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TRP GENES FAMILY EXPRESSION IN COLORECTAL CANCER

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Colorectal cancer (CRC) is the most common cancer of the gastrointestinal tract. Different factors are responsible for the development of CRC. Transient Receptor Potential (TRP) which is an important component of calcium channel is associated with several pathological conditions like cancer, neurodegenerative and cardiovascular diseases. Thirty members of the family of TRP ion channel in mammals have been determined till now. The *aim* of this study is to investigate *TRPM*, *TRPV* and *TRPC* gene expression levels in tumor tissues of CRC patients and to analyze the relationship of expression in tumor tissue of CRC with other known prognostic factors. *Material and Methods*: In this study, 93 CRC patients were included. The level of TRP gene expression in paraffin blocks of normal and cancerous colorectal tissue samples were studied at the level of mRNA with Real-time PCR. *Results*: The mRNA expression level of *TRPV3*, *TRPV4*, *TRPV5*, *TRPM4* and *TRPC6* genes in 37 female and 56 male patients diagnosed with CRC was revealed lower in tumor tissue as compared to normal tissue ($p < 0.05$). No statistically significant differences of mRNA expression levels of other TRP genes were found. *Conclusions*: TRP gene family like *TRPV3*, *TRPV4*, *TRPV5*, *TRPM4* and *TRPC6* may be thought as potential genes contributing to tumorigenesis as their expression decreases in CRC as compared to normal tissues.
***Key Words*: colorectal cancer, TRP genes family: *TRPV3*, *TRPV4*, *TRPV5*, *TRPM4*, *TRPC6*, mRNA expression.**

Colorectal cancer (CRC) is the most common cancer of the gastrointestinal tract. When evaluated along with rectal cancer, it ranks third as the most common cancer being observed after prostate and lung cancer in males and breast and lung cancer in females.

Genetic and environmental factors have an influential role in the development of CRC. Colorectal cancers are sporadic cancers even though the genetic predisposition is the most prominent risk factor in the majority of CRC. Cellular oncogenes, growth factors and receptors play a pivotal role in the development and growth of the CRC [1]. Alike other types of cancer, the development and progression of CRC also results from multiple genetic variations.

In most of the recently conducted studies, it has been found that that the ion channels play a critical role in cell proliferation. Blockade of these channels have been found to inhibit the development of cancer cells. Therefore ion channels have received a considerable attention for therapeutic targets or prognostic biomarkers [2].

Being an ion channel, the family of Transient Receptor Potential (TRP) channels also comprise of seven different subsets. These are TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolip), TRPP (polycys pyridine), TRPE (ankyrin transmembrane protein) and TRPN (nompc-like) which are associated with many pathological and physiological functions. The relationship between TRP channels and cancer suggests that TRP is effective in many ongoing cancer processes like tumor invasion, migration, angiogenesis, apoptosis, differentiation and proliferation [3].

TRPM8, *TRPM1*, and *TRPV6* are highly expressed in cancer cells and the amount of protein being expressed changes with progression from normal to tumorigenic and then to metastatic cells. The expression levels of some other TRP channels, including *TRPC1*, *TRPC6*, *TRPM5* and *TRPV1* is also increased in cancer tissues [4]. In a recent study, *TRPC5* was found to be overproduced at the mRNA and protein levels in 5-Fu-resistant human CRC cells [5]. *TRPM8*, *TRPM1* and *TRPV1* are potential diagnostic markers for the prognosis of tumor development especially the degree of tumor aggression, and are potential targets for pharmaceutical interventions [4].

In this study, our aim is to find out gene expression levels of *TRPM*, *TRPV*, *TRPC* in cancerous tissues of colorectal cancer patients. We also target to analyze the relationship of expression in tumor tissue of CRC with other known prognostic factors like age, gender, stages of cancer, location and size of tumor, vascular invasion, perineural invasion, lymph node involvement and to investigate the usability as a therapeutic target.

MATERIALS AND METHODS

Selection of Patients. This study was performed during 2001–2010 and was undertaken in correlation with Department of Medical Oncology, Medical Faculty Hospital, Gaziantep University which was responsible for patients follow-up and their treatment and Department of Pathology, Medical Faculty, Gaziantep

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Abbreviation used: CRC – colorectal cancer; TRP – transient receptor potential.

