



Original Research

Gene variants of TCF7L2 are histopathologically important in colorectal cancers but do not have direct association with MYC expression

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Received August 24, 2018; Accepted November 1, 2019; Published December 31, 2019

Doi: <http://dx.doi.org/10.14715/cmb/2019.65.8.1>

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Abstract: Rapidly accumulating preclinical and clinical studies have helped us to unveil underlying mechanisms of colorectal cancer development and progression. Deregulated signaling pathways play instrumental role in carcinogenesis, drug resistance and metastasis. Wnt signaling cascade has attracted considerable attention in colorectal cancer as many ground-breaking researches have highlighted central role of Wnt pathway in pathogenesis of colorectal cancer. T-Cell Transcription Factors (TCFs) have been shown to work synchronously with β -catenin to fuel colorectal cancer development and progression. Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-Seq) data sets has deepened our knowledge about critical role of risk-associated SNPs. Increasingly it is being reported that many risk-associated SNPs are located within binding sites for transcription factors and consequently risk status of these SNPs may modify binding pattern of transcriptional factors and thus rewire the transcriptional regulation. DNA was extracted from peripheral blood samples of 117 colorectal cancer patients and 127 healthy subjects. TCF7L2 variants (rs6983267, rs7903146) were examined by the PCR-RFLP method. Tumor and the surrounding tissues were dissected from 37 CRC patients and RNA isolation was performed. The gene expression of c-myc was determined by RT-PCR. T allele carriage of rs6983267 variant was found to be associated with CRC ($p=0.042$). TT genotype of rs7903146 was associated with late tumor stage (T3+T4) ($p=0.037$) and presence of mucinous component ($p=0.031$). TTCT haplotype was found to be statistically higher in CRC compared to the control group ($p=0.007$). There was no statistically significant difference in c-myc gene expression. TCF7L2 gene variants may play an important role in histopathologic aspects associated with CRC and it is independent of c-myc gene expression.

Key words: TCF7L2; Colorectal Cancers; Tumor stage; Gene expression; Signaling.

Introduction

It is becoming progressively more understandable that colorectal cancer is therapeutically challenging and recent advancements in high-throughput technologies have helped us to uncover extra-ordinary complexity and suggest multiple cancer-related functions for many genes, which are often cancer stage-dependent or tissue-dependent (1,2). Large-scale sequencing of human cancers has unveiled a myriad of genomic alterations. Furthermore, the past few years have witnessed tremendous breakthroughs in the biology of colorectal cancer and development of multiplexed and quantitative approaches has revolutionized the field of molecular oncology (3,4,5).

Wnt- β -catenin signaling is involved in modulation of different molecular mechanisms (6,7). β -catenin is shipped to the nucleus where it interacts with the TCF family of transcription factors (TCF1, LEF, TCF3, and TCF4). T-Cell Transcription Factors (TCFs) have gained

considerable attention because of their ability to transcriptionally modulate wide ranging target genes. TCF proteins have been shown to physically interact with transcriptional repressors, such as Groucho/Grg/TLE (transducin-like enhancer of split) CtBP, histone deacetylases (HDACs) and other factors and induce transcriptional inactivation of different genes (6,7). However, intriguingly, interaction of TCF proteins with β -catenin converted TCFs into transcriptional activators (6,7).

It has recently been convincingly revealed that TCF7L2 with rs138649767-A allele harbored the ability to activate the MYC enhancer with rs6983267-G allele and enhanced proliferation of colorectal cancer cells (8). Functional silencing of TCF7L2 provided evidence of its pro-metastatic role in proliferation, adhesion and migration of colorectal cancer cells (9). TCF7L2 has previously been reported to be frequently overexpressed in therapy-resistant colorectal cancer cells (10, 11). TCF7L2 inhibition re-sensitized colorectal cancer cells to

therapeutic strategies (10, 11).

Accumulating evidence has started to shed light on involvement of long non-coding RNAs in facilitating Wnt-catenin and TCF7L2 driven pathway.

