**ASSOCIATION OF TNF-α EXPRESSION WITH CLINICOPATHOLOGICAL VARIABLES AND SURVIVAL IN COLORECTAL CANCER PATIENTS: A STUDY FROM WESTERN INDIAN REGION**

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**ABSTRACT**

Tumor Necrosis Factor alpha (TNF-α), a proinflammatory cytokine, is recognized as a significant contributor to tumor progression. Higher TNF-α expression has been reported to promote growth and invasion, and correlated with unfavorable clinical outcomes in colorectal cancer (CRC) patients. Therefore, present study investigated TNF-α mRNA expression in CRC patients and assessed its correlation with clinicopathological parameters and prognosis in western region of India. Total 40 primary CRC patients were enrolled and tumor tissues were collected. Out of 40, 10 adjacent normal tissues were collected as controls. TNF-α mRNA expression was evaluated by reverse transcription real time PCR (RT-qPCR) method and relative TNF-α expression was determined by 2-ΔΔCT method. The data was analyzed using SPSS software. Out of 40 CRC patients, upregulation of TNF-α mRNA expression was detected in 63% of patients, while 37% showed TNF-α downregulation as compared to controls. Further, relative TNF-α expression was significantly higher in patients with positive lymphnode status (P=0.041), advanced stage (P=0.041), and larger tumor size (P=0.032). Relative TNF-α expression was not significantly associated with relapse-free survival (P=0.873) and overall survival (P=0.581). The study concludes that higher TNF-α mRNA expression correlated with biologically aggressive prognostic factors suggesting its role as a useful biomarker to identify high-risk group of CRC patients.

**Keywords:** TNF-α, mRNA expression, Real time PCR, Clinicopathological parameters, Survival, Colorectal cancer

1. **INTRODUCTION**

Colorectal cancer (CRC) remains the major health concern being the third most frequently diagnosed cancer accounting for approximately 10% of all cancer cases and the second leading cause of cancer-related death with 9.4% mortality rate worldwide according to Globocan 2020 [1]. The main reason for colon cancer-related mortality is resistance to current standard chemotherapeutic treatments, resulting in high incidence of metastatic recurrence. Hence, development of novel therapeutic approaches, which may enhance chemosensitivity and suppress metastatic recurrence are required to improve overall survival rates in CRC [2].

Rising evidence suggests that treatment using anti-inflammatory medication approach may prevent or delay the development of CRC in hereditary and sporadic cases, emphasizing a potentially extensive role of inflammation in the development and progression of all types of CRC [3]. Inflammation influences various stages of carcinogenesis, such as initiation, promotion, and progression. Additionally, epithelial inflammation has been regarded as one of the hallmarks of CRC pathogenesis factors, just next to genetic abnormalities. Among other mediators of inflammation, cytokines appear to have a remarkable but complex role in driving or preventing malignant transformation [4].

Tumor necrosis factor alpha (TNF-α) is one of the most important proin­flammatory cytokines in the immune system [5]. It is predominantly produced by macrophages as well as tumor cells, and is a cytokine ligand of the TNF family that interacts with different receptors of the TNF receptor superfamily [6]. TNF-alpha acts on cells via three major signaling pathways: the mitogen-activated protein kinase (MAPK), the nuclear factor kappa B (NF-κB) and apoptosis signaling pathways. Through these wide signaling pathways, TNF-α can cause either neoplastic cells promotion or destruction. It can contribute to the development of neoplasms in several ways, including cellular transformation, proliferation, angiogenesis, migration, and invasion [5]. It regulates cellular communication within the tumour microenvironment and is associated with the progression of the metastatic phenotype [7]**.** On the other hand, it can induce apoptosis, necrosis, and disruption of tumor vasculature in therapeutic dosage [5]. Thus, TNF-α has been found to have both tumor promoting and tumor suppressing effects.

