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Chapter 1. MODULATION OF CYTOKINES' EXPRESSIONS IN COLORECTAL CANCER (CRC) PATIENTS IN TERTIARY CARE HOSPITALS OF PAKISTAN

1.1. Introduction

Cancer is the undifferentiated rapid growth of cells that can invade the adjoining parts of the body through a process called "metastasis" [1]. Around 600,000 new cases are diagnosed each year throughout the world [2], and in 2020 there were 10.3 million cancer deaths and 19.3 million new cancer cases [3]. Common types of cancers are lung, breast, colorectal, prostate, skin, and stomach [4]. Colorectal cancer (CRC) deaths are estimated to be 9.2% which makes it one of the leading causes of cancer deaths [3–5]. The tumorous growth in the rectum, colon, and appendix are all included in colorectal cancer, also known as large bowel cancer. For both men and women, it is the second and third most prevalent malignant condition, respectively [6]. CRC has become more prevalent in Pakistan, particularly among people over 50 [7]. According to a meta-analysis of seven different studies completed in Pakistan, the prevalence of CRC is 5% nationwide [8]. Many environmental and genetic factors contribute to the development of CRC [9]. In between 50 and 80 percent of identified cases, environmental variables have an impact on its development and 50% of colorectal cases can be attributed to dietary choices [10]. Inactivity, smoking, obesity, and low consumption of fruits and vegetables are some of its risk factors [11, 12]. Fatty acids can promote the progression of CRC [13], and it has been discovered that there is a link between obesity and colorectal cancer [14].

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New predictive biomarkers are required for CRC given that the disease has a poor prognosis [15, 16]. Although molecular-level diagnostics for targeted therapy for colorectal cancer are receiving more attention in research, it is still challenging to incorporate this technology into routine procedures [17]. Activated immune cells release growth factors and cytokines to regulate the inflammatory conditions in tumor tissues [18]. By accelerating metastasis and tumor growth, chronic inflammation greatly contributes to malignancies [19, 20]. Cytokine networks have a major role in the pathogenesis of colorectal cancer [21]. They are expressed by tumors with either pro- or anti-tumor characteristics [22, 23]. Many alterations have been found in cytokine profile upon CRC [24]. Variety of interleukins are involved [25, 26] with roles in tumor growth [27], invasion of cancer cells, and metastasis [28]. They may also act as biomarkers to detect cancer and predict its outcomes because they are frequently seen in blood circulation [29].

When compared to healthy individuals, CRC patients had higher serum levels of interleukin-17 (IL-17A) which is significantly correlated with tumor size [30]. The high expression of genes associated to Th17 [31] suggested that patients will have a poor prognosis for survival [32]. Interleukin-22 is another important player in the development of CRC [33]. Patients with CRC who have high serum or tissue levels of IL-22 have a poor prognosis because the IL-22 reduces the effectiveness of treatment [34]. Similarly, the level of FOXP3 is much higher in CRC tissues than in normal tissues of the colon and rectum [35] playing a prime role in cancer progression [36]. Tumor necrosis factor-stimulates invasion and epithelial-mesenchymal transitions which enhance CRC metastases [37]. Compared to normal, its expression is also noticeably greater in CRC [38,39]. Stages three and four of neoplasms exhibit higher expression than do earlier stages [38]. The risk of colorectal cancer is discovered to be connected with genetic variations in interferon-gamma (IFN-γ) [39]. Polymorphisms in genes of IL-1β also elevate the risk of colon cancer development [40]. It is a proinflammatory cytokine secreted by macrophages to propagate the inflammatory response [41, 42]. Patients with advanced colon cancer usually cannot go through surgery, therefore effective biomarkers and therapeutic targets are needed for advanced colorectal cancer [43]. This study aims to discover such cytokines in the blood serum of CRC patients in comparison to regular blood.

1.2. Experimental

1.2.1. Study Design

In this study, the expression of a genes with inflammatory cytokines in colorectal cancer was investigated through qPCR expression analysis. The extracted RNA was used to synthesize complementary DNA (cDNA). In qPCR, expression profiles of selected genes were studied using cDNA as a template. The target gene was thought to involve in the progression of gastric and colorectal cancer.

