Elevated expression levels of COX-2, IL-8 and VEGF in colon adenocarcinoma

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ARTICLE INFO	ABSTRACT
	There is growing evidence of a connection between inflammation and tumor development and NF-κB is an important transcription factor in the inflammation pathway. Genetic approaches have proven the role of NF-κB responsive genes in tumorigenesis. The NF-κB responsive genes products such as IL-8, VEGF and COX-2 are the key components of angiogenesis. MMP-2 and MMP-9 are playing important roles in the disruption of the extracellular matrix that may contribute to the metastasis of tumor cells. This study aimed to investigate gene expression levels of COX-2, IL-8, VEGF, MMP-2 and MMP-9 in colon tumors. A total of 34 fresh colon carcinoma specimens and paired normal adjacent tissues (NAT) were collected during the surgery and RNA isolations were carried out from specimens. Synthesis of cDNA was carried out from these RNAs with oligo dT18 primers. The transcribed cDNA was used for PCR amplification reactions for the investigated genes with β-actin being the internal reference via the semi-quantitative RT-PCR method. A statistically significant difference was observed for COX-2, IL-8 and VEGF which were all upregulated in colon tumors compared with adjacent normal tissues ($p<0.05$). However, MMP-2 and MMP-9 expression levels did not change between tumor and normal tissues ($p>0.05$). Upregulated expression levels of COX-2, IL-8 and VEGF might occur in the early stages of tumorigenesis and detection of these mRNA levels may be beneficial for early diagnosis and management of colon tumors.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in developed countries. Adenocarcinomas constitute the majority of CRCs (98%) (1). Approximately 1.2 million new cases of CRC and 600.000 associated deaths are reported worldwide annually (2). The development of colorectal cancer is a multi-step process consisting of mutations in oncogenes such as K-RAS, adenomatous polyposis coli (APC) gene and tumor suppressor p53 gene. These multi-step mutations finally result in cellular degeneration and uncontrolled cell proliferation (1-3). Currently, radical surgery represents the standard therapy method and adjuvant therapy approaches such as chemotherapy and radiation therapy are mostly beneficial at the early stages. Even after radical surgery and adjuvant chemotherapy, nearly half of CRC patients relapsed and 5-year survival rate for patients with distant metastasis was reported to be as low as 19% (4-5). These data highlight the significance of early diagnosis and associated molecular markers.

Cell proliferation is pivotal in the tumorigenesis process and cyclooxygenases (COXs) are significant enzymes for this purpose. The human COX family involves three members (COX 1-3). They catalyze the conversion of free arachidonic acid into prostaglandin H2, which is the precursor of other prostaglandins and thromboxanes. These compounds play significant roles in both cell proliferation and inflammation. COX-2 expression can be affected by pro-inflammatory and mitogenic stimuli. It is involved in tumor growth and invasion (2-3).

Interleukin-8 (IL-8) has been implicated in chronic inflammatory processes and a link has been proposed between inflammation and cancer for many years (6-7). IL-8 induces angiogenesis and the chemotaxis of monocytes, neutrophils, and basophils. It can also bind to the G-protein coupled receptors CXCR2 and CXCR1 expressed on neutrophils and endothelial cells. Although IL-8 is hardly detectable in healthy tissues, it is rapidly induced as a response to pro-inflammatory cytokines (7-8).

Neoangiogenesis is the key event in tumor invasion and metastasis. VEGF belongs to the family of platelet-derived growth factors including members of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factors. The predominant factor inducing neoangiogenesis is VEGF-A which is a heparin-binding glycoprotein. It has angiogenic and mitogenic properties specific to endothelial cells (9-11). VEGF expression has been shown to be upregulated in many tumors (5, 12).

Matrix metalloproteinases (MMPs) are zinc-dependent metalloendopeptidases. They are responsible for mediating metastasis. Tumor cells employ MMPs to cleave the extracellular matrix and thus invade the nearby tissues. High expression levels of several MMPs were reported in various human tumors. In colorectal cancer, overexpression of MMP-2, MMP-7, MMP-9 and MMP-11 was repor-

ted (13-14).

