Expression of Endoglin and Vascular Endothelial Growth Factor as Prognostic Markers in Experimental Colorectal Cancer

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Abstract. *Background/Aim: Endoglin (CD105) is a receptor for the transforming growth factor-beta 1 (TGFβ1) with crucial role in vascular development and angiogenesis. Additionally, vascular endothelial growth factor (VEGF) overexpression has been associated with advanced stage and poor survival for several cancer types. These molecules have been shown to be useful markers for identifying proliferating endothelium involved in tumor angiogenesis, especially in patients with cancer at risk of developing metastases. The aim of this study was to evaluate the relationship between VEGF and endoglin expression in an experimental model of colorectal cancer, as well as to investigate the effect of cyclooxygenase-2 (COX2) inhibitors on tumor development incidence. Materials and Methods: Colon cancer was induced with 1,2-dimethylhydrazine dihydrochloride (DMH). Celecoxib and diclofenac treatment was started simultaneously with DMH induction. Endoglin protein expression was performed using western blot analysis. VEGF plasma concentrations were measured by enzyme-linked immunosorbent assay. Results: In histopathological evaluations, no pathological change was observed in control rats, while adenocarcinoma (62.5%), dysplasia (31.25%) and inflammation (6.25%) were detected in the group given DMH. In treatment groups, a marked decrease was observed in adenocarcinoma rate. Expression of endoglin protein was significantly elevated in the DMH group compared to controls ($p<0.001$). No statistically significant difference was noted between treatment groups and DMH group regarding endoglin expression but a decrease was detected in the celecoxib-treated groups. Conclusion: It was confirmed by histopathology and western blotting that COX2 inhibitors, particularly celecoxib, decrease the rate of disease and slow-down progression of existing CRC. These data show that endoglin expression may have an important role in tumor angiogenesis and predict of tumor invasion.*

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Colorectal cancer (CRC) is one of the most common neoplasias and a leading cause of cancer-related death in developed countries (1). Identifying effective preventive strategies aimed at inhibiting the development and progression of CRC is critical for reducing the incidence and mortality of this malignancy. Cancer development and metastasis are dependent on angiogenesis. Therefore, certain angiogenesis markers may be useful as metastasis markers or targets for anti-angiogenic therapy. Vascular endothelial growth factor (VEGF) plays a very prominent role in vasculogenesis and angiogenesis. Many of the currently available anti-angiogenic agents target the VEGF pathway (2).

The transforming growth factor-beta (TGFβ) pathway has also been implicated in proliferation, migration and invasion of various cancer types. Endoglin (CD105), a co-receptor involved in TGFβ signaling, is a proliferation-associated antigen of endothelial cells and essential for tumor angiogenesis. Angiogenesis is associated with the expression of many markers of this process, among them VEGF, endoglin, TGFβ, as well as neuropilin (NRP), the co-receptor for VEGF (3-6). Expression of endoglin in vessels is increased during different pathological conditions such as vascular injury, tumor-associated angiogenic vasculature. Endoglin represents an ideal target for anti-angiogenic therapy and is a good indicator of tumor prognosis and developing metastases (7-9).

Conventionally, the prognosis of neoplastic disease and its treatment are mainly based on exact clinical and histopathological staging. However, this prognosis could be improved by measuring molecular and cellular markers which play key roles in tumor progression. Understanding of the

cellular processes responsible for tumor dissemination can be useful not only in diagnosis and prognosis of treatment results, but also in the development of targeted drugs, selectively directed towards those factors responsible for tumor invasiveness, as well as in creating new therapeutic strategies permitting the use of such drugs. Numerous experimental, epidemiological, and clinical studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) have promise as anticancer agents. Theoretically, NSAIDs may be candidates for chemopreventive agents for the suppression of tumor progression induced by the inflammatory response by inhibiting cyclooxygenase-2 (COX2) (10). They may exert their effects by down-regulating pro-inflammatory cytokines or growth factors and transcription factors. However, the mechanism of chemoprevention by NSAIDs is still a matter of debate and is not fully understood. Celecoxib, a specific COX2 inhibitor, and diclofenac, a selective COX2 inhibitor, reduce colon adenoma development in individuals with familial adenomatous polyposis (11). In individuals with non-familial sporadic colon adenomas, celecoxib reduces the risk of developing future adenomas by 33-45%, and the risk of developing adenomas with advanced histology by 57-64% (12).