1.2.2. Sample Collection

Colorectal blood samples were collected with patient's consent from Victoria hospital, Bahawalpur and Pakistan's Institute of Medical Sciences, Islamabad after ethical approval from ethical review board. RNA was extracted manually by Trizol LS method. Nanodrop (Nanodrop™ 2000 Spectrophotometer of thermo fisher) was used to quantify RNA and stored at -20°C for further processing. All participants relevant data was tabulated such as of age, gender, region and stage.

1.2.3. cDNA synthesis

cDNA synthesis is done from extracted RNA using reverse transcriptase enzyme. The first strand complementary DNA was synthesized by using kit of Thermo Fisher Scientific "RevertAid First Strand cDNA Synthesis".

1.2.4. Amplification

RNA expression of the target gene was analyzed using PCR through cDNA synthesis with set of primers. The conditions of the PCR were optimized by applying various cycles and conditions.

The following steps were performer

- Initial denaturation at 95ºC for 5 minutes
- Denaturation at 95ºC for 30 seconds
- Annealing at XºC for 30 seconds
  (According to each primer)
- Incubation of 72ºC for 45 seconds
- Final extension 10 min at 72ºC

} 35 cycles

For each primer, the annealing temperature of PCR was optimized to obtain appropriate annealing conditions.

1.2.5. Gel visualization and Gel Electrophoresis

To perform PCR amplification of the target genes, post amplification was performed on gel electrophoresis. 2% agarose gel was used for PCR products for which agarose to 70 ml of 1X TBE electrophoresis buffer was adding to prepared the gel and agarose was melted at high temperature for 2 minutes in microwave oven. Visualized the DNA under digitally photographed using Gel Doc imaging or UV light.

1.2.6. RT PCR

WizPure qPCR (SYBR) with ROX Dye is an optimized ready-to-use solution for real-time quantitative PCR assays.

1.2.7. Real time PCR Preparation

By using Step One qPCR system (Applied Biosystems), all real time reactions were carried out. The qPCR was performed to analyze the expression of genes in gastric and colorectal tumor versus normal cell. ß-actin was used as a reference or endogenous control. The qPCR master mix was prepared using the kit components in appropriate quantities. Appropriate quantity of cDNA template was added along with master mix and were run-in Real-time PCR system.

Transfer tubes into a Real-time PCR instrument and run as following:

By $2^{-\Delta\Delta CT}$ analysis method, the relative expression of *IL-22* and housekeeping gene ß-actin were calculated (Livak and Schmittgen 2001).

$$\frac{X_{Test}}{X_{Control}} = 2^{\Delta\Delta}C_T = 2(C_{T,X-}\ C_{T,R})_{control}\text{-}\ (C_{T,C-}\ C_{T,R})_{test} \quad (1)$$

where: X_{Test} = Value of expression of interest gene $\mathbf{X}_{Control}$ = Expression value of reference gene e.g., ß-actin $C_{T,X}$ = Threshold cycles of gene of interest $C_{T,R}$ = Threshold cycle of the reference gene.

The PCR reaction were run on Applied Biosystems® StepOnePlus™. The qPCR steps were as follow:

- Step 1 at 95ºC for 10 minutes
- Step 2 at 95 º C for 30 seconds
- Step 3 at 55 º C for 20 seconds
- Step 4 at 72 º C for 20 seconds

} 40 cycles

1.3. Statistical Analysis

Statistical analysis was done using OriginPro 2016 (Origin Lab, Northampton, MA). For expression analysis of data, the relative expression of the target gene *IL-22* was calculated using the endogenous control gene (ß-actin). The statistical importance was determined using either the Mann-Whitney test with a significance level of $P< 0.05$.

1.4. Results

1.4.1. Amplification of ß-actin and TNF-α

We used the Invitrogen ladder of 100 base pair (bp) to estimate the size of the product of specific gene which is 120 base pair. The bands indicate that the amplification of ß-actin (Figure 1). PCR amplification of TNF-α is done at the annealing temperature of 59°C (Figure 2).

