Original paper

# Influence of TNF-α promoter variability on stage and grade in individuals with colorectal cancer

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Carcinogenesis is a multistep process in which inflammation plays an important role. Tumour necrosis factor α (TNF-α) is a cytokine that plays a major role in inflammation. Activity of the TNF cytokine family could influence progression of colorectal cancer (CRC). The aim of the study was to establish an association between TNF-α promoter variability and stage/grade in individuals with sporadic CRC. The study included 152 CRC patients and 107 healthy volunteers. Four single nucleotide polymorphisms (rs361525, rs1800629, rs1799724, and rs1799964) located at the promoter of *TNFA* gene were genotyped using commercially available TaqMan allelic discrimination assays by real-time PCR. CRC stage was described on the basis of preoperative imaging studies and postoperative histopathological report. The grade was described on the basis postoperative pathological examination of the specimen. In the case of rs361525, there was a statistically significant association with M-score ($p = 0.0209$). Rs361525 has significant association with tumour grade ($p = 0.0260$). We failed to demonstrate significant association between the other 3 SNPs and cancer grade. Rs361525 potentially could be under consideration when the survival rate and prognosis is assessed.

**Key words:** colorectal cancer, TNF-α, promoter, SNP, stage, grade.

## Introduction

Colorectal cancer (CRC) is one of the leading causes of death among patients with neoplasms [1]. About eight million new CRC cases are diagnosed each year worldwide [2]. Carcinogenesis is a multistep process, in which inflammation plays an important role. The presence of inflammation in the tumour microenvironment is well described [3, 4, 5]. Tumour necrosis factor α (TNF-α) is a cytokine that plays major role in the inflammatory process. It is produced mainly by macrophages, but secretion of TNF-α by tumour cells has also been described [6].

Cancer progression is a process described in stages. According to the American Joint Committee on Cancer (AJCC 2010 ed. with update), colorectal cancer has 5 stages, starting with 0 (cancer *in situ*), when neoplastic cells are confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa, and ending with stage 4, where distant metastases (M1) are present. Neoplastic grade is a description of cell anaplasia. According to the AJCC, grades range from 1 to 4 (well differentiated to undifferentiated, respectively). The stage is strongly associated with the prognosis. Individuals with a tumour TisN0M0 according to AJCC have a 5-year survival rate of 100%, while patients with distant metastases

(M1) have an average 5-year survival rate of approximately 5%. It is important to identify the patients who are likely to have more aggressive course of the disease and try to tailor their treatment. High-sensitivity *KRAS* detection methods are an example [7].

The activity of the TNF cytokine family could influence the progression of CRC [8, 9]. Tumour necrosis factor α is encoded by the *TNFA* gene. Promoter of *TNFA* has single nucleotide polymorphism (SNP). The question arises whether this variability has an influence on carcinogenesis and prognosis in patients with CRC. The promoter region in DNA initiates transcription. It has a binding site for RNA polymerase and transcription factors that facilitate the process. Variability of this sequence could alter affinity of transcription factors and therefore enhance or suppress gene expression in certain SNP variants. This may have a link to cancer advancement and, as a consequence, prognosis.

The aim of the study was to establish if there is an association between TNF-α promoter variability and stage/grade in case of individuals with sporadic colorectal cancer.

## Material and methods

The study enrolled consecutive patients admitted for surgery to the 2nd Department of Surgery, University Hospital, Krakow, Poland, between 2013 and 2016. Patients' data collected at the admission included medical anamnesis and family history. Patients with a history of systemic diseases of immune inflammatory mechanism were excluded from the study. The study group consisted of 152 patients (82 males and 70 females) with colorectal cancer. The average age of the studied group was 67.2 years. Clinicopathological characteristics of the study group is summarised in Table I.