CCAT2, a long non-coding RNA has been shown to promote TCF7L2 binding to the MYC promoter. Chromatin-immunoprecipitation analysis demonstrated higher binding of TCF7L2 to promoter region of MYC in CCAT2-overexpressing colorectal cancer cells. Data clearly suggested that TCF7L2 worked synchronously with versatile regulators to promote genomic instability and colorectal cancer (12). In accordance with this approach, another long non-coding RNA, colorectal neoplasia differentially expressed (CRNDE) sequestered TCF7L2 away from miRNA-217. CRNDE promoted Wnt signaling mainly through interfering with miR-217 mediated targeting of TCF7L2 in colorectal cancer cells (13). Stable knockdown of TCF7L2 exerted inhibitory effects on anchorage-independent growth of colorectal cancer cells (14).

Previously published high-impact research has provided evidence of central role of Wnt signaling in regulation of MYC-335 (15). MYC-335 elements containing the T and G alleles of rs6983267 were cloned into a reporter vector. It was observed that MYC-335 had Wnt-responsive enhancer activity and interestingly, G allele demonstrated 1.5-fold stronger Wnt responses as compared to T allele (15). It has been experimentally verified that rs6983267-containing region showed notable binding affinity with TCF7L2 and the transcriptional activator β -catenin (15).

Furthermore, circumstantial evidence indicated that rs6983267 risk region physically interacted with MYC in colorectal cancer cell lines (16).

Certain hints have emerged which highlighted that some splicing forms of TCF7L2 might play functionally important role in transcriptional regulation of MYC in colon tissue but this regulation was not dependent directly on rs6983267 (17). MYC expression was studied in colorectal cancer tissue samples and data clearly suggested that although MYC expression was associated with some of the TCF7L2 splicing forms but not with genotypes of rs6983267, or relationship of rs6983267 with TCF7L2 expression (17). These exciting findings suggested some splicing forms of TCF7L2 might play functionally important role in transcriptional regulation of MYC in colon tissue but this regulation was not dependent directly on rs6983267.

We aimed to investigate the distribution of variants of TCF7L2 (rs6983267, rs7903146) between Turkish colorectal cancer patients and healthy individuals. We also aimed to study any possible effect of these variants on c-myc gene expression, and the contribution of both of these conditions to tumor histopathology.

Materials and Methods

Subjects

In our study, peripheral blood samples of 117 patients, 42 female and 75 male, who were diagnosed as Colorectal Cancer by Clinic of General Surgery, Istanbul Training and Research Hospital were taken. The tumor tissues samples of 36 patients were resected at these clinics. Our work was carried out by the permis-

sion of the Ethics Committee of Istanbul University Istanbul Medical Faculty with the permission number 1671-1265.

Histopathological examinations

Patients with colorectal cancer, who were diagnosed with the radiological, and routine pathological and histopathological examination of the tissue, were enrolled into the study. The stage of the tumors which is determined according to the last edition of 7th staging system (pTNM) was determined as a result of the pathological examination of the resected tissue, and the grade of the tumor detected in the pathological examination.

Surrounding colonic tissue far from the tumor without the findings of the macroscopic invasion were included in the study as the control group.

Genotyping

DNA isolation was performed by salting out the peripheral blood samples from Colorectal Cancer patients and healthy subjects. For the rs6983267 of TCF7L2 polymorphism, the forward primer 5'- ATGAAGGC-GTCGTCCAAATGA -3' and reverse primer 5'- TTG-GCTGGCACTGTCTGTAT -3' were used at a concentration of 10 pmol/ μ l for each primer. Also for the rs7903146 of TCF7L2 polymorphism, the forward primer 5'- AATTAGAGAGCTAAGCACTTTTATTAGG-TA-3' and reverse primer 5'- CAAGCTTCTCAGTCA-CACAGG-3' were used at a concentration of 5 pmol/ μ l for each primer.

The PCR reaction mixture contained 150 ng DNA template, 1.75 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 600 μ M dNTP (iNtRON Biotechnology Co., Korea) and 0.06 unit Taq DNA polymerase (iNtRON Biotechnology Co., Korea). The PCR conditions were determined as first denaturation at 95 °C for 5 minutes, 35 cycles at 94 °C for 45 seconds, 56 °C for 45 seconds, 72 °C for 45 seconds, and a final extension at 72 °C for 5 minutes. PCR product of rs7903146 was digested by RsaI (MBI Fermentas, CA) at 37°C for 2.5 hours. Also, PCR product of rs6983267 was digested by Tsp45I (MBI Fermentas, CA) at 37°C for 2.5 hours.