Amplification of ß-actin

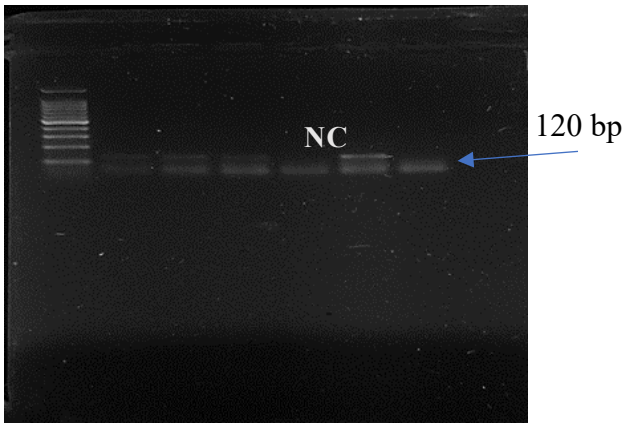

Fig. 1. Confirmatory PCR result of cDNA with ß-actin PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product was loaded in each well. As for size marker 100 bp ladder was used. NC represents the negative control for the PCR reaction. Bands were visible which indicates that amplification of gene ß-actin occurred at 120 bps size

Rys. 1. Badania PCR cDNA z ß-aktyną prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. NC oznacza próbkę kontrolną-negatywną dla reakcji PCR. Widoczne prążki wskazują, że amplifikacja genu ß-aktyny zachodziła przy 120 bp

TNF-α

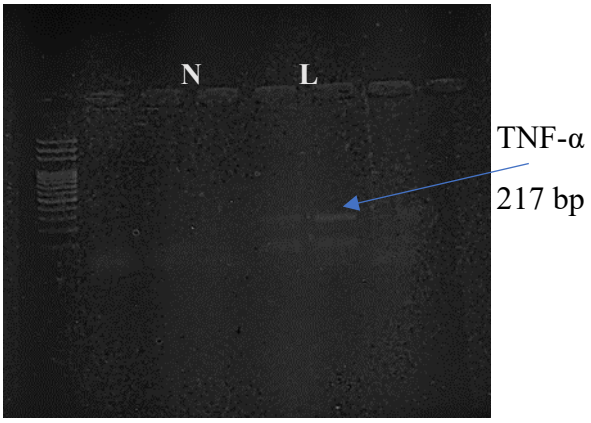

Fig. 2. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product was loaded in each well. As for size marker 100 bp ladder was used. Fig shows the amplification of gene TNF-α having 217 bps size without the non-specific bindings. L5 shows amplification while NC is showing the nagative control

Rys. 2. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. Rysunek przedstawia amplifikację genów TNF-α o wielkości 217 bps bez niespecyficznych wiązań. L5 pokazuje amplifikację, podczas gdy NC oznacza próbkę kontrolną-negatywną

1.4.2. Amplification of IL-17 and IL1-ß

The estimated size of the *IL-17* is 156 bps for comparison we used 100 bp marker. The bands indicate that the amplification of *IL-17* in Figure 3. After the primer optimization, PCR amplification was performed at a specific annealing temperature that is 60^{o}C of or both tumor and control tissue samples. The bands indicate that the amplification of IL1-ß (Figure 4).

IL-17

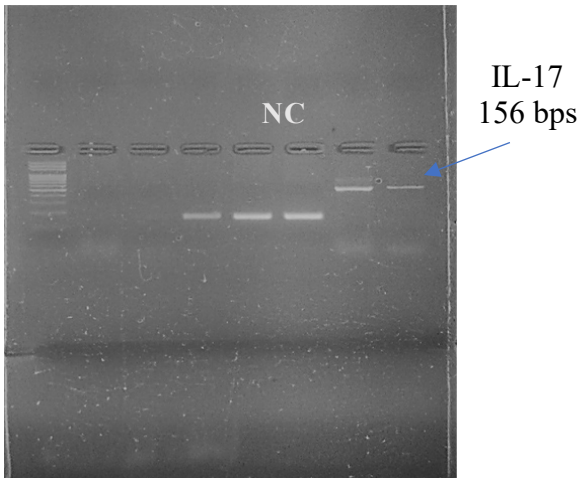

Fig. 3. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product was loaded in each well. As for size marker 100 bp ladder was used. NC represents the negative control for the PCR reaction. The amplified PCR product of IL-17 with the size 156 bps

Rys. 3. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. NC oznacza próbkę kontrolną-negatywną dla reakcji PCR. Rysunek wskazuje amplifikowany produkt PCR IL-17 o wielkości 156 pz