**Table I.** Clinical and pathological characteristics of the study population

| Variable | | Number of patients |
|---|---|---|
| Gender | Male | 82 (54%) |
| | Female | 70 (46%) |
| Localization | Caecum and ascending colon | 20 (13%) |
| | Transverse colon | 15 (10%) |
| | Descending and sigmoid colon | 64 (42%) |
| | Rectum | 53 (35%) |
| TNM classification | T1 | 9 (6%) |
| | T2 | 28 (18%) |
| | T3 | 92 (60%) |
| | T4 | 20 (13%) |
| | N0 | 90 (60%) |
| | N1 | 30(20%) |
| | N2 | 27 (18%) |
| | M0 | 126 (83%) |
| | M1 | 26 (17%) |
| Stage according to AJCC | I | 29 (19%) |
| | II | 49 (32%) |
| | III | 48 (32%) |
| | IV | 26 (17%) |
| Grade | G1 | 23 (15%) |
| | G2 | 114 (75%) |
| | G3 | 15 (10%) |

Blood samples were collected from all the subjects of the study. The samples were kept at 4°C until DNA extraction, which was performed within 6 hours of blood collection. DNA was extracted from peripheral leucocytes using DNAzol reagent (Invitrogen Life Technologies, USA). Four SNPs located in the promotor of *TNFA* gene were selected: rs361525, rs1800629, rs1799964, and rs1799724. SNPs were genotyped with commercially available TaqMan allelic discrimination assays (Life Technologies) using 7900HT Real-time PCR System (Applied Biosystems, Foster City, CA). The mix of unlabelled PCR primers and TaqMan MGB probes labelled with FAM or VIC dye were used. The reaction was performed in a 10-µl solution that contained 0.5 µl of 40 × ologonucleotides mix, 5 µl of 2 × TaqMan Genotyping Master mix (Applied Biosystems), and 2 µl of 50 ng genomic DNA. PCR conditions were as follows: initial denaturation at 95°C for 15 min; 40 cycles at 95°C for 10 s and 60°C for 45 s. Genotyping was performed on coded and blinded samples in the presence of negative ones (no DNA) included in each 96-well plate for quality control. The genotyping results were determined by using SDS 2.3 Allelic Discrimination Software (Applied Biosystems).

Tumour stage was described on the basis of preoperative imaging studies (abdominal and thoracic computed tomography, pelvic magnetic resonance imaging, abdominal ultrasound, chest X-ray), intraoperative assessment, and postoperative pathological report. Grade was described on the basis of postoperative pathological examination of the specimen.

After surgical procedure all specimens were fixed with 10% formaldehyde solution. Materials were handled according to the AJCC 2010 ed. Guidelines (with updates). The sections were taken within the standard procedure [10] and processed in Shandon Excelsior processors (Thermo Fisher Scientific

Inc., UK), embedded in paraffin, cut into 4-μm sections, stained, and coverslipped in a Tissue-Tec Prisma device (Sakura Finetek Europe B.V., Netherlands). Standard staining included haematoxylin-eosin (HE); if needed, other stains (mucin, PAS, Trichrome, Alcian blue) or immunohistochemistry were performed. The WHO Classification of Tumours of the Digestive System – 2010 was used and pTNM was assessed as described in AJCC (2010 ed. with updates) guidelines (T1 defined as involvement of the submucosa; T2 into but not through the muscularis propria; T3 penetration through the muscularis propria and T4 as involvement the serosal surface or direct invasion of adjacent organs; the N category according to the number of metastatic lymph nodes/presence of tumour deposits; the M category due to the presence of distant metastases). The tumour grade was assessed as the tendency to form gland-like structures (WHO).

Statistical analysis was performed using Statsoft Statistica v.12 software. Comparisons of the SNPs frequency in the TNF-α gene in different grade and TNM groups were calculated according to Pearson's goodness-of-fit $\chi^2$. In the case of multiple testing with statistically significant results, Bonferroni correction was applied. A p-value $< 0.05$ was considered significant.

Informed, written consent to participate in the study was obtained from all the participants. All procedures followed the ethical standards of the responsible committee on human experimentation (institutional and national) and the 2008 revision of the 1975 Declaration of Helsinki. The protocol of the study was accepted by the Bioethics Committee of Jagiellonian University Medical College (KBET/376/B/2012).

## Results

Table II presents frequencies of particular genotypes and alleles in the whole study population. The allele and genotype frequencies of rs1799724 and rs1799964 are in Hardy-Weinberg equilibrium. The population is not in Hardy-Weinberg equilibrium for the genotypes rs361525 and rs1800629 (Table III).