After enzymatic restriction; For rs6983267, two fragments having 334 and 198 bp (G allele) or one fragment having 498 bp (T allele) were identified. For rs7903146, one fragment having 176 bp (T allele) or two fragments having 149 and 27 bp (C allele) were identified with agarose gel electrophoresis.

Gene expression

Tumor tissue and the tumor surrounding tissue samples were surgically dissected. Samples were stored in liquid nitrogen until use. Total RNA was isolated from tissues using the TRIzol method. cDNA synthesis from total RNA was performed by using the High-Capacity cDNA Reverse Transcription Kit (LifeTech, Applied Biosystem, USA). As a result of our literature researches and pilot studies, it was determined that β -Actin gene is suitable as housekeeping gene. PCR primers and probes for the c-MYC and β -Actin gene were selected using the Single Tube TaqMan Gene Expression Assay (LifeTech, Applied Biosystem, USA).

The real-time PCR reaction mixture contained 100 ng cDNA, 2X PZR Master Mix (LifeTech, Applied

Biosystem, USA) and 20X Primer and Probe ready mix (LifeTech, Applied Biosystem, USA) for both primers. Real-time PCR conditions were determined as UDG incubation at 50° C for 2 minutes, AmpliTaq Gold UP enzyme activation at 95 ° C for 10 minutes, and 40 cycles at 95° C for 15 seconds and at 60° C for 1 minute. Real-time PCR analysis was performed using Stratagene Mx3005p instrument (Agilent Technologies, CA, USA) with TaqMan Gene Expression Master Mix (LifeTech, Applied Biosystem, USA).

Statistical analysis

In our study, Chi square and further tests were applied by using SPSS 7 package program for statistical analysis of TCF7L2 variants, c-myc gene expression values, demographic data and histopathological parameters. Mann-Whitney U test, which is one of the non-parametric tests, was applied by using GraphPad Prism 5 program for the statistical analysis of gene expression levels of tumors and control tissues. The data were verified with both statistical programs. P values that are lower than 0.05 were regarded as statistically significant.

Results

The demographic data of the patients are shown in Table 1 and the allele and genotype distribution of variants Rs6983267 and rs7903146 are shown in Table 2. There was no statistical difference between patient and control groups in terms of genotype and allele distributions in both variants (rs6983267 and rs7903146).

However, the T allele carrier (TT-GT) for the rs6983267 variant was found to be higher in CRC patients compared to the control group ($p=0.042$; OR=1.130; CI:1.005-1.270).

The distribution of rs6983267 and rs7903146 variants according to tumor histopathology is shown in Table 3. When rs6983267 variant is examined histopathologically, there are some differences which are close to statistical significance. It is observed in our study that in the advanced tumor stage (T3-T4) the G allele carrier is higher than the early tumor stage (T1-T2) ($p = 0.054$, OR = 1.407, CI: 0.936-2.113). Similarly, when patients were examined according to node metastasis, the presence of G allele (GG-GT) in patients with node metastasis was found to be higher (74.1%) than in patients without node metastasis (57.6%) ($p = 0.060$, OR = 1.287, CI: 0.986-1.679).

The TT genotype carriage of rs7903146 was not detected in all early-onset (T1-T2) patients. It was seen in 15.4% of patients with advanced tumor (T3-T4) ($p=0.037$; OR=0.846; CI:0.775-0.924). CT genotype carriage of rs7903146 was 69.2% in early tumor stage (T1-T2) and 39.6% in advanced tumor stage (T3-T4). The frequency of the patients with advanced tumor stage who have CT genotype was lower than those with early tumor stage ($p=0.007$; OR=0.571; CI: 0.398-0.820).

The CT genotype for the rs7903146 variant was higher in patients without node metastasis (N0) (55%) than patients with node metastasis (N1,N2,N3) (36.8%) and statistical significance were found to be close ($p = 0.049$, OR = 0.670, CI: 0.445-1.009). The patients with rs7903146 TT genotype were found higher in tumors

Table 1. The demographic data of working groups.