IL1-ß

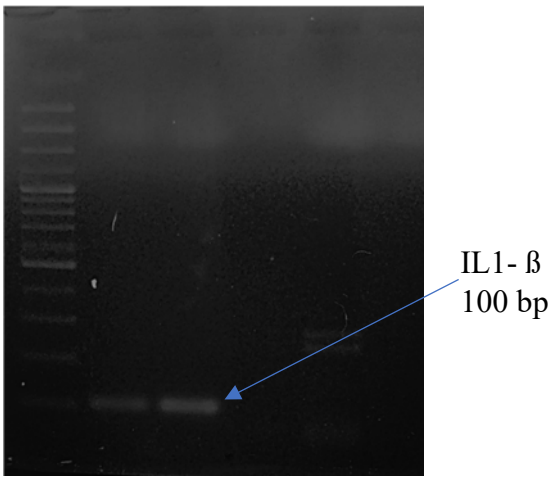

Fig. 4. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product along with loading dye 2 µl was loaded in each well. As for size marker 100 bp ladder was used. Bands were visible which indicates that amplification of target gene IL1-ß occurred at 100 bp size

Rys. 4. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR wraz z nakładającym barwnikiem 2 µl. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. Widoczne prążki wskazują, że amplifikacja docelowego genu IL1-β zachodziła przy wielkości 100 bp

1.4.3. Amplification of IFN-γ and TNF-α

PCR amplification was performed at of or both tumor and control tissue samples at the annealing temperature of 58°C (Figure 5). PCR amplification of TNF-α at 59°C, the gene shows clear bands of 217 bps size (Figure 7).

IFN-γ

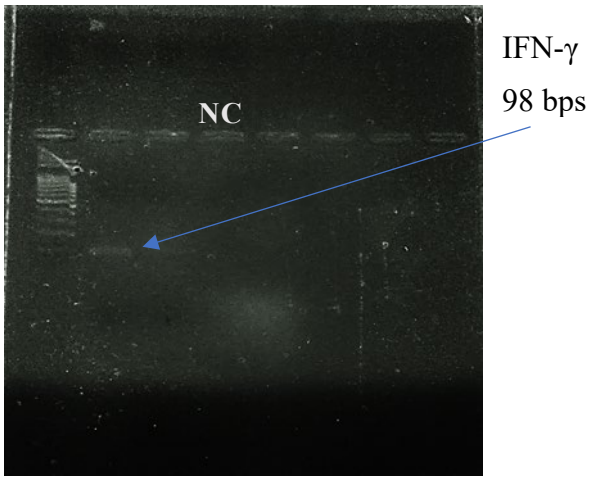

Fig. 5. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product was loaded in each well. As for size marker 100 bp ladder was used. NC represents the negative control for the PCR reaction. The amplified PCR product of IFN-γ with the size 98 bps

Rys. 5. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. NC oznacza kontrolną próbkę negatywną dla reakcji PCR. Zaobserwowano amplifikowany produkt PCR IFN-γ o wielkości 98 bps

TNF-α

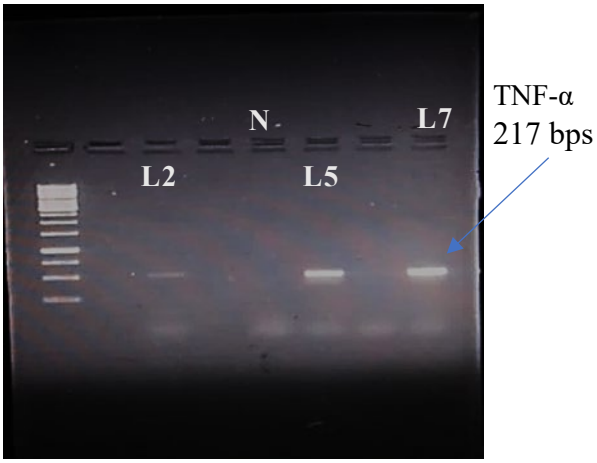

Fig. 6. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product was loaded in each well. As for size marker 100 bp ladder was used. L2,L5 and L7 show the amplified gene while NC is negative control

Rys. 6. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. L2, L5 i L7 przedstawiają zamplifikowany gen, podczas gdy NC jest kontrolą probką negatywną

1.4.4. Amplification of FOX P3

FOX P3 was amplified at 59.5°C and the desired band size (227 bps) was obtained (Figure 7). The gradient PCR carried out at different temperatures for different genes i.e. at 60°C and 59.5°C for ß-actin and FOX P3 respectively. The amplified products are shown in Figure 8.