Though some studies focused on the expressions of the above-mentioned genes in colon cancer, none included all of the genes as far as we know. Therefore, we aimed to determine the expressions of COX-2, IL-8, VEGF, MMP-2, and MMP-9 in primary colon tumors and revealed their possible roles as molecular indicators in the development of the malignancy.

Materials and Methods

Patient population

The present study was based on a consecutive series of patients with colon cancer. A total of 34 fresh colon carcinoma specimens and paired normal adjacent tissues (NAT) were collected in Eppendorf tubes during the surgery and snap-frozen in liquid nitrogen for preservation. The specimens were stored at -80°C until further use for RNA extraction. Inclusion criteria: Patients pathologically diagnosed with colon adenocarcinoma. Patients with other malignant tumors and patients who had received radiotherapy and chemotherapy before surgery were excluded from the study. The study was approved by the Medical Ethics Committee of Dicle University (Decision no: 57-18.03.2011) and all parts of the study were conducted in accordance by the Declaration of Helsinki.

RNA extraction and semi-quantitative RT-PCR

Total RNA was prepared from frozen tissues using Trizol and chloroform/isoamyl alcohol. RNA concentrations were quantified with a UV-VIS spectrophotometer in 10 mM Tris-HCl pH 7.5. Evaluation of gel images of 28S/18S bands enabled the analysis of the integrity of the specimens. Since only high-purity RNA specimens were selected for further analysis, some of the specimens were excluded and the study was followed by 34 fresh colon carcinoma specimens and paired normal adjacent tissues (NAT). For cDNA synthesis, RNA samples were treated with RNase-free DNase I to remove all potential genomic DNA molecules. First-strand cDNA was synthesized from 1000 ng total RNA with oligo dT18 primer (20 µl total volume) with RevertAid First Strand cDNA Synthesis Kit. The transcribed cDNA was used for PCR amplification with β-actin being the internal reference. Data were normalized by using beta-actin gene expressions. Beta-actin gene is one of the housekeeping genes whose expression should be stable and not dependent upon some other conditions. In this study, normalizations were done by using beta-actin gene expression of the same sample both for tumor tissue and normal tissue. Gene expression levels were determined via semi-quantitative RT-PCR with primer pairs specific for each gene analyzed as mentioned below:

COX-2 (NM_000963.2): F: 5'-GCTCAAACATGA-TGTTTGCATTC-3'; R: 5'-TCATAAGCGAGGGC-CAGC-3'; IL-8 (BC013615.1): F: 5'-AAGTTTTTGAA-GAGGGCTG-3'; R: 5'-ATTGTACCATGAAATATCC-3'; VEGF (NM_001204385.1): F: 5'-GCACCCATGG-CAGAAGG-3'; R: 5'-AGCTACTGCCATCCAATC-GAG-3'; MMP-2 (BC002576.2): F: 5'-TGATCTTGAC-CAGAATACCATCGA-3'; R: 5'-AACTTCTTCCCTCG-CAAGCC-3'; MMP-9 (BC006093.1): F: 5'-TGGGGGG-CAACTCGGC-3'; R: 5'-CTGGGCTTAGATCATTCC-3'; β-actin (NM_001101.3): F: 5'- CTGGAACGGTGAAG-GTGACA-3'; R: 5'- AAGGGACTTCCTGTAACAA-TGCA-3'. PCR products obtained from normal and tumor tissues were run on the same agarose gel. Analyses were made with the size and brightness of the bands with the 'Image J' program.

Statistical analysis

Because of the experimental design, nonparametric tests were used to determine the statistical outcome. Nonparametric Wilcoxon Signed Rank Test was used to determine the statistical difference with SPSS 16.0 program. $P < 0.05$ was considered as the statistically significant difference.

Results

In order to determine COX-2, IL-8, VEGF, MMP-2 and MMP-9 expression levels and β-actin expression in colon tumors, semi-quantitative RT-PCR was performed. Analyses were made by calculating the average of the obtained band densities/brightness via the 'Image J' program.