Groups	Control (n=127) %	CRC Patient (n=117)	p value
Gender			
Woman, n(%)	59 (46.9)	42(35.9)	
Men, n(%)	68(53.5)	75(64.1)	
Average age (year)	58.0	61.2	
Family cancer story			
Positive, n(%)	8(6.3)	8(6.8)	>0.05
Negative, n(%)	119(93.7)	109(93.2)	
Alcohol Use			
Positive, n(%)	4 (3.1)	4(3.4)	
Negative, n(%)	123 (96.9)	113(96.6)	
Smoking ^{xxx}			
Positive, n(%)	4 (3.1)	6(5.1)	^{xxx} p=0.029
Negative, n(%)	123(96.6)	111(94.9)	

Table 2. Allele and genotype distribution of Rs6983267 and rs7903146 variants in CRC patient and control group.

Genotypes or Alleles	CRC Patient (n=117), %	Control (n=127), %	p value
Rs6983267			
GG	17(14.5)	29(23)	
GT	64(54.7)	64(50.8)	
TT	36(30.8)	33(26.2)	
G	98 (41.9)	122 (48.4)	
T ^{xxx}	136 (58.1)	130 (51.6)	>0.05
Rs7903146			
CC	54(46.2)	54(42.9)	^{xxx} p=0,042
CT	50(42.7)	47(37.3)	
TT	13(11.1)	25 (19.8)	
C	158(67.5)	155(61.5)	
T	76(32.5)	97 (38.5)	

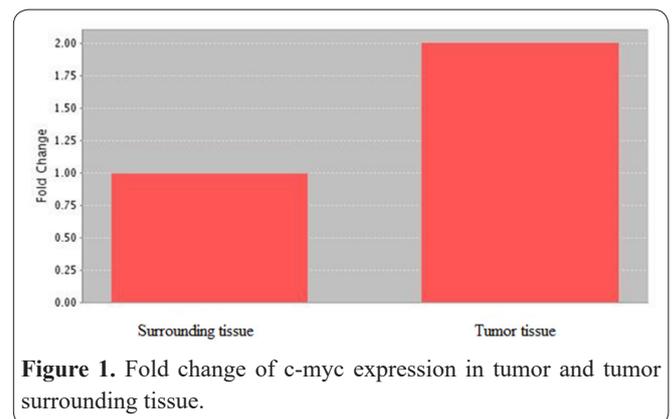


Figure 1. Fold change of c-myc expression in tumor and tumor surrounding tissue.

with mucinous component than in those without mucinous component ($p = 0.031$, OR = 3.268, CI: 1.245-8.576). Similarly, C allele carriers were found to be higher in smokers compared to non-smokers ($p=0,022$; OR=0,555; CI:0,249-1,238).

The TTCT haplotype of both variants (rs6983267TT ve rs7903146CT) was significantly higher in CRC patients than in the control group ($p=0.007$; OR=2.653; CI:1.274-5.527). The same haplotype (TTCT) was found to be statistically significant in the low tumor stage ($p = 0.001$, OR = 0.286, CI = 0.140-0.583) and without node metastasis ($p = 0.007$; OR = 0.310, CI = 0.122-0.784) (Table 4).

Table 3. The Genotype Distribution of *c-myc* and TCF7L2 gene variants according to the CRC patient's histopathological data.

	TCF7L2 rs7903146			8q24 rs6983267		
	CC n(%)	CT n(%)	TT n(%)	GG n(%)	GT n(%)	TT n(%)
Gender						
women	13 (31,0)	26(61,9)	3(7,1)	7(16,7)	19(45,2)	16(38,1)
men	36(48,0)	28(37,3)	11(14,7)	8(10,7)	43(57,3)	24(32,0)
T stage						
T1	4(50,0)	4(50,0)	0 (0,0)	0(0,0)	3(37,5)	5(62,5)
T2	4(22,2)	14(77,8)	0(0,0)	1(5,6)	9(50,0)	8(44,4)
T3	34(47,2)	29(40,3)	9(12,5)	11(15,3)	37(51,4)	24(33,3)
T4	7(36,8)	7(36,8)	5(26,3)	3(15,8)	13(68,4)	3(15,8)
Lymph Node Involvement						
N0	22(36,7)	33 (55,0)	5(8,3)	6(10,0)	29(48,3)	25(41,7)
N1	16(50,0)	12(37,5)	4(12,5)	7(21,9)	17(53,1)	8(25,0)
N2	6(33,3)	7(38,9)	5(27,8)	1(5,6)	12(66,7)	5(27,8)
N3	5(71,4)	2(28,6)	0 (0,0)	1(14,3)	4(57,1)	2(28,6)
Distant metastasis						
Yes	11 (42,3)	11 (42,3)	4(15,4)	4(15,4)	14(53,8)	8(30,8)
No	38 (41,8)	43(47,3)	10(11,0)	11 (12,1)	48 (52,7)	32(35,2)
Angiolymphatic Invasion						
Positive	14(38,9)	16(44,4)	6(16,7)	4(11,1)	23(63,9)	9(25,0)
Negative	35(43,2)	38(46,9)	8(9,9)	11(13,6)	39(48,1)	31(38,3)
Perineural Invasion						
Positive	16 (42,1)	18(47,4)	4(10,5)	5(13,2)	23(60,5)	10(26,3)
Negative	33 (41,8)	36(45,6)	10(12,7)	10(12,7)	39(49,4)	30(38,0)