FOX P3

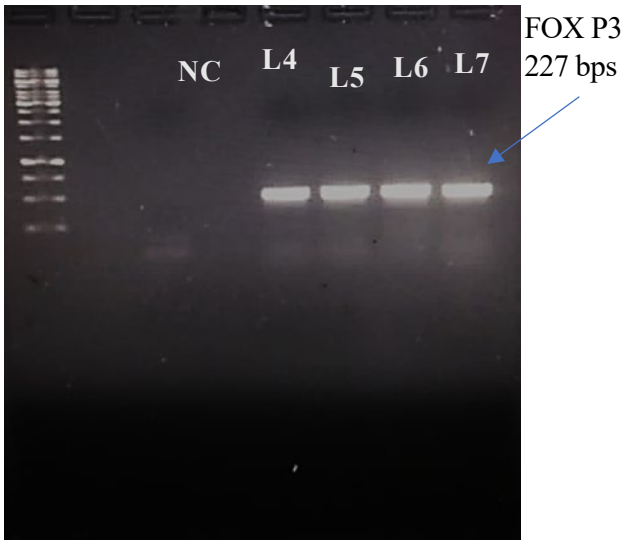

Fig. 7. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product along with loading dye 2 µl was loaded in each well. As for size marker 100 bp ladder was used. Lane 4, 5, 6, 7 show positive samples with the amplified gene while NC is negative control

Rys. 7. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR wraz z barwnikiem (2 µl). Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. Ścieżki 4, 5, 6, 7 pokazują pozytywne próbki ze zamplifikowanym genem, podczas gdy NC jest kontrolą próbką negatywną

GRADIENT PCR

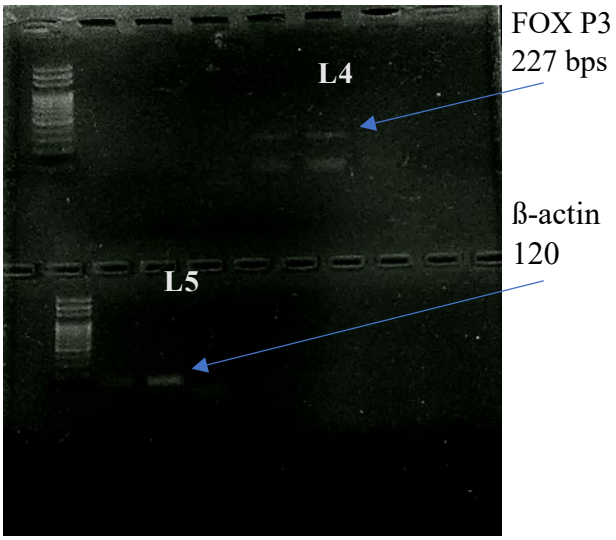

Fig. 8. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product along with loading dye 2 µl was loaded in each well. As for size marker 100 bp ladder was used. FOX P3 with 227 bps shown in L4 while NC1 is its negative control while ß-actin with 120 bps size band is shown in L5 while NC2 is its negative control

Rys. 8. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR wraz z barwnikiem (2 µl). Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. FOX P3 z 227 bps pokazano w L4, podczas gdy NC1 jest jego kontrolą próbką negatywną. ß-aktyna z prążkiem wielkości 120 bps jest pokazana w L5, podczas gdy NC2 jest jego kontrolą próbką negatywną

1.4.5. Amplification of *IL-22*

The PCR amplification occurred at 60°C, IL-22 shows its expression as shown in Figure 9.