In the case of rs361525, there was a statistically significant association with M-score. Allele A was more frequent in cases with no distant metastases, whereas allele G was more frequent in patients with dissemination ($p = 0.0209$). No other association between studied SNPs and TNM classification was observed (Table IV-VII).

Statistical analysis showed that rs361525 has significant association with tumour grade. Allele A has the highest frequency in the case of grade 1. Allele G has the highest frequency in individuals with grade 3 ($p = 0.0260$). We failed to demonstrate any significant association between the other 3 SNPs and cancer grade (Table VIII).

**Table II.** Frequencies of particular genotypes and alleles in the study population

| SNP | Genotype/ allele | Number | Percentage (%) |
|---|---|---|---|
| rs361525 | AA | 13 | 8.67 |
| | AG | 35 | 23.33 |
| | GG | 102 | 68 |
| | A | 61 | 20.33 |
| | G | 239 | 79.67 |
| rs1800629 | AA | 13 | 8.67 |
| | AG | 33 | 22 |
| | GG | 104 | 69.33 |
| | A | 59 | 19.67 |
| | G | 241 | 80.33 |
| rs1799724 | CC | 98 | 65.33 |
| | CT | 43 | 28.67 |
| | TT | 9 | 6 |
| | C | 239 | 79.67 |
| | T | 61 | 20.33 |
| rs1799964 | CC | 5 | 3.33 |
| | CT | 59 | 39.33 |
| | TT | 86 | 57.33 |
| | C | 69 | 23 |
| | T | 231 | 77 |

**Table III.** Hardy-Weinberg equilibrium analysis

| SNP | $\chi^2$ | p-value |
|---|---|---|
| rs361525 | 11.7420 | 0.0006 |
| rs1800629 | 13.8395 | 0.0002 |
| rs1799724 | 1.9895 | 0.1584 |
| rs1799964 | 1.8310 | 0.1760 |

## Discussion

In this study we have investigated whether SNPs in the promoter of TNF-α gene have an association with clinical advancement and the level of neoplasia in individuals with sporadic colorectal cancer. We found that rs361525 has a statistically significant association with metastasis ($p = 0.0209$). No other association between studied SNPs and TNM classification was observed. Although it is described in the literature that increased TNF-α gene expression is connected with stage [9], it seems that the contribution of TNF-α gene promoter variability is limited.

**Table IV.** rs361525 stage analysis

| **rs361525** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **AG** | **GG** | **AA** | **p** | **A** | **G** | **p** |
| T1 | 28.57% | 71.43% | 0.00% | 0.3222 | 14.29% | 85.71% | 0.1765 |
| T2 | 36.36% | 59.09% | 4.55% | | 22.73% | 77.27% | |
| T3 | 22.54% | 64.79% | 12.68% | | 23.94% | 76.06% | |
| T4 | 13.33% | 86.67% | 0.00% | | 6.67% | 93.33% | |
| N0 | 22.86% | 67.14% | 10.00% | 0.8401 | 21.43% | 78.57% | 0.9624 |
| N1 | 21.74% | 69.57% | 8.70% | | 19.57% | 80.43% | |
| N2 | 33.33% | 61.90% | 4.76% | | 21.43% | 78.57% | |
| M0 | 24.11% | 65.18% | 10.71% | 0.1749 | 22.77% | 77.23% | **0.0209** |
| M1 | 20.00% | 80.00% | 0.00% | | 10.00% | 90.00% | |

**Table V.** rs1800629 stage analysis

| **rs1800629** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **AG** | **GG** | **AA** | **p** | **A** | **G** | **p** |
| T1 | 14.29% | 85.71% | 0.00% | 0.0974 | 7.14% | 92.86% | 0.0852 |
| T2 | 36.36% | 63.64% | 0.00% | | 18.18% | 81.82% | |
| T3 | 19.72% | 63.38% | 16.90% | | 26.76% | 73.24% | |
| T4 | 20.00% | 80.00% | 0.00% | | 10.00% | 90.00% | |
| N0 | 21.43% | 71.43% | 7.14% | 0.5431 | 17.86% | 82.14% | 0.1724 |
| N1 | 21.74% | 60.87% | 17.39% | | 28.26% | 71.74% | |
| N2 | 28.57% | 57.14% | 14.29% | | 28.57% | 71.43% | |
| M0 | 20.54% | 69.64% | 9.82% | 0.4634 | 20.09% | 79.91% | 0.5379 |
| M1 | 32.00% | 60.00% | 8.00% | | 24.00% | 76.00% | |