Recent epidemiological and clinical data supports the concept that TNF-α acts as a key endogenous tumor promoter that connects chronic inflammation and carcinogenesis [8]. TNF-α has been described to present at high levels in solid tumours and the serum of CRC patients that have been implicated in metastatic development [7]. Previous clinical investigations showed that serum TNF-α levels in CRC patients were significantly elevated, and patients with higher TNF-α levels had unfavorable prognosis [9]. Moreover, higher expression of TNF-α mRNA could be an independent diagnostic indicator of CRC, and targeting TNF-α might be a promising prognostic tool by assessment of the clinical stages of CRC [6]. Further, TNF-α was highly expressed in colon cancer cell lines and described to be an independent adverse prognosticator of colon cancer, and anti-TNF-α might benefit colon cancer patients [10]. Therefore, understanding the molecular mechanisms underlying TNF-α dysregulation in CRC may provide insights into novel therapeutic strategies targeting the tumor microenvironment to improve patient outcomes. Hence, present study aimed to explore the role of TNF-α mRNA expression in CRC patients in western Indian region. Further, its association with clinicopathological parameters and survival in CRC patients was evaluated.

1. **METHODOLOGY**
	1. **Subjects**

A total of 40 histopathologically confirmed patients of primary colorectal cancer at The Gujarat Cancer & Research Institute between 2017 to 2021 were enrolled for the study. The study was approved by the Institutional Review Committee and Ethics Committee. The detailed clinical history such as patient’s age, gender, habit, and histopathological findings were recorded from the case files maintained at the Institutional Medical Record Department. Patients were monitored for at least 24 months or until death during this period. Follow-up data could be collected for 33 patients and utilized for overall survival (OS) analysis. Of these, 2 patients were excluded from recurrence-free survival (RFS) analysis due to persistent disease. Thus, 31 CRC patients were considered for RFS analysis.

* 1. **Sample collection**

Informed consent of all patients was obtained before collection of the samples. Tumor tissue samples of all 40 CRC patients were collected. Of 40 patients, adjacent normal colon/rectal tissues from 10 patients were available and taken as controls. For sample collection, tissue specimens were collected on ice directly from the operation theatre. Then, tumor/normal portion from collected specimen was selected by a pathologist and snap frozen in liquid nitrogen and preserved at -80°C till further analysis.

* 1. **RNA extraction and cDNA synthesis**

Total RNA was extracted from tissue samples using RNA isoplus reagent (Takara Bio Inc) and RNA quantification was performed by Qubit fluorimeter 3.0 (Invitrogen). Reverse transcription for cDNA synthesis was carried out from 1 µg RNA samples using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer’s protocol.

* 1. **Real time PCR**

Real time quantitative PCR of cDNA samples was performed by using QuantiNova SYBR Green PCR Kit (Qiagen). The primer sequences for TNF-α and GAPDH (used as housekeeping reference gene for normalization) were as follows: TNF-α- Forward: 5’ TCT GGG CAG GTC TAC TTT GG 3’ Reverse: 5’ TCTTCTCAAGTCCTGCAGCA3’ and; GAPDH Forward: 5’ AAGGTCGGAGTCAACGGATTTG 3’ Reverse: 5’ GCCATGGGTGGAATCATATTGG 3’. The cycling procedure began with PCR activation at 95°C for 2 minutes, followed by 40 cycles comprising denaturation at 95°C for 5 seconds and combined annealing/extension at 60°C for 10 seconds. The relative quantification of TNF-α was performed by 2-∆∆Ct method.

* 1. **Statistical Analysis**

Data was evaluated statistically using SPSS (Statistical Package for the Social Sciences) software. Association of relative expression of TNF-α with the clinicopathological parameters of CRC patients was calculated using Independent-Samples T-test. RFS and OS analysis were performed using Kaplan-Meier method and Log rank test. P values ≤0.05 were considered significant.