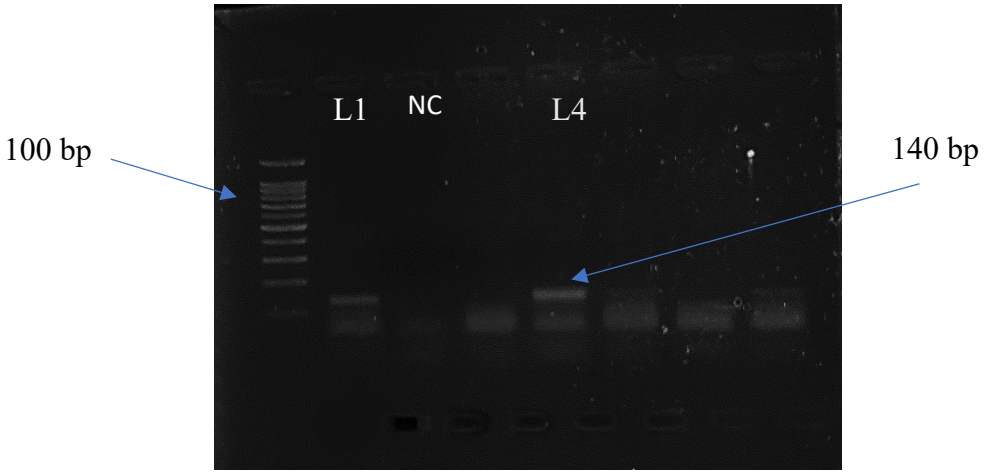

Fig. 9. PCR product was run on 2% agarose gel for 30 minutes at 90 V and 400 mA. 5 µl PCR product was loaded in each well. As for size marker 100 bp ladder was used. NC represents the negative control for the PCR reaction. L1 and L4 show 140 bps bands

Rys. 9. Reakcję PCR prowadzono na 2% żelu agarozowym przez 30 minut przy 90 V i 400 mA. Do każdej studzienki załadowano 5 µl produktu PCR. Jako marker wielkości zastosowano wzorzec 100 bp DNA Ladder. NC oznacza kontrolna próbkę negatywną dla reakcji PCR. L1 i L4 pokazują pasma 140 bps

1.4.6. Quantitative PCR

StepOne Plus™ Real Time PCR system was used. The result was determined as a ratio of relative expression level mRNA. ß-actin was used as an endogenous control. For real time assay, we used 3 technical replicates of each sample to minimize the biasness and errors that could be introduced by sample handling i.e., pipetting. Cyclic Threshold (CT) values were obtained for each sample and further normalized against the control gene (ß-actin). Relative fold changes in expression mRNA levels of *IL-22* were calculated for normal and tumor tissues.

1.4.7. Expression of *IL-22* in Colon and Gastric tumor

The expression pattern of *IL-22* mRNA, in high grade tissues of colon cancer and its normal counterparts. Our data show that relative expression levels of IL-22 gene is relatively upregulated which is significantly increase of 3-fold in mRNA levels of *IL-22* as compared to normal counterparts shown in figure 10. The significant P-value of normal and cancerous which is ≤ 0.05 are shown in Table 1 along with the standard deviation Table 2.