Gene expression results based on band densities are presented in Table 1. According to the statistical analysis between normal and tumor tissues, a significant difference was observed for COX-2, IL-8 and VEGF which were all upregulated in colon tumors. However, MMP-2 and MMP-9 expression levels did not change between tumor and normal tissues (p>0.05) (Figure 1).
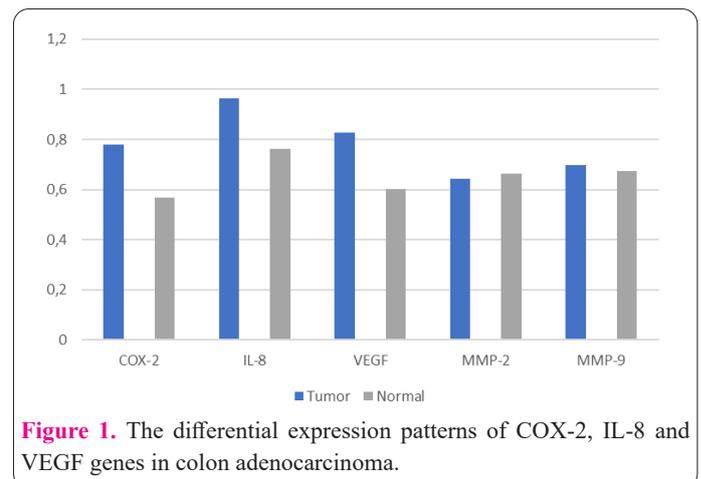

Figure 1. The differential expression patterns of COX-2, IL-8 and VEGF genes in colon adenocarcinoma.

Table 1. Gene expression results in colon tumors and adjacent normal tissues.

Gene analyzed	Mean tumor tissue value	Mean normal tissue value	p-value
COX-2	0.779	0.569	<0.05
IL-8	0.965	0.762	<0.05
VEGF	0.827	0.602	<0.05
MMP-2	0.643	0.665	>0.05
MMP-9	0.699	0.673	>0.05

Discussion

The development and progression of colon cancer are mediated via various molecular changes accumulating. An understanding of the nature of fluctuating expression levels of some significant genes in primary colon tumors can aid muchly in the early diagnosis and treatment of tumors. In this study, we aimed to evaluate the expression levels of COX-2, IL-8 and VEGF whose activations are mediated through the NF-k pathway. In addition, MMP-2 and MMP-9 expression levels were also evaluated.

COX-2 can be induced as a response to various pro-inflammatory cytokines, growth factors and tumor promoters. Overexpression of COX-2 protein has been reported in the development of colon carcinogenesis (6, 15-18). Besides protein overexpression, higher levels of COX-2 mRNA were also demonstrated in tumors compared with the normal tissues. COX-2 mRNA levels were reported to be higher in 77% of the carcinoma tissues compared to the adjacent normal mucosa (3). A recent study supported these data emphasizing higher mRNA levels of most of the interleukins including IL-8 and COX-2 in adenomas than in adjacent tissues (19). Another study reported overexpression of COX-2 both at mRNA and immunohistochemical levels (2). COX-2 also seems to be displaying changes according to the metastasis status since a study reported the positivity ratios as 70.8% in primary tumors, 92.0% in lymph node metastases and 100% in hepatic metastases (20). Together with TIMP-1 and MMP-7, COX-2 expression levels in colon cancer tissues were reported to be higher than both normal tissue and polyp tissue (1). COX-2 can have a role in tumor metastasis via epithelial-to-mesenchymal transition (EMT) (21). A recent pilot study aiming to evaluate the impact of perioperative combined COX-2 and beta-adrenergic blockade in colorectal cancer patients undergoing curative surgery revealed improvements in 5-year disease-free survival (22). In coherence with the above reports, our study also determined the upregulated expression level of COX-2 in colon tumors.

A profound link between colon cancer and chronic inflammation was established. Along with other members of the TNF superfamily, IL-8 is involved in various physiopathological processes and thus can affect tumor growth, survival and invasion. IL-8 network activation in the niche of cancer stem-like cells (CSCs) could serve as a switch from the precancerous adenoma stage to the colorectal cancer stage (23). IL-8 mRNA expression was found to be upregulated in tumor tissues compared with normal tissues. Moreover, a higher expression ratio was reported in metastatic tissues (7). This result is coherent with a previous report in which increased expression of IL-8 was found in colon tumors compared to normal colon tissue (8). A recent meta-analysis study reported that high IL-8 levels were significantly correlated with shorter overall survival and progression-free survival (24). IL-8 expression was also proposed as a biomarker for telomerase-targeted cancer therapies since expression of IL-8 mRNA and protein was inhibited in colon cancer cells upon treatment with telomerase inhibitor imetelstat (25). Increased serum level of IL-8 was reported to have potential as a non-invasive diagnosis marker of colorectal cancer (26). In line with previous IL-8 expression reports, our study demonstrated the elevated expression levels of IL-8 in colon tumors.