In the present study, we aimed to investigate the effect on angiogenesis of endoglin and VEGF expression in 1, 2-dimethylhydrazine dihydrochloride (DMH)-induced rat colon carcinogenesis and evaluated the implementation of COX2 inhibitors for molecular targets on tumor considering the above-mentioned mechanisms.

Materials and Methods

Animals. This study sample comprised 48 male Sprague-Dawley rats [Firat University Experimental Research Center (FUERC), Elazig, Turkey] whose weights were between 200 and 250 g. All animals were housed in wire-bottomed cages at the FUERC at 25± 3˚C, 50-60% humidity, under a 12/12-h light/dark cycle. Rats were acclimatized to the control diet (standart rat chow) and water *ad libitum* for at least 1 week. All studies were performed with the approval of the animal experimental Ethics Review Committee of Fırat University (number 11/100, 08.11.2012).

Animal groups. The rats were divided randomized into four groups: Control group (n=8): Animals were administered the vehicle (1 mM EDTA-saline) subcutaneously (*s.c.*) in weekly injection for 12 weeks and 1 ml dimethyl sulfoxide (DMSO) *per os* (*p.o.*), daily throughout the experiment (25 weeks).

DMH group (n=16): Animals were administered DMH (Sigma Chemicals, St Louis, MO, USA) *s.c.* weekly at a dose of 25 mg/kg body weight for 12 weeks and DMSO (*p.o.*, daily) for 25 weeks. DMH was freshly prepared in 1 mM EDTA-saline.

DMH plus diclofenac group (n=12): Diclofenac (Dikloron tablet; Deva Holding A.Ş, Turkey) was given orally on a daily basis (8 mg/kg body weight in DMSO, once a day for 25 weeks) to the animals along with the weekly administration of 25 mg/kg body weight of DMH for 12 weeks;

DMH plus celecoxib group (n=12): Celecoxib (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was administered on a daily basis (for rats, 6 mg/kg body weight in DMSO, once a day for 25 weeks) to the animals along with the weekly administration of 25 mg/kg body weight of DMH for 12 weeks.

Diclofenac and celecoxib were given daily by oral gavage within its therapeutic anti-inflammatory dose (ED_{50} for rats) to the animals. At the end of this study, animals were kept for overnight fasting with drinking water *ad libitum* and sacrificed the next day under ether anesthesia. The body weights of animals in all the groups were recorded once in a week until the termination of the experiment.

Histopathological analysis. The colons were removed and flushed clear with ice-cold physiological saline (NaCl solution, 9 g/l). The colons were divided into proximal, medial and distal segments for the examination. Colonic pieces were cut from the killed rats and immediately fixed in 10% buffered formalin for 24 h. The tissues were embedded in wax and 5 μm sections were cut using a hand-driven microtome. Sections were then dewaxed in xylene, stained in hematoxylin and eosin, viewed under a light microscope and photographed. Lesions were classified histologically (as inflammation, varying degrees of mild to severe dysplasia, and adenocarcinoma). The quantitative assessment of endoglin protein expression was performed on each colorectal tissue specimen by western blot analysis.

Western blot analysis. Frozen colon tissues were weighed and homogenized with RIPA lysis buffer with Cocktail protease inhibitor using a Bullet Blender tissue homogenizer (Next Advanced Inc., Averill Park, NY, USA). All procedures were carried out at 4˚C in accordance with the manufacturer's instructions. Homogenized samples were centrifuged at 10,000×g for 10 minutes to obtain the supernatant and then recentrifuged to form a clear lysate. Samples were stored at −80˚C until analysis. The amount of protein in the samples was determined with a Qubit® 2.0 fluorometer (Invitrogen™, Carlsbad, CA, USA) using a Qubit® protein assay kit. Fifty micrograms of the total protein was loaded on gels (NuPAGE 4%-12% Bis-Tris; Life Technologies Corporation, Carlsbad, CA, USA) for electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with rabbit polyclonal antibody against endoglin (rabbit IgG, Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution. The blot was incubated with a horseradish peroxidase–conjugated secondary antibody and the protein bands were visualized by chromogenic substrates using a kit (WesternBreeze Chromogenic Immunodetection Kit; Invitrogen). Endoglin protein expression levels were assessed using Image J software (http://rsb.info.nih.gov/ ij/index.html; National Institute of Mental Health, Bethesda, MD, USA). This software was used to compare the density of bands on western blots.