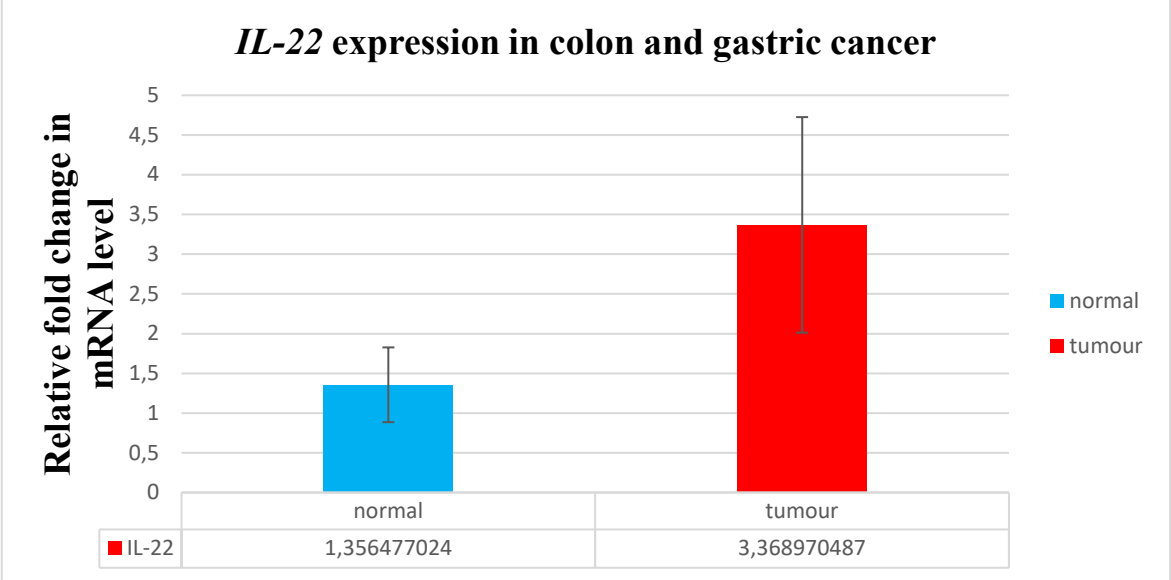

Fig. 10. Fold change expression of *IL-22* in colon and gastric cancer cases. Normal and tumor stages are indicated on x-axis and fold change mRNA expression is shown on y-axis to compare the expression of the gene with the control samples after the qPCR determination

Rys. 10. Zmiana krotności ekspresji IL-22 w przypadkach raka okrężnicy i żołądka. Etapy normalne i nowotworowe są wskazane na osi x, a krotność zmiany ekspresji mRNA jest pokazana na osi y, aby porównać ekspresję genu z próbkami kontrolnymi po określeniu qPCR

Table 1

P-value of tumor versus normal

Samples	P-Value
Tumor	0.04732836
Control	0.04732836

Table 2

Standard deviation (S.D) of tumor vs normal

Samples	Standard deviation
Tumor	1.37004959
Control	0.47046447

1.5. Discussion

The prevalence of CRC has been growing in Pakistan but, unfortunately there are no proper programs for prevention or screening [44]. Clinical techniques including colonoscopy and fecal occult blood testing have been used to screen blood, with some limitations [45–47]. Therefore, non-invasive diagnostic techniques are needed for early detection of CRC [48]. It is important to study the specific role of each cytokine in CRC development and survival as many tumorigenic cytokines are being expressed in the tissues of CRC patients [22].

In our study, statistical analysis was done using OriginPro 2016 (Origin Lab, Northampton, MA) and $P< 0.05$ was used to determine the Mann-Whitney test's significance threshold as a ratio of relative expression level mRNA. Using a Real-Time PCR technique, the relative expression of the IL-22 gene was estimated using *ß-actin* as the control gene. We observed that mRNA levels of IL-22 have increased by three times indicating the gene's relative expression levels being relatively up-regulated. Its elevated expression has already been recorded in various cancers [49] and linked to the development of colorectal/stomach cancer [50, 51]. Studies show that IL-22 is involved in the invasion of gastric cancer cells by modulating the signaling pathway of IL-22R1/AKT/MMP-9 [52]. The high expression of IL-22RA1 indicates the tumor's unfavorable prognosis [53]. An examination of IL-22 expression in human colon cancer tissue revealed that IL-22 mRNA expression was more than two times higher in the colon cancer tissue than in the adjacent normal tissues [54]. Similar findings from [55] show that it is considerably ($P<0.05$) greater than the average. An oncogene called STAT3 controls the tumorigenic potential of IL-22 by regulating the expression of several genes involved in tumor formation [56–58].

The levels of various plasma cytokines varied significantly between CRC patients and control subjects, suggesting the possibility of differently expressed plasma cytokines as potential biomarkers for detecting the existence of CRC. We found that cytokines and interleukins (IL-17, IL1-ß, TNF-ß, IFN-ß, and FOX P3) are expressed in the patient’s blood serum of the study population. These cytokines are already been reported in different populations of the world [59]. Numerous investigations have identified cytokine levels in plasma and their relationship to colorectal cancer [60].

Even with a localized tumor, interleukins are frequently identified in the bloodstream. As a result, they can serve as biomarkers to detect cancer, foretell its

effects, and manage treatment alternatives [29]. For each cytokine primer, we ran PCR amplification at a certain annealing temperature. Numerous bands of them showed their amplification in tumor samples as compared to control samples. Using a marker of 100 bp, we were able to get Interleukin 17 at a size of 156 bps. The pro-inflammatory cytokine IL-17 is up-regulated in the serum and tissues of CRC patients. Studies show that IL-17A with chemokine ligand 20 (CCL20) in serum has been found a potential diagnostic biomarker for CRC in humans [61]. Several studies have shown that IL-17 has an important role in the metastasis and prognosis of CRC [62]. It also acts as an important tumor marker in CRC patients [63] as it nurtures angiogenesis by inducting various tumorous angiogenic factors [64]. It promotes the expression and formation of other proinflammatory cytokines like IL1-ß and TNF-α [37].

