucleotide sequence of the gene. *BUB1B* rs1031963 polymorphism is in 5'-promoter region, which harbours binding sites for C/EBPbeta, GR, HNF-4alpha, LEF-1, SRY, TCF-4E, and TCF4 if T allele is present (PROMO, ALGGEN server).[32,33] Interestingly, if C allele is present, the DNA sequence harbours no transcription factor motifs. In our study the T/T genotype was associated with well differentiated tumours in total population, whereas in female population, when analysed separately, it was associated with moderately differentiated tumours. Well differentiated adenocarcinomas tend to have a better prognosis than infiltrative poorly differentiated adenocarcinomas. Furthermore, T/T+C/T genotypes were nominally associated with reduced risk of gastric cancer in male population, whereas C/C genotype was more common in male patient population. Comparisons with European population showed similar results. *BUB1B* rs1801376 A/A genotype was significantly higher in female patients with diffuse gastric cancer. A/A genotype was also increased in samples, which were characterized by invasion of tumour cells into subserosa in male population, and was associated with tumours, growing throughout whole stomach tissue. The consequence of this functional polymorphism is amino acid substitution Q349R in conserved region KEN, which is the binding site for CDC20.[43] CDC20 is co-activator of anaphase promoting complex APC/C.[24] Impaired function of KEN region in *BUB1B* could thus affect the regulation of anaphase delay, which ensures genome stability by providing time for correct spindle assembly, chromosome alignment and segregation. In addition, A/G genotype showed significant association with gastric cancer risk in female population of gastric cancer patients compared to Slovenian control group and European population.

In conclusion, our study provides evidence that polymorphisms in mitotic kinases *TTK* rs151658, *BUB1B* rs1031963 and rs1801376 could have an effect on gastric tumorigenesis and risk of adenocarcinoma development. In addition, we observed differences in genotype distributions between certain clinic-pathological features in patient populations, which could be used as the diagnostic aid in clinical setting; however, a large scale evaluation of these polymorphisms and functional analyses of their effect on protein products are needed to confirm their role in gastric carcinogenesis.

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Funkcionalni polimorfizmi antioksidativnih genov pri neoplazmi Huerthlejevih celic ščitnice - povezava med polimorfizmom gena *GPX1* in ponovitvijo raka Huerthlejevih celic ščitnice

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Izhodišča. Za Huerthlejeve celice ščitnice je značilno veliko število mitohondrijev in oksidativnih encimov. Ker povečan oksidativni metabolizem lahko vodi v povečan oksidativni stres oziroma ga lahko povezujemo z večjo verjetnostjo razvoja raka, smo v naši raziskavi preverjali, ali obstaja povezava med funkcionalnimi polimorfizmi antioksidativnih genov (*SOD2*, *CAT*, *GPX*, *GSTP1*, *GSTM1* in *GSTT1*) in nastankom ali kliničnim potekom raka Huerthlejevih celic ščitnice (HCTC).

Bolniki in metode. Retrospektivno raziskavo smo izvedli pri 139 bolnikih, pri katerih smo zaradi suma na neoplazmo Huerthlejevih celic ščitnice opravili operacijo ščitnice. Diagnozo HCTC, adenoma Huerthlejevih celic ščitnice (HCTA) ali gomolja Huerthlejevih celic ščitnice (HCTN) smo postavili s histopatomorfološko analizo. DNA smo izolirali iz stebričkov histološko potrjenega zdravega dela ščitnice, pridobljenega iz arhiviranih parafinskih blokov tumorjev, fiksiranih v formalinu. S postopki genotipizacije smo določali prisotnost polimorfizmov v antioksidativnih genih. Z logistično regresijo pa smo primerjali porazdelitve posameznih genotipov med različnimi skupinami bolnikov.

Rezultati. HCTC smo ugotovili pri 53, HCTA pri 47 in HCTN pri 21 bolnikih. Pri 20 bolnikih s HCTC smo ugotovili prisotnost zasevkov, pri 16 pa ponovitev bolezni. Pri skupinah bolnikov s HCTC, HCTA in HCTN frekvence genotipov in alelov preučevanih polimorfizmov niso odstopale od Hardy-Weinbergovega ravnotežja. Dominantni genetski model ni pokazal povezave med porazdelitvijo frekvenc genotipov preučevanih polimorfizmov in prisotnostjo HCTC v primerjavi s HCTA in HCTN, prav tako ni bilo povezave s prisotnostjo zasevkov pri HCTC. Ugotovili pa smo povezavo med polimorfizmom *GPX1* in ponovitvijo HCTC (p = 0,040).

Zaključki. Polimorfizem *GPX1* lahko vpliva na možnost ponovitve HCTC.

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Vpliv polimorfizmov v segregacijskih genih BUB1B in TTK na dovzetnost za razvoj želodčnega raka

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Izhodišča. Maligna preobrazba normalnih želodčnih celic je zapleten večstopenjski proces, ki vodi v nastanek heterogenih tumorjev. Na razvoj želodčnega raka vplivajo poleg dejavnikov okolja tudi genetsko ozadje in genetske spremembe. Polimorfizmi enega baznega para (*angl. Single nucleotide polymorphisms*, SNP) v mitotskih segregacijskih genih bi lahko bili odgovorni za počasno kopičenje genetskih sprememb, ki vodijo v genomsko nestabilnost.

Bolniki in metode. V raziskavi primerov s kontrolami smo opredelili vpliv polimorfizmov rs151658 v mitotski kinazi *TTK* in rs1031963 ter rs1801376 v kinazi *BUB1B* na razvoj želodčnega raka. Z metodo imunskega odtisa smo določili količino TTK v rakavih tkivih bolnikov.

Rezultati. Odkrili smo, da genotipa C/G in G/G polimorfizma rs151658 značilno vplivata na dovzetnost za razvoj difuzne oziroma intestinalne oblike želodčnega raka (p = 0,049). Genotip A/A polimorfizma rs1801376 je bil značilno povezan z višjim tveganjem za razvoj želodčnega raka pri bolnicah (0,007), medtem ko se je pri moških z želodčnim rakom pogosteje pojavljal le pri preiskovancih, pri katerih so tumorske celice preraščale v subserozo (0,009). Pri nosilcih genotipa T/T polimorfizma rs1031963 so se pogosteje razvili dobro diferencirani tumorji (0,035). V dominantnem modelu sta bila genotipa TT+CT polimorfizma rs1031963 (razmerje obetov [OR] = 2,929, 95 % interval zaupanja [CI]: 1,281–6,700; p = 0,017) in genotipa GG+AG polimorfizma rs1801376 (OR = 0,364, 95 % CI: 0,192–0,691; p = 0,003) značilno povezana z višjim tveganjem za razvoj bolezni.

Zaključki. Rezultati raziskave kažejo, da polimorfizmi v mitotskih kinazah *TTK* in *BUB1B* v naši skupini preiskovancev statistično značilno prispevajo k povišanemu tveganju za razvoj želodčnega raka in mogoče vplivajo na potek razvoja tumorjev. Za opredelitev njihove klinične uporabnosti so potrebne nadaljnje raziskave v večjih skupinah bolnikov z želodčnim rakom različnih ras.