University which carried out pathological testing for different stages of colon cancer. This study was carried out on 96 patients for which approval of the local ethics committee have been made retrospectively. Three out of 96 paraffin blocks of cancer tissue could not later be found and thus were excluded from the study. As a result, 93 cases of colon cancer were included in this study.

The study of the patient demographics (age, gender) and their different pathological features (tumor location, tumor size, histological type, differentiation, vascular and perineural invasion, involvement of lymph node), their different clinical stages, follow-up and their survival span have been registered in a standard form.

The stored paraffin blocks of 93 patients were obtained from the archives of Department of Pathology, Faculty of Medicine, Gaziantep University for further study. The obtained samples were revaluated under light microscope. For each subject, separate paraffin blocks containing normal colon mucosa and infected colon cancerous tissue slides were selected.

RNA isolation from FFPE tissue. The total RNA was obtained from samples embedded in paraffin using RNeasy FFPE Kit (Qiagen Sample and Assay Technologies, Hilden, Germany, Cat. No: 73504). The quantity and integrity of the RNA was measured using Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, USA). After total RNA isolation and purification, samples were diluted to a concentration 50 ng/μl and stored at –80 °C until further use. RNA was converted to cDNA using Ipsogen Reverse Transcription-Dx Kit (Qiagen, Hilden, Germany, Lot no: 11–11–11) following the manufacturer's protocol.

qPCR. Gene expression analysis was done on a BioMark™ HD System (Fluidigm Corporation, CA, USA). Samples were preamplified for 14 cycles using TaqMan® PreAmp Master Mix (Life Technologies, Foster City, CA, USA, Cat. No: 4391128) prior to real-time qPCR. Real-time qPCR was done in BioMark 96.96 Dynamic Array (Fluidigm Corporation, CA, USA) using a set of TaqMan Gene Expression Assays (Life Technologies, Foster City, CA, USA, Cat. No: 4333458).

Statistical analysis. Real-time PCR was performed using Fluidigm Dynamic Array along with BioMARK HD System and the gene expression measurements and their data analysis was evaluated using Biogazel and qBasePLUS software program. ACTB was used as housekeeping gene for the normalization of the target genes.

Significance level was considered as $p < 0.05$ using Mann — Whitney test to analyze data. The dispersion values were also taken into account during comparison of Mann — Whitney average test values. When "datapoints" corresponding to the sample number measured in the assay fall below 12, significance test became quite sensitive and apart from the means, the distribution analysed for statistical significance of the test was probably found insufficient to generate a standard curve. Therefore, *p* value expressing the significance level of the test would be very high in this group and a significant comparison would not be expressed statistically.

RESULTS

A total of 93 patients including 37 women and 56 men who were pathologically diagnosed with CRC were included in study. The ratio of men to women was 1.51. The patients were in the age group of 12–87 years and the mean age of patients was found to be 60 ± 16.1 years. The general demographic, clinical and pathological characteristics are shown in Table 1. The smallest and largest tumor size was 1.5 cm and 16 cm, respectively. The mean tumor size was 5.8 ± 2.6 cm. According to the TNM classification, 9 (9.7%) patients were in stage I, 33 (35.5%) patients were in stage II, 26 (27.9%) patients were in stage III and the remaining 25 (26.9%) patients were in stage IV (Fig. 1).

Table 1. Distribution of general demographic, clinical and pathological characteristics of patients

Characteristics	Number of patients, n (%)
Gender	
Female	37 (39.8)
Male	56 (60.2)
Localization	
Rectosigmoid	9 (9.7)
Rectum	12 (12.9)
Sigmoid	18 (19.4)
Right Colon	31 (33.3)
Left Colon	11 (11.8)
Transverse Colon	12 (12.9)
Histology	
Adenocarcinoma	87 (93.5)
Mucinous carcinoma	6 (6.5)
Vascular invasion	
Negative	78 (83.9)
Positive	15 (16.1)
Perineural invasion	
Negative	86 (92.5)
Positive	7 (7.5)
Differentiation	
Unknown	64 (68.8)
High	16 (17.2)
Medium	10 (10.8)
Less	3 (3.2)
Lymph node involvement	
Negative	52 (55.9)
1–3 positive	29 (31.2)
4 and above positive	12 (12.9)