1. **RESULTS**
	1. **TNF-α mRNA expression and its association with clinicopathological parameters in CRC patients**

Among the studied 40 CRC patients, 63% (25/40) of patients had upregulation of TNF-α mRNA expression, while 37% (15/40) showed downregulation as compared to controls.Further, TNF-α mRNA expression was not significantly correlated with clinical parameters such as age, gender, habit, family history, diet, tumor site and tumor location (Table 1). However, when correlated with pathological parameters, TNF-α mRNA expression showed significant association with tumor size, nodal status, and tumor grade. The fold change expression of TNF-α mRNA was significantly higher in patients with large tumor size as compared to those with small tumor size (P=0.032). A significant increase in the expression of TNF-α mRNA was noted in CRC patients with lymph node metastasis as compared to those without lymph node metastasis (P=0.041). Moreover, relative TNF-α expression was significantly higher in advanced stage patients as compared to early stage patients (P=0.041). Although not significant, the fold change expression of TNF-α was found to be higher in patients with presence of lymphatic permeation, presence of vascular permeation, presence of perineural invasion, and presence of mucin as compared to their respective counterparts. (Table 2).

**Table 1**: Correlation of TNF-α expression with clinical parameters in CRC patients

| **Characteristics** | **N (%)** | **TNF-α mRNA expression** |
| --- | --- | --- |
| **Age (years)****Median: 56 years** |  | **Mean ± SE** | **P value** |
| < 56 | 18 (45) | 8.20 ± 2.96 | 0.339 |
| ≤ 56 | 22 (55) | 13.20 ± 3.98 |  |
| **Gender**  |  |  |  |
| Female  | 12 (30) | 7.81 ± 4.04 | 0.431 |
| Male | 28 (70) | 12.30 ± 3.24  |  |
| **Habit** |  |  |  |
| No | 25 (62) | 10.00 ± 3.27 | 0.641 |
| Yes | 15 (38) | 12.53 ± 4.25 |  |
| **Family History** |  |  |  |
| No | 36 (90) | 11.47 ± 2.83 | 0.551 |
| Yes | 04 (10) | 6.27 ± 2.50 |  |
| **Diet** |  |  |  |
| Vegetarian | 29 (73) | 11.34 ± 2.94 | 0.809 |
| Vegetarian + Non-Vegetarian | 11 (27) | 9.92 ± 5.41 |  |
| **Tumor site** |  |  |  |
| Colon | 28 (70) | 11.85 ± 3.14 | 0.601 |
| Rectum | 12 (30) | 8.86 ± 4.54 |  |
| **Tumor location** |  |  |  |
| Right side (Cecum+ Ascending colon+ Transverse colon) | 15 (38) | 14.91 ± 4.81 | 0.238 |
| Left side (Descending colon+ Sigmoid colon+ Rectum) | 25 (62) | 8.58 ± 2.90 |  |

**Table 2**: Correlation of TNF-α expression with pathological parameters in CRC patients

|  |  |  |
| --- | --- | --- |
| **Characteristics** | **N (%)** | **TNF-α mRNA expression** |
| **Tumor size** |  | **Mean ± SE** | **P value** |
| Small (T1+T2) | 05 (12) | 3.73 ± 2.16 | **0.032** |
| Large (T3+T4) | 35 (88) | 11.98 ± 2.88 |  |
| **Nodal status** |  |  |  |
| Negative | 18 (45) | 5.49 ± 1.94 | **0.041** |
| Positive | 22 (55) | 15.41 ± 4.20 |  |
| **TNM stage** |  |  |  |
| Early stage (Stage I+II) | 18 (45) | 5.49 ± 1.94  | **0.041** |
| Advanced stage (Stage III+IV) | 22 (55) | 15.41 ± 4.20  |  |
| **Histological grade**  |  |  |  |
| Well differentiated  | 05 (12) | 20.06 ± 8.86 | 0.184 |
| Moderately/Poorly differentiated  | 35 (88) | 9.65 ± 2.53 |  |
| **Histologic type** |  |  |  |
| Adenocarcinoma | 33 (82) | 9.28 ± 2.49 | 0.161 |
| Mucin/Signet ring cell adenocarcinoma | 07 (18) | 18.82 ± 8.74 |  |
| **Mucin** |  |  |  |
| Absent | 32 (80) | 9.18 ± 2.56 | 0.170 |
|  Present | 08 (20) | 18.04 ± 7.61 |  |
| **Lymphatic permeation**  |  |  |  |
|  Absent | 20 (50) | 8.81 ± 3.14 | 0.413 |
|  Present | 20 (50) | 13.08 ± 4.08 |  |
| **Vascular permeation** |  |  |  |
| Absent | 27 (68) | 8.71 ± 2.60 | 0.213 |
| Present | 13 (32) | 15.61 ± 5.72 |  |
| **Lymphocytic stromal response** |  |  |  |
| Absent | 21 (53) | 11.95 ± 3.91  | 0.688 |
| Present | 19 (47) | 9.85 ± 3.34 |  |
| **Perineural invasion** |  |  |  |
| Absent | 33 (83) | 9.09 ± 2.61 | 0.118 |
| Present | 07 (17) | 19.70 ± 7.63 |  |
| **Necrosis** |  |  |  |
| Absent | 30 (75) | 10.56 ± 2.86 | 0.798 |
| Present | 10 (25) | 12.11 ± 5.88 |  |
| **Pre-operative circulating CEA levels (ng/ml)** |  |  |  |
| ≤ 5.0  | 19 (48) | 11.08 ± 3.75 | 0.962 |
| > 5.0 | 21 (52) | * 1. ± 3.61
 |  |