**Table VI.** rs1799724 stage analysis

| **rs1799724** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **CC** | **CT** | **TT** | **p** | **C** | **T** | **p** |
| T1 | 57.14% | 42.86% | 0.00% | 0.3352 | 78.57% | 21.43% | 0.4537 |
| T2 | 50.00% | 45.45% | 4.55% | | 72.73% | 27.27% | |
| T3 | 71.83% | 21.13% | 7.04% | | 82.39% | 17.61% | |
| T4 | 53.33% | 40.00% | 6.67% | | 73.33% | 26.67% | |
| N0 | 57.14% | 34.29% | 8.57% | 0.0637 | 74.29% | 25.71% | 0.1643 |
| N1 | 86.96% | 8.70% | 4.35% | | 91.30% | 8.70% | |
| N2 | 61.90% | 38.10% | 0.00% | | 80.95% | 19.05% | |
| M0 | 66.96% | 27.68% | 5.36% | 0.7674 | 80.80% | 19.20% | 0.4432 |
| M1 | 60.00% | 32.00% | 8.00% | | 76.00% | 24.00% | |

Statistical analysis showed that rs361525 also has a significant association with tumour grade ($p = 0.0260$). We failed to demonstrate significant association between grade and other investigated SNPs.

Tumour necrosis factor α is a proinflammatory cytokine, which has a wide range of activities, among them inflammation, cell proliferation, differentiation, and apoptosis [5]. It is believed that cytokines from the TNF family play an important role in oncogenesis [2]. It is very interesting that this cytokine has paradoxical role in tumour development. In high concentration TNF-α can destroy tumour blood vessels, but during chronic inflammation it can promote cancer growth and spread by remodelling tissue and stroma [9]. Chronic inflammation is connected with

**Table VII.** rs1799964 stage analysis

| **rs1799964** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **CC** | **CT** | **TT** | **p** | **C** | **T** | **p** |
| T1 | 0.00% | 71.43% | 28.57% | 0.1744 | 35.71% | 64.29% | 0.2231 |
| T2 | 0.00% | 50.00% | 50.00% | | 25.00% | 75.00% | |
| T3 | 4.23% | 33.80% | 61.97% | | 21.13% | 78.87% | |
| T4 | 0.00% | 20.00% | 80.00% | | 10.00% | 90.00% | |
| N0 | 4.29% | 44.29% | 51.43% | 0.1832 | 26.43% | 73.57% | 0.0678 |
| N1 | 0.00% | 30.43% | 69.57% | | 15.22% | 84.78% | |
| N2 | 0.00% | 23.81% | 76.19% | | 11.90% | 88.10% | |
| M0 | 3.57% | 35.71% | 60.71% | 0.3738 | 21.43% | 78.57% | 0.6910 |
| M1 | 0.00% | 48.00% | 52.00% | | 24.00% | 76.00% | |

**Table VIII.** Grade analysis

| **rs361525** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **AG** | **GG** | **AA** | **p** | **A** | **G** | **p** |
| grade 1 | 47.06% | 11.76% | 41.18% | 0.0873 | 35.29% | 64.71% | **0.0260** |
| grade 2 | 20% | 7.5% | 72.5% | | 17.5% | 82.5% | |
| grade 3 | 9.09% | 81.82% | 81.82% | | 13.64% | 86.36% | |
| **rs1800629** | | | | | | | |
| | **AG** | **GG** | **AA** | **p** | **A** | **G** | **p** |
| grade 1 | 35.29% | 58.82% | 5.88% | 0.3331 | 23.53% | 76.47% | 0.3659 |
| grade 2 | 17.5% | 70% | 12.5% | | 21.25% | 78.75% | |
| grade 3 | 18.18% | 81.82% | 0% | | 9.09% | 90.91% | |
| **rs1799724** | | | | | | | |
| | **CC** | **CT** | **TT** | **p** | **C** | **T** | **p** |
| grade 1 | 64.71% | 29.41% | 5.88% | 0.4359 | 79.41% | 20.59% | 0.1463 |
| grade 2 | 61.25% | 32.5% | 6.25% | | 77.5% | 22.5% | |
| grade 3 | 90.91% | 9.09% | 0% | | 95.45% | 4.55% | |
| **rs1799964** | | | | | | | |
| | **TT** | **CT** | **CC** | **p** | **T** | **C** | **p** |
| grade 1 | 41.18% | 52.94% | 5.88% | 0.4741 | 67.65% | 32.35% | 0.2277 |
| grade 2 | 63.75% | 33.75% | 2.5% | | 80.63% | 19.38% | |
| grade 3 | 63.64% | 36.36% | 0% | | 81.82% | 18.18% | |