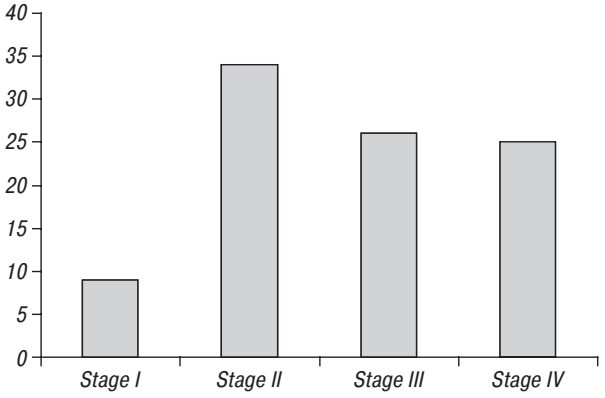

Fig. 1. Distribution of patients according to TNM staging

Real-time PCR was performed using "Fluidigm Dynamic Array" along with BioMARK HD System and the gene expression measurements and their data analysis were evaluated using Biogazel and qBasePLUS software program. ACTB was used as housekeeping gene for the normalization of the targets mentioned in Table 2.

The obtained gene expression data from normal and cancerous tissues of the same patient were compared (Fig. 2, Table 3). For this purpose, Real Time PCR was carried out of the mRNA obtained from 93 patients for both tumor tissue and the normal ones. The expression of *TRPV4*, *TRPM4*, *TRPV3*, *TRPC6* and *TRPV5* in tumor tissues were found to have lesser gene expression as compared to normal tissues ($p < 0.05$). When expression levels of other *TRP* genes in tissues were compared, any significant difference was not found ($p > 0.05$).

Table 2. List of target genes to be investigated for gene expression in TRP channels

TRPA1	*TRPC6*	*TRPM4*	*TRPV1*	*TRPV6*
TRPC1	*TRPC7*	*TRPM5*	*TRPV2*	*ACTB*
TRPC3	*TRPM1*	*TRPM6*	*TRPV3*	–
TRPC4	*TRPM2*	*TRPM7*	*TRPV4*	–
TRPC5	*TRPM3*	*TRPM8*	*TRPV5*	–

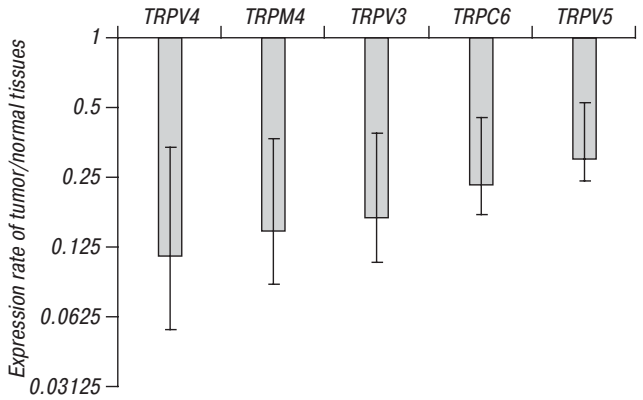

Fig. 2. Expression rate of tumor/normal tissues

Table 3. Comparison of gene expression data from normal and cancerous tissues

Target gene	Tissue type	Gene expression level: Normalized mean value	95% CI lower limit	95% CI higher limit	Number of patients	Tumor/normal ratio of gene expression: Mean value	95% CI lower limit	95%CI higher limit	*p*
TRPV4	Normal	1.000	0.656	1.524	76	0.116	0.06	0.222	3.26E-06
	Tumor	0.116	0.070	0.193	57				
TRPM4	Normal	1.000	0.666	1.502	76	0.148	0.074	0.294	6.41E-05
	Tumor	0.148	0.082	0.266	47				
TRPV3	Normal	1.000	0.655	1.527	73	0.169	0.085	0.336	1.28E-04
	Tumor	0.169	0.096	0.298	48				
TRPC6	Normal	1.000	0.610	1.640	54	0.234	0.111	0.493	3.75E-03
	Tumor	0.234	0.132	0.415	41				
TRPV5	Normal	1.000	0.651	1.536	55	0.303	0.152	0.605	1.44E-02
	Tumor	0.303	0.171	0.535	45				

For the comparison of data related to different cancer stages, the number of patients in different groups was counted. In stage I, 9 patients; in stage II, 33 patients; in stage III, 26 patients; in stage IV, 25 patients were found out. However, for the comparison of average Mann — Whitney test values, the dispersion values should be taken into account. When "datapoints" corresponding to the sample number measured in the assay fall below 12, significance test became quite sensitive and apart from the means, the distribution analysed for statistical significance of the test was probably found insufficient to generate a standard curve. As the total number of patients in stage I was 9, therefore total number of patients in stage I and II were combined together in one group for their evaluation. As a result, stage I + II (42 patients), stage III (26 patients), stage IV (25 patients) were evaluated.