* 1. **Survival Analysis**

For survival analysis, median relative quantification was used as cut-off to divide the patients into low and high TNF-α mRNA expression groups. Univariate Kaplan-Meier survival analysis for RFS showed no significant difference in the incidence of disease relapse between patients with low TNF-α mRNA expression (19%) and high expression (20%) (P=0.873). For OS also, no significant difference in the incidence of death was observed between patients with low TNF-α mRNA expression (12%) and those with high expression (19%) (P=0.581) (Table 3). Kaplan-Meier survival curves are depicted in Figure 1.

**Table 3**: Univariate survival analysis for RFS and OS in relation to TNF-α mRNA expression in CRC patients

|  |  |  |
| --- | --- | --- |
| **TNF-α mRNA expression** | **RFS** | **OS** |
| **N** | **No recurrence****N (%)** | **Recurrence** **N (%)** | **N** | **Alive****N (%)** | **Dead****N (%)** |
| CRC patients | 31  |  |  | 33  |  |  |
| Low | 16 | 13 (81) | 03 (19) | 17 | 15 (88) | 02 (12) |
| High | 15 | 12 (80) | 03 (20) | 16 | 13 (81) | 03 (19) |
|  |  | Log rank=0.025, df=1, P=0.873 |  | Log rank=0.305, df=1, P=0.581 |

|  |  |
| --- | --- |
| A graph of a patient's expression  Description automatically generated | A graph of a patient's disease  Description automatically generated |

**Figure 1:** Kaplan-Meier survival curves for RFS and OS in relation to TNF-α mRNA expression in CRC patients

1. **DISCUSSION**

Chronic inﬂammation is a known risk factor for CRC development, progression, metastasis, and resistance to cancer therapy [7]. It is the most important promoter of tumorigenesis in colon cancer. Inflammation induced by tumor-released cytokines has been suggested as a crucial factor in colorectal carcinogenesis [6]. TNF-α, a proinflammatory cytokine, is one of the key chemical mediators of inflammation in cancer, associated with a poor prognosis. TNF-α has a wide range of biological activities, including apoptosis, inflammation, cell proliferation and differentiation [11]. Increased TNF-α levels were found in serum samples and tumors of CRC patients [6,10,12,13], contributing to metastatic development. Previous studies have identiﬁed strong association of inﬂammatory mediators with the progression of CRC [14] and the ability of TNF-α to promote DNA damage in CRC cells. Hence, present study was conducted to identify the clinical value of TNF-α mRNA expression in CRC patients of western Indian region.