Measurement of plasma VEGF by enzyme-linked immunosorbent assay (ELISA). Plasma VEGF measurement (taken within 2 months) was performed according to the manufacturer guideline, using commercially available solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (Boster, Boster Biological Technology Ltd, Pleasanton, CA, USA). Samples were prepared in duplicate and results were read at 450 nm absorbance. The sensitivity and interassay coefficients of variation for VEGF as per the manufacturer's specifications was <1 pg/ml and 15.6-1,000 pg/ml, respectively.

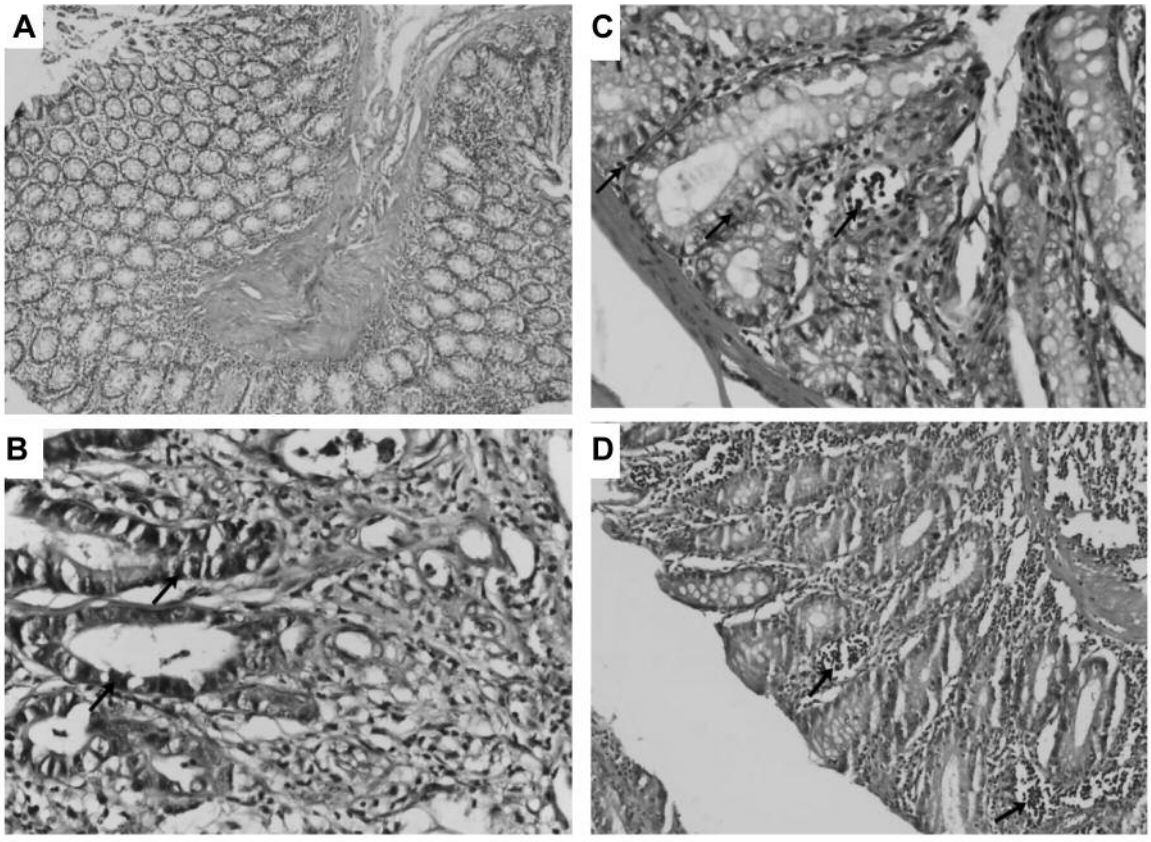

Figure 1. *Histopathological analysis of colonic tissue from different groups after treatment or not with 1,2-dimethylhydrazine dihydrochloride (DMH) for 12 weeks with or without cyclo-oxygenase-2 inhibitor for 25 weeks. Severe dysplasia and hyperplasia were observed in the DMH-treated group (B) compared to the control (A). Crypts were largely disturbed, goblet cells were lacking and the tumor was highly invasive with DMH treatment (arrows). Intact crypt architecture and large number of goblet cells are prominently visible in the sections from the control. Groups given DMH and diclofenac (C) or celecoxib (D) showed much less dysplastic changes. There was inflammation and diffuse polymorphonuclear leukocyte infiltration in colon tissues of these groups. The epithelial layer was also less disturbed in groups treated with inhibitors as compared to those treated with DMH alone (arrows).*