When the gene expression of cancerous tissues for different groups of patients for various stages as stage I + II, stage III and stage IV were compared with each other, there was no significant difference being found ($p > 0.05$).

The gene expression data obtained from the tumor tissues showed no significant differences statistically for tumor localisation, histological types, perineural invasion, vascular invasion, lymph node involvement, differentiation grade, patients gender, tumor size, status of recurrence and metastasis ($p > 0.05$).

DISCUSSION

The tumor formation involves the conversion of normal cells into hyperplastic, dysplastic, neoplastic and finally metastatic cancerous cells. This transformation is triggered by accumulation of certain mutated key signalling proteins which are encoded by oncogenes and tumor suppressor genes and as a result more aggressive cells are formed in order to compete with adverse local surroundings.

Expression of many proteins in cancer cells increases or decreases as compared to normal cells. Some of the proteins which are encoded by oncogenes in majority and tumor suppressor genes play a crucial role in tumor growth and metastasis formation. Although the other proteins which are responsible for intracellular Ca^{2+} homeostasis take role in cancer progression, they are not associated with the development of tumor and/or malignant cells [6–8](Rosado JA, 2004, #66).

Ca^{2+} is known to be a versatile carrier of cell regulating information right from the formation of cell till its death. In recent studies, the role of calcium messenger has been shown in several cell processes like cell division, cell motility, hormone secretion, metabolism, nervous system functioning, protein turnover, gene expression, developmental regulation and programmed cell death (apoptosis) [9–11].

Many members of the TRP family of Ca^{2+} and Na^{+} permeable channels show altered expression in cancer cells. The most studied proteins of TRP family are *TRPM8*, *TRPV6*, *TRPM1* and *TRPV1* [12–14]. To date, most changes involving TRP proteins do not involve mutations in the TRP gene but rather increased or decreased levels of expression of the normal (wild type) TRP protein, depending on the stage of the cancer

In a number of studies during the past 20 years, the expression and activity of TRP channels have been shown to change during cancer. In particular, TRPC, TRPM and TRPV have been reported to be associated with tumorigenesis and the growth and development of cancer cells [4]. As a result of these findings, TRP channel expressions have been proposed as means of prognosis or diagnosis of some cancers and targeting of TRP channels have been proposed as a novel therapeutic strategy. For example, TRPV6 and TRPM8 have been proposed as an indicator (marker)

for prostate cancer development and TRPC6 as a therapeutic target for oesophageal cancer [15–17].

As there is an association between TRPM, TRPV, TRPC of TRP family especially with malignant cell division and its progression, therefore we targeted to find out the gene expression levels of *TRPM*, *TRPC* and *TRPV* in CRC.

As far as we know, there is no literature that shows the gene expression of the extensive TRP gene family in CRC. Our study is the most comprehensive study on this issue and can pave a way to future works related to this. In literature we find only the expression study of *TRPM8*, *TRPV1* and *TRPV6* in CRC [18–21].

In this particular study, comparative study has been done between the gene expression data of cancerous tissues and normal tissues of the same patient suffering from CRC. According to the data obtained by using Real-time PCR reaction, the gene expression of *TRPV4*, *TRPM4*, *TRPV3*, *TRPC6* and *TRPV5* in cancerous tissues were found to be lower as compared to normal tissues ($p < 0.05$). When expression levels of other TRP genes in tissues were compared, no significant difference was found statistically ($p > 0.05$).

Duncan et al. conducted a research on human melanoma specimens using *in situ* hybridisation method and found that there was an increased *TRPM1* expression in benign cells (melanocytic nevi) but there was no decrease in expression in primary melanoma as well as metastatic melanomas [22]. In our study, we also noticed a decrease in synthesis of *TRPV4*, *TRPM4*, *TRPV3*, *TRPC6*, *TRPV5* in cancerous cells as compared to normal cells. *TRPM1* showed a correlation with mRNA expression of melanocytic tumor progression, tumor thickness and its aggressiveness. In aggressive tumors, the mRNA expression of *TRPM1* was contemplated to be lower or in an undetectable amount. These observations showed *TRPM1* to be a tumor suppressor gene [23–25].