In present study, TNF-α mRNA expression was found to be upregulated in 63% of CRC patients, while it was downregulated in 37% of patients as compared to controls. Accordingly, TNF-α mRNA expression was found to be more abundantly expressed in CRC tissues compared with adjacent normal mucosa [6, 15]. In non-small cell lung cancer (NSCLC) also, TNF-α mRNA expression as well as protein expression in tumor tissues were significantly higher compared with adjacent tissues. Additionally, in these patients, a significant higher serum TNF-α levels were noted than those of the control group [16]. Further, majority (94%) of the patients with CRC expressed TNF-α [17]. Moreover, mRNA expression of TNF-α was 3.4 fold higher in CRC cases as compared to controls [18]. In addition, other reports demonstrated that TNF-α serum levels in a group of CRC patients were significantly higher than those in the control group [13,19]. In vitro studies also showed significantly elevated TNF-α expression in A498 renal cell carcinoma cell lines [20]. Previous reports in other cancer types such as multiple myeloma and non-hodgkin’s lymphoma also described high expression levels of TNF-α [21,22]. In line with these findings, present study conducted in western part of India, also showed greater incidence of higher TNF-α expression in CRC patients.

Further, present study correlated relative TNF-α mRNA expression with clinicopathological parameters in CRC patients and higher TNF-α mRNA expression was found to be significantly correlated with lymph node involvement (P=0.041), advanced tumor stage (P=0.041) and larger tumor size (P=0.032). Concordant to present results, high TNF-α expression was significantly associated with positive lymph node stage and recurrence of tumor [17]. Previously, a significant higher TNF- α mRNA expression was noted in late CRC stage compared to early stage (P=0.004). Moreover, although not significant, increased gene expression was seen in the advanced stages, with lymph node metastases compared with patients with no lymph node metastasis. Additionally, immunohistochemistry results indicated that TNF- **α** is significantly expressed in later stages (Stage Ⅲ, Ⅳ) of CRC compared to early stages (Stage Ⅰ, Ⅱ). Both immunohistochemistry and RT-PCR results were associated with tumor progression [6]. Recent report also revealed a significant relationship between the stages in CRC patients and TNF-α level in blood plasma (P=0.0001) [23]. Moreover, present study demonstrated a non-significant higher fold change expression of TNF-α in patients with older age, presence of mucin, presence of lymphatic permeation, presence of vascular permeation, presence of perineural invasion as compared to their respective counterparts. Association of high-risk clinicopathological factors with higher TNF-α expression suggests that there is a link between inflammation and cancer [24], and TNF-α might serve as an important indicator of disease progression [6]. It acts not only as a pro-inflammatory cytokine but can also cause tumor development. Moreover, TNF-α is highly related to all stages in the tumorigenesis process, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [23]. Consequently, the use of TNF-α inhibitors as cancer therapeutics has been proposed [25]. These results could contribute to a further understanding of CRC pathogenesis. However, several studies could not find any significant relationship of TNF-α mRNA expression with gender, age, tumor localization, lymph node metastasis and the presence of distance metastases [18,23].

Present study also examined prognostic value of TNF-α mRNA expression in CRC patients however, no significant association was found with RFS or OS. It is well documented that elevated TNF-α might be useful prognostic factor in CRC as well as other malignancies like in renal cell carcinoma, non-Hodgkin lymphoma (NHL), and sarcomas [26-29]. Previous report in CRC revealed high TNF-α expression as an independent prognostic factor and increased TNF-α expression was correlated with tumor recurrence in CRC patients with metastases [17]. Furthermore, the survival rate of CRC patients with low TNF-α serum level, estimated as median survival, was significantly higher than that of patients with high levels of TNF-α (38.4 vs. 7.761 months; P = 0.00015) [13]. Another study demonstrated that patients with high TNF-α expression were significantly associated with decreased disease-free survival (P = 0.0209) and overall survival (P = 0.0163) [30]. However, present study could not find any significant correlation of TNF-α mRNA expression and survival in CRC patients which might be due to small number of patients. Hence, further studies with large cohort are required to evaluate the prognostic role of TNF-α mRNA expression as a powerful biomarker for predicting survival in CRC.

1. **CONCLUSION**

Higher TNF-α mRNA expression was correlated with high-risk prognostic factors such as larger tumor size, lymphnode involvement and advanced stage in CRC patients. It suggests association of high TNF-α expression with disease aggressiveness in CRC particularly in western Indian region. Hence, increased TNF-α mRNA expression might have a role in CRC progression and may identify high risk group of CRC patients for therapeutic management.

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