Vascular endothelial growth factor (VEGF)-A, also known as VEGF is an angiogenic factor. Upregulated levels of VEGF expression were reported in various human tumors. The protein expression rate of VEGF was found as 55.5% in colon tumors (12). VEGF mRNA expression levels were found significantly higher in colon cancer samples when compared with normal colonic tissue. Also, high VEGF expression (65%) was observed with the immunohistochemistry method (5). This ratio is highly close to the staining ratios previously reported in colon tumors (67.3%) (27), (73.9%) (28) and a bit lower than a recent report (84%) (29). Increased expression of VEGF was found to be correlated with increased expression of microvessel density (MVD) in primary colon tumors (11). To accelerate further research in the future, the CXC chemokine-VEGFA network would propose as a prognostic biomarker in colon adenocarcinoma (30). VEGF expression was also determined to be associated with several clinicopathological factors such as metastatic sites and status (4, 9), stage (10) and recurrence (31). Our study analyzed expression levels in primary colon tumors and evaluation of the clinicopathological findings was out of scope. Compatible with previous reports, we found a significantly higher VEGF mRNA level in colon tumors compared with adjacent normal tissues.

The invasion of cancer cells is dependent upon the degradation of the extracellular matrix and this process is mediated by various extracellular proteases. Matrix metalloproteinases (MMPs) have central significance for this purpose. Especially MMP-2 and MMP-9 have drawn considerable attention in carcinoma studies (13, 32). MMP-2: The TIMP-2 ratio was proposed as a beneficial local invasion marker in colorectal cancer (32). MMP-2 expression in stromal cells was a high-risk indicator of recurrence in stage II colon cancer (33). MMP-9 expression was increased in colorectal tumors compared to matched normal adjacent tissues and 100% of patients with MMP-9 overexpression exhibited lymph node involvement (13). MMP-9 expression positively correlated with the depth of invasion, lymph node metastasis and distant metastasis (14). Compared to normal colon mucosa tissues, high levels of MMP-9 mRNA were also reported in colon adenocarcinomas (34). In our study design consisting of primary colon tumors, a significant expression difference was not found in terms of MMP-2 and MMP-9. It seems like MMP-2 and MMP-9 are important for tumor progression and are rather involved in the metastasis process than the tumorigenesis process itself. All the same, this issue merits further investigations and we are chary of drawing any assertive conclusion.

Our study comprehensively evaluated the expression levels of COX-2, IL-8, VEGF, MMP-2 and MMP-9 in 34 colon tumors and matched normal adjacent tissues. Though our sample number seems like a minor limitation factor, we only preferred high-quality RNA samples and thus we lowered the initial specimen numbers (n=50) on purpose. One of the powerful ways of our study design is the use of paired samples of normal and cancerous tissue from the same patient which was sometimes neglected by other studies.

To sum up, increasing expression levels of COX-2, IL-8 and VEGF were found in colon tumors compared with paired normal adjacent tissues. MMP-2 and MMP-9 mRNA levels did not differ between normal and tumor specimens. Upregulated expression levels of COX-2, IL-8 and VEGF

might occur in the early stages of tumorigenesis and detection of mRNA levels of these genes may be beneficial for early diagnosis and management of colon tumors.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships.

Authors' Contribution

Conceptualization: I.H.Y. and O.U.; Methodology: O.U., Z.Y., F.T., I.H.Y.; Investigation: O.U., Z.Y., F.T., I.H.Y.; Data curation: I.H.Y. and Z.Y.; Writing—original draft: Z.Y. and O.U.; Writing—review and editing: I.H.Y. and F.T.; Project administration: I.H.Y.; Funding acquisition: I.H.Y. All authors have read and agreed to the published version of the manuscript.

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Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards of the Ethical Committee at the Dicle University Hospital (Decision no: 57-18.03.2011).

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