Statistical analyses. The data are expressed as the mean±SD of four independent observations for each group. Data were tested for normality. Pairwise comparisons of group means for parametric data were performed using either Student's *t*-test or analysis of variance (ANOVA) with post-hoc analysis (Tukey HSD and Bonferroni for groups with equal variances, as appropriate). All statistical analyzes were performed with SPSS 22.0 statistical software package (IBM, Armonk, NY, USA) for this purpose. A value of $p<0.05$ was considered significant in the present study.

Results

There were no significant differences in the body weights of the animals among four groups during the 25-week treatment schedule.

Macroscopic analysis of colon for tumor development. No tumor was found in the control group. The number of tumors was found to be lower in the groups co-administered COX2 inhibitor with respect to that treated with DMH alone. The visible size of the tumors produced was also relatively larger in the DMH-treated groups compared to the COX2 inhibitor-treated groups. The tumor burden and multiplicity were

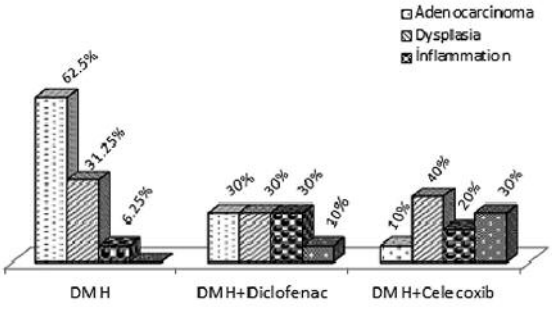

Figure 2. *Quantitative analysis of tumor formation rate after 25 weeks in all groups. The values are given as a percentage of animals with tumor.*

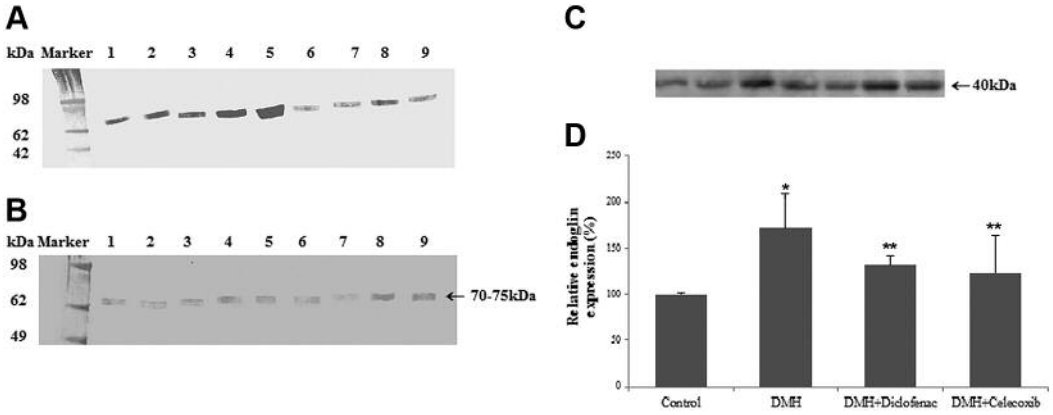

Figure 3. *Western blot results for endoglin. The analysis of endoglin cleavage showing the effect of 1, 2-dimethylhydrazine dihydrochloride (DMH) and co-administered diclofenac and celecoxib on endoglin protein expression. A: Lanes 1-3: DMH+diclofenac, lane 4 and 5: DMH, lanes 6-9: DMH+celecoxib. B: Lanes 1-7: Controls band pattern, lane 8 and 9: DMH+ celecoxib-treated group. Expression of β-actin is included as loading control (C). Marker: Molecular weights of the protein bands in the SeeBlue®Plus2Pre-Stained Standard. Western blotting was normalized to actin expression and expressed relative to the control sample. The corresponding densitometric analysis is also shown (D). Data are means±SD of three independent experiments. *p<0.001, compared with the control; **p<0.05 compared with DMH alone.*

observed to be lower among the COX2 inhibitors co-administered groups compared to the DMH alone due to the less number of tumors.