Table 4. The distribution of *c-myc* and TCF7L2 genotype combinations in our study groups.

Groups/ <i>c-myc</i> and TCF7L2 genotype combinations n(%)	CCGG	CCGT	CCTT	CTGG	CTGT	CTTT	TTGG	TTGT	TTTT
CRC patients	7 (6%)	27 (23.1%)	15 (12.8%)	6 (5/1%)	26 (22.2%)	22 (18.8%)	2 (1.7%)	9 (7.7%)	3 (2.6%)
Controls	10 (7.9%)	24 (18.9%)	20 (15.7%)	14 (11%)	25 (19.7%)	9 (7.1%)	5 (3.9%)	16 (12.6%)	4 (3.1%)

The *c-myc* gene expression levels of tumor and surrounding tissue are shown in figure 1. The fold changes of different genotypes of variants rs6983267 and rs7903146 on *c-myc* gene expression are shown in Table 5. When *c-myc* gene expression levels between tumor and surrounding tissue were compared, it was observed that *c-myc* expression in tumor tissue was increased 2-fold compared to surrounding tissues, but no statistically significant difference was found ($p > 0,05$).

There was no statistically significant difference between rs6983267 and rs7903146 variants and *c-myc* expression levels in tumor tissue ($p > 0,05$). When the GG (wild type) genotype was referenced for rs6983267, the expression of *c-myc* was increased 1.36-fold in TT (homozygous mutant type) genotypes and 1.53-fold with GT (heterozygous) genotypes ($p > 0,05$). When the

CC genotype was referenced for the rs7903146 variant, the *c-myc* gene expression level decreased by 1.14 fold in the CT (heterozygous) genotype and 2.14 fold in the TT genotype ($p > 0,05$). There was no statistically significant correlation between the level of *c-myc* gene expression and histopathologic factors. Similarly, *c-myc* gene expression increased 1.2-fold in patients with lymphatic involvement and 1.07-fold in patients with N2+N3 nod phase. When *c-myc* gene expression is assessed for metastasis, it is seen that *c-myc* expression in metastatic patients is 1.1 times lower ($p > 0,05$).

Discussion

Advent of next generation sequencing technologies has leveraged our understanding related to risk-associ-

Table 5. The fold changes of different genotypes of variants rs6983267 and rs7903146 on *c-myc* gene expression.

Variant/Genotyp	Fold Change	%95 CI	Fold Regulation	p value
rs6983267 TT	1,3624	0.00001-2.88	1,3624	
rs6983267 GT	1,5268	0.00001-3.17	1,5268	
rs6983267 GT-TT	1,4458	0.00001-2.89	1,4458	
Rs7903146 CT	1,1482	0.18-2.12	1,1482	
Rs7903146 TT	2,1443	0.55- 3.74	2,1443	>0.05
Rs7903146 CT-TT	1,3192	0.31-2.33	1,3192	

ted SNPs to a next level. Substantial fraction of information is continuously being added into pre-existing pool of cancer risk associated SNPs and we now know that risk allele (G) of SNP rs6983267 has been noted to demonstrate preferential binding affinity for the TCF7L2 as compared to (T) allele.

In our study, rs6983267, T allele carrier was found to be higher in the patient group compared to the control group and it was found to be related to cigarette use in GT genotype carriers ($p > 0.05$). There was no correlation between c-myc gene expression and rs6983267 variant ($p > 0.05$).