In the literature, there are no studies showing the expression of *TRPM1* in CRC. We did not find any statistical difference of *TRPM1* expression between tumor and normal tissues. This can be explained due to difference in tissues and diversity in cells.

Tsavaler et al. [21] investigated the relationship of *TRPM8* expression between normal prostate tissue and prostate cancer tissue using *in situ* hybridisation analysis and concluded that there was a significant increase of *TRPM8* expression in prostate cancer tissues as compared to normal ones. *TRPM8* expression was also studied in a number of non prostatic primary tumors of breast, colon, lung, and skin origin and a significant increase in its expression was found as compared to normal tissues. But in our study, there was no statistically significant difference of *TRPM8* expression between tumor and normal tissues. We used RT-PCR from paraffin blocks as a technique while Tsavaler et al. employed *in situ* hybridisation technique as method of analysis [21].

Suguro et al. [26] stated that diffuse large B-cell lymphoma accounts for 30% of non-Hodgkin's lymphomas and noticed an increase of *TRPM4* mRNA expression in $CD5^+$. *TRPM4* being one of the genes involved in the development of a "CD5 signature", therefore it can be used as a prognostic clinical marker. In our study, *TRPM4* expression was significantly lower in tumor tissue than in normal tissue. The reason for this may be due to difference in types of tissues.

Prawitt et al. [27] showed a sharp increase in *TRPM5* mRNA expression in Wilms' tumor and rhabdomyosarcoma. There was no statistically significant difference of *TRPM5* expression between tumor and normal tissues in our study.

As far as we know there has been no work done related to expression study of *TRPV4*, *TRPV3*, *TRPV5* in CRC. We found a significant decrease in expression of *TRPV4*, *TRPV3*, *TRPV5* in cancerous tissues than normal tissues. When compared with normal tissues or cells, the *TRPV6* mRNA expression and/or *TRPV6* protein expression shows a significant increase in prostate cancer tissue, human colon cancer, breast cancer, thyroid cancer and ovarian cancer tissues [18, 28–30]. As compared to normal tissues, increase in *TRPV6* mRNA expression is observed in prostate cancer LNCaP and PC-3 cell line, CRC SW480 cell line and chronic myelogenous leukaemia K-562 cell lines [18, 19].

Immunohistochemical studies demonstrated that TRPV1 is expressed in adenocarcinoma [20]. Extracellular polyamines which are agonists of TRPV1 are present in considerable concentration in the gastrointestinal tract and at synapses and these levels increase during inflammation and cancer [31]. As compared to this, our study showed no significant difference statistically of *TRPV1* expression between tumor and normal tissues.

The present study detected an increase in gene expression of *TRPM8*, *TRV6*, *TRPV1* in CRC tissues as compared to normal tissues which makes it different from others studies. Channel expression in tumor is performed by Western blot assay, immunohistochemical studies, RT-PCR or *in situ* hybridisation techniques. In order to prevent differences in results arising due to different operating methods, it is proposed to carry out comparison studies of these four techniques simultaneously from the same tissues. Gene expression studies should be carried out from paraffin blocks or fresh obtained tissues. We performed this study on paraffin blocks but further studies can be carried out using fresh tissues because ambient conditions during preparation of paraffin blocks can be an influential factor in channel expression studies. In addition, studies on fresh tissues will certainly aid in better understanding of TRP expression pattern in CRC because of good correlation between mRNA and protein levels in fresh tissues.

In the present study there is markedly lower expression of *TRPV4*, *TRPM4*, *TRPV3*, *TRPC6*, *TRPV5* in tumor tissues of CRC than normal tissues. There may arise some questions related to present study like what about effectiveness of these genes in CRC, what are the related mechanisms related to its suppression. Epigenetic factors may be involved in the lower expression of *TRPV4*, *TRPM4*, *TRPV3*, *TRPC6* and *TRPV5* in these results.

In conclusion, genes like *TRPV4*, *TRPM4*, *TRPV3*, *TRPC6* and *TRPV5* which are showing lower expression in CRC tissues are considered to be prominent gene candidates for potential tumor growth. This hypothesis needs to be supplemented and supported with further studies for its verification. New targeted therapeutic agents can be developed by employing TRP channel inhibitors in further studies.

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CONFLICT OF INTEREST

Authors have no conflicts of interest regarding the subject of this manuscript.

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