Histopathological examination. After hematoxylin and eosin staining, sections were observed under light microscopy for histological changes (Figure 1). Severe dysplasia and hyperplasia were observed in the DMH-treated group compared to the control. In the groups to which COX2 inhibitors were co-administered, tumor regression was detected, with changes reverting towards normal crypt structures and epithelial layer of the tumor.

In histopathological evaluation, no pathological change was observed in control rats, while adenocarcinoma, dysplasia and inflammation were detected in the DMH-treated group (Figure 2).

Effect of COX2 inhibitors on endoglin expression and plasma VEGF level. The expression of endoglin protein was higher in the DMH-treated group with respect to the control ($p<0.001$). Diclofenac and celecoxib administrations led to a significant decrease in expression levels of endoglin with respect to the group treated with DMH alone (Figure 3), which suggests that endoglin may essential for tumor angiogenesis.

Plasma VEGF levels were found to be statistically significantly higher in the DMH-treated group (64.19±33.33 pg/ml) compared to the control group (16.11±5.09 pg/ml) ($p<0.001$). Compared to the DMH-treated group, VEGF levels statistically significantly decreased ($p<0.001$) in groups treated with diclofenac and celecoxib (23.91±5.81 and 24.34±5.25 pg/ml, respectively).

Discussion

The growth of tumor requires angiogenesis, that is also required for invasion and metastasis. Many complex biochemical pathways can participate in tumor cell proliferation and angiogenesis. The inhibition of angiogenesis is an important strategy for cancer treatment. CRC has several characteristics to justify population-wide screening: a high prevalence, the occurrence of precancerous lesions, and the existence of treatment that is effective when applied in the early stages (13). In our study, we examined the antitumor and anti-angiogenetic efficacy of diclofenac and celexocib in DMH-induced rat colorectal cancer.

Endoglin is a TGFβ co-receptor that has been shown to regulate TGFβ signaling in various normal and cancer cells. TGFβ plays an important role in angiogenesis, by promoting proliferation and migration of endothelial cells or promoting vessel maturation (5, 14). Endoglin is expressed in angiogenic blood vessels, and is a specific marker of newly formed blood vessels. The inhibition of angiogenesis is an emerging strategy for cancer treatment.

In the present study, endoglin expression was observed to be higher in the DMH-treated group compared to the control group ($p<0.001$). Western blot analysis of endoglin also showed that NSAIDs co-administration reduced the expression of endoglin with respect to the group treated with DMH alone, which suggests that endoglin may be essential for tumor angiogenesis. This suggests that NSAIDs may have a suppressive effect on the endoglin expression by negatively regulating the proliferation and migration of endothelial cells by TGFβ signaling.

Takahashi *et al*. observed that increased serum endoglin was associated with metastasis in patients with CRC patients, the difference in endoglin levels between the metastasis-negative patients and the metastasis-positive patients was statistically significant (15). In other studies, the presence of endoglin also had a prognostic meaning, showing a positive correlation with the presence of angiolymphatic invasion, lymph node metastasis, tumor stage and hepatic metastasis, reinforcing the premise that endoglin might be considered for further therapeutic trials as anti-angiogenic therapy (16, 17). Furthermore, TGFβ is known to induce VEGF expression (18), and high-level VEGF expression is accompanied by high endoglin expression (19).

Several reports have demonstrated that malignant neoplastic cells in CRC promote angiogenesis by secreting angiogenic growth factors such as VEGF and TGFβ (20-24). VEGF may contribute to angiogenesis by stimulating endothelial cell mitogenesis and inducing microvessel permeability (25). VEGF and its receptors are expressed at high levels in metastatic human colon carcinomas and in tumor-associated endothelial cells (26). High levels of VEGF expression were associated with advanced cancer stage and related with poor prognosis (27-29). Chung *et al*. also showed that VEGF expression was significantly associated with prognosis and hematogenous spread in colorectal carcinoma (24).