solid tumours of the digestive tract, including colorectal cancer [2, 3, 11]. Due to this fact, TNF-α and its gene are a target for scientific research. An interesting issue, for which we do not have any answers, is the influence of TNF-α promoter polymorphism on CRC stage and grade.

Although TNF was initially described as a cytokine responsible for tumour necrosis, further study has revealed that cytokines from the TNF family can promote tumour growth [9]. It has been reported that TNF-α serum level is increased in patients affected by cancer [12]. Elevated TNF-α expression may promote growth and invasion of CRC [13]. It is well documented that elevated TNF-α serum level is connected with cancer stage and progression, and it is linked with poor prognosis. Stanilov *et al*. [11] demonstrated that TNF-α serum levels in a group of CRC patients were significantly higher than those in the control group. The highest TNF-α level was found in stage IV of CRC, and it was significantly higher when compared to the earlier stages of CRC and the control group. Al Obeed showed that high TNF-α gene expression was associated with stage III and IV of CRC when compared with earlier

tumour stages [13]. In the case of breast cancer, TNF-α induces an epithelial-mesenchymal transition, the process in which cancer cells of the primary tumour undergo a phenotypic conversion to invade and metastasize through the circulation [14]. A similar process could occur in CRC.

In our study we demonstrated that there is some connection with TNF-α promoter SNP and colorectal cancer stage and grade. A stronger association was described previously in the case of other tumours [15]. Promoter is a region of DNA that initiates transcription of a particular gene. Variability of this region could alter the affinity of transcription factors and therefore expression of a gene. It was reported previously that expression could depend on a certain SNP variant [11, 16, 17]. There is no report of any association of that kind in the case of TNF-α and CRC. We found that only one of 4 SNPs has an association with the presence of distant metastases and only one has an association with tumour grade. On the basis of that, we could assume that the impact of TNF-α promoter SNP on CRC stage and grade is limited. High TNF-α serum levels in the case of advanced CRC mentioned above probably has a different aetiology. It seems that in opposition to TNF-α serum level, SNPs in the promoter of this gene are not very good candidates to be a tool that helps predict stage and grade, and indirectly survival rate.

In previous studies so-called high plasma TNF producer was described [17]. It is a GA genotype of rs1800629 (-308 A/G). This genotype was found to be significantly associated with a poor disease outcome in breast cancer patients [18]. In our research, rs1800629 had no connection with stage and grade, and therefore, indirectly, with prognosis.

Although the problem of association between CRC stage/grade and TNF-α promoter SNPs is not widely discussed in the literature, research that describes the relation between polymorphisms of TNF-α promoter and colorectal cancer itself was widely conducted. Some studies demonstrated an association between TNF-α promoter SNP and colorectal cancer [19, 20, 21]; however, there are studies that contradict this [19, 22, 23]. Results of the research in this field are inconclusive. The differences could be caused by ethnicity and, more importantly, lifestyle. Most CRCs are sporadic, and genetic burden plays a minor role in such cases [24]. We could assume that in some populations, genetic screening for TNF-α promoter SNPs could be a tool for stratifying risk.

To conclude, it seems that single-nucleotide polymorphisms of TNF-α gene are not good markers when the survival rate and prognosis is assessed. From 4 SNPs in the promoter of TNF-α, rs361525 could be under consideration because of its association with M-score and grade; however, it should be mentioned that the results presented by us are restricted to the Polish population and had a limited sample size. Further investigations on larger groups from different populations are needed to confirm the results presented herein.

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*The authors declare no conflict of interest.*

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