Cytokine IL-17 along with TNFα collectively prompt glycolysis in CRC cells [65]. In cancer samples, we discovered amplification of the TNF-α gene with a 217-bps size. It is a crucial inflammatory cytokine in the tumor's microenvironment and promotes colon cancer invasion [66]. It also influences cancer development having an impact on CRC survival [67]. TNF-α in plasma can act as a diagnostic factor to estimate the prediction of CRC patients before other invasive tests [68]. Receptor TNF-R1 can be an optimistic biomarker of colorectal cancer playing a key role in CRC's earlier stages [69]. It regulates the gene induction of MACC1 (MET Transcriptional regulator) and displays a potential target for the treatment of CRC patients [70]. Our research revealed that the Fox P3 gene is also overexpressed at 59.5°C and has a 227-bps size. Patients with colorectal cancer have tissue with higher levels of FOXP3 than in normal [71]. Its elevated expression is related to bad prognosis in patients and is moderated by cancerous cells to progress the disease [72]. Its high level has been seen in metastasis conditions more than in patients without metastasis. It also hinders immune reaction which results in immune escape [71, 73]. Similarly, the percentage of FOXP3+Treg cells is higher in the blood of CRC patients as compared to control individuals [35].

The targeted gene of IL-1ß of 100 bp size was amplified at 58°C. Polymorphism of the IL-1β gene, accompanied by elevated levels of IL-1β has been associated with an increased risk of colon cancer development [40]. High IL-1β levels are reported in colon cancer [74, 75]. These studies have shown an association between colon IL-1β levels, the severity of IBD, and increased development and invasiveness of CRC [76]. Elevated IL-1β levels have been associated with increased colon tumor growth and invasion [77, 78]. In addition, IL-1β also inhibited the growth of xenografts *in vivo*, and may be suitable as a new therapeutic drug for CRC patients [79]. The amplified PCR product

of IFN-γ with the size 98 bps was found in our study. IFN-γ mediated by NK cells regulates IL-15 in colorectal cancer [80]. IFN-gamma leads to cell death and hinders growth [81]. The cytokine has an anti-pathogenic and immune regulatory role-playing a crucial role in organizing immune responses [82]. The control of epigenetics of the signaling pathway IFN affects tumor immunity [83].

Circulating cytokines or systemic inflammatory markers have been suggested as prognostic markers in CRC as a part of systemic inflammation [84, 85, 86]. The development and implementation of new, specific, and more sensitive biomarkers will improve diagnostic strategies soon, and allow clinicians to detect CRC cases early in the disease, thereby prognosis of thousands of patients will be improved. In this regard, more detailed research is needed on the relationship between diet, microbiota, and CRC. The upregulation of these immune cytokines can serve as an important indicator of disease progression.

1.6. Conclusion

Quantification of cytokines is still in progressing stage and there is a need to find an effective solution for the real-time identification of cytokines in vivo. In-depth study is needed to comprehend the mechanisms underlying immunological biomarkers and how they impact the onset and progression of colorectal cancer. The improvement of antitumor responses and reduction of the immune cells supporting tumor growth must be anticipated by cytokine-based cancer therapy.

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MODULATION OF CYTOKINES' EXPRESSIONS IN COLORECTAL CANCER (CRC) PATIENTS IN TERTIARY CARE HOSPITALS OF PAKISTAN

Abstract

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths, including 935000 colorectal cancer (CRC) cases in 2020 (WHO). One of the most prevalent forms of cancer, colorectal cancer is the second and third most common malignancy in men and women, respectively. Chronic inflammation, activated immune cells, and growth factors modulate the release of cytokines in tumor tissues. CRC has been linked, in particular, to alteration in the expression of interleukins including IL-17, IL-22, and IL-1ß. The objective of the current investigation was to identify the expression of cytokines implicated in CRC tumor development and progression. We discovered that the IL-22 gene's relative expression level was increased in CRC by three times and was consequently linked to the development of the tumor. The immune system releases these interleukins at different phases of CRC, and they can serve as biomarkers.

Keywords: Colorectal Cancer, Immunity, Biomarker, Interleukins, Cytokines, Therapy, Diagnostics