Plasma levels of VEGF were significantly increased in DMH-induced rats compared to control and treatment groups in the present study. Our results showed that up-regulation of endoglin resulted in increased VEGF level; moreover, a non-statistically significant correlation between VEGF levels and rates of endoglin expression was observed in the DMH-treated group ($r=0.556$, $p=0.06$). Considering that increased endoglin and VEGF expression were associated with metastasis in patients with CRC, the assessment of VEGF and endoglin expression may be useful for predicting metastasis from CRC.

Overexpression of COX2 in colon cancer cells increases various angiogenic factors such as VEGF, the migration of endothelial cells through a collagen matrix, and the formation of capillary-like networks *in vitro* (30). Therefore, inhibition of COX-mediated prostaglandin formation can be an efficient way to decrease of VEGF and endoglin expression, and might offer a useful treatment option for CRC clinical chemoprevention (31-33). The preventive efficacy of these agents have been accepted to exert their anti-neoplastic effects by inducing apoptosis of malignant cells (34). Numerous studies have demonstrated that COX2-selective or non-selective inhibitors suppressed intestinal polyp formation and colorectal adenoma recurrence by several mechanisms. Sheng *et al*. demonstrated that the apoptotic effects of a selective COX2 inhibitor were reversed by the *in vitro* addition of prostaglandin E2 to colonic cancer cells (35). Yoshinaka *et al*. showed that celecoxib inhibited angiogenesis and VEGF expression, and induced the mitochondrial pathway of apoptosis in a murine mammary cancer model (36). In other studies, it was observed that selective COX2 inhibitors significantly reduced the risk of colorectal adenoma recurrence and the number of colon tumors if treatment was started early after exposure to the carcinogen (37-39). Our study also showed that celecoxib reduced the plasma VEGF level and endoglin expression. This may be a result of the anti-angiogenic and pro-apoptotic effects of celecoxib mentioned in the previous literature.

Diclofenac was included as it is a widely used, well tolerated drug for which there is only scanty evidence for anti-CRC activity (40). Diclofenac treatment with was concentration-dependently associated with a reduction in nuclear and cytoplasmic β-catenin immunofluorescence signal and apoptosis, with a corresponding increase in membranous β-catenin localization and decrease in apoptosis in human colorectal cancer cells (41). The earlier studies had observed that diclofenac negatively regulates angiogenesis in advanced colon cancer *via* inhibiting COX2 and other chemokines (42).

We observed a significantly higher protein expression of endoglin *via* western blot in the group treated with DMH alone with respect to the controls, whereas COX2 inhibitors helped in reducing such expression. This suggests that endoglin may be involved in cancer progression mediated by DMH or its metabolites and indicates the anti-neoplastic and anti-inflammatory effects of diclofenac and celecoxib. While higher plasma VEGF levels were observed in the group treated with DMH alone with compared to the controls, lower plasma levels of VEGF were observed in the groups treated with COX2 inhibitor.

Several studies revealed high angiogenic activity in CRC, which was likely correlated with aggressive histopathological features that included presence of goblet cell, crypt formation, parietal invasion, tumor stage, grade of tumor differentiation, metastatic potential and poor patient survival (26,43). Tanigawa *et al*. observed the inverse relationship between tumor vascularity and patient survival (44). Gurzu *et al*. showed augmented angiogenesis in CRC was higher in early-stages of tumoral proliferation but was not a progressively increasing process (43). Another study revealed that angiogenesis does not provide any significant information (45). There is no consensus among researchers due to a significant variation in patient populations, diagnostic techniques and different cut-off values.

Our study showed that VEGF and endoglin expression may be a useful marker for prognosis by correlating and expression of endoglin and levels of VEGF with DMH-induced rat colon histopathological results (endothelial dysfunction, malignant transformation and tumor invasion). Most studies report that expression of endoglin and VEGF family members are indicators of poor prognosis in patients with CRC. Beyond

these controversies, the clinical implication of tumor angiogenesis is the development of anti-angiogenic therapy, targeting tumor vasculature. However, further study is required to evaluate the combination of different markers with the aim of improving reliability and sensitivity.

Conclusion

It was confirmed by histopathological and western blotting that COX2 inhibitors particularly celecoxib, reduce the rate of CRC and slow-down progression of existing diseases. These data show that COX2 inhibitors may have significant influence on the endoglin expression and plasma VEGF levels, having an important role in tumor angiogenesis.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

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