According to our study, no correlation was found between c-myc gene expression and rs6983267 variant. In this respect, this data does not support the work of Poerantz *et al.* Although we did not find it statistically significant, we observed that the expression of c-myc gene in T-allele carriers of the Rs6983267 variant was slightly lower in tumor tissue ($p > 0.05$). Accordingly, our opinion is that the related genotype does not have a great effect on c-myc gene expression. Prokunina *et al.* (17) obtained data that c-myc expression was independent from rs6983267 variant and that co-expression was not associated with colorectal cancers. Our results are in accordance with the findings reported by Prokunina *et al.*

In our study, T allele carriage of Rs6983267 variant was statistically high in patients with colorectal cancer. The results of Folsom *et al.* are parallel to our results (18). Nan *et al.*'s study examined the association of aspirin use and rs6983267 variant and found that T allele carriage is associated with a lower risk of colorectal cancer (19). It is noteworthy that the T allele carriage of rs6983267 variant is important in both studies, although it is not appropriate to compare the results with our results due to the regulatory effects of aspirin on Wnt/ β -catenin pathway.

In the rs7903146 variant, CT genotype and C allele carriage was correlated histologically with early tumor stage (T1-T2), mucinous component carrier and cigarette smokers in colorectal cancer cases. In the rs7903146 variant, TT genotype was correlated histologically with late tumor stage (T3+T4).

This data is of original value because there is no similar data in the related literature. According to the meta-analysis results of Zhang *et al.* (20), The variants of TCF7L2 are associated with different cancers. Part of the evidence of our work supports this belief. In another study, similarly, the study of Mexican colorectal carcinoma cases shows that TT genotype of rs7903146 is associated with rectal localization of the tumor, and at the same time, there are indications that multiple gene variants of TCF7L2 are important for TNM staging (21). In a study of colorectal cancer patients with type 2 diabetes, it was determined that the T allele carriage of rs7903146 variant was higher in the patient group than in the control group (22).

Similarly to these studies, for rs7903146 in our study, genotypes bearing the TT genotype were found higher in tumors with mucinous component than in those without mucinous component. We think that the difference between the studies is due to different ethnicity and case selection criteria.

Mucinous component presence in colorectal cancers

is used as an important clinical parameter in the prognosis and histopathologic condition. One of the genes encoding the mucinous component, MUC1-C, was found in breast cancer cases where the TCF7L2 gene could be regulated by the β -catenin pathway (23,24). In our study, the relationship between the TT genotype carrier of the rs7903146 variant and the presence of the mucin component in the tumor tissue was a supporting factor in terms of the common carcinogenesis mechanisms reported in the literature.

There was no statistical difference between c-myc gene expression level and rs7903146 ($p > 0.05$). Although there was no statistically significant difference in our study, there was a decrease in the gene expression of c-myc in CC-CT genotypes of rs7903146 (CC-CT) and an increase in gene expression in T-allele carriers ($p > 0.05$). This is the first study in the literature examining the relationship of rs7903146 variant of TCF7L2 to c-myc gene expression. The TTCT haplotype of two polymorphisms (rs7903146 and rs6983267) that we studied in cases with colorectal cancer in our study was found to be statistically high in early tumor stage (T1-T2) and colorectal cancer patients ($p < 0.05$). It has been observed that these variants are associated with Turkish colorectal cancer cases, as the cases of other literature. However, in our study no statistically significant effect of TCF7L2 variants on c-myc expression was detected. It is noted that different alleles and genotypes of TCF7L2 variants may cause slight alterations in gene expression of c-myc ($p > 0.05$). Overall our findings provided clues of association between rs7903146 and c-myc expression however, we did not find any correlation between rs6983267 and c-myc in colorectal cancer patients.

Immunohistochemical studies are planned to be carried out by increasing the number of cases with the support of statistically significant data obtained in our study. This will provide a more detailed look at the clinicopathological and histopathological effects of TCF7L2 and other TCF gene family members in colorectal cancers. Thus, the role of the TCF gene family in the tumor stage will be demonstrated tissue-specific. The relationship of TCF7L2 protein level with T cell clustering will be determined. At the same time, the effect of TCF7L2 level change on both epithelial tissue and T-lymphocyte proliferation will be observed separately.

Our study was the first study to examine the histopathological relationship of gene variants of TCF7L2 in Turkish cases and this study will support the researcher who is studying Wnt and Wnt/ β -catenin pathways.

Funding

The present work was supported by the (Project No. 32513) Bilimsel Araştırma Projeleri Birimi, İstanbul Üniversitesi [32513].

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