Interleukin-17 may be a valuable serum tumor marker in patients with colorectal carcinoma


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Received Mai 29, 2009

The promotion of tumor growth is due to a combination of several mechanisms, including angiogenesis and the abundance of cell-derived inflammatory cytokines. The aim of this study was to investigate the serum levels of interleukin 17 (IL-17) and the expression of p53 and Vascular Endothelial Growth Factor (VEGF), in order to determine the relationship between these markers and serum IL-17 levels in patients with colorectal carcinoma.

Serum levels of the proinflammatory cytokine IL-17 in patients with colorectal carcinoma (CRC) \( (n=40) \) and in a healthy group \( (n=37) \) were analysed by ELISA. Surgically resected specimens of 59 colorectal carcinomas were studied by immuno-histochemical staining for VEGF and p53.

Analyses by ELISA showed significantly higher IL-17 serum levels in patients with colorectal carcinoma than in control subjects (IL-17; mean 128.52±47.62 pg/ml vs. mean 101.91±22.46 pg/ml; \( p=0.022 \)). We also found an inverse correlation between p53 expression and the level of IL-17 in the serum of patients with CRC. In fact, the serum concentration of IL-17 was significantly higher in patients who did not express p53 (\( p=0.023 \)). There was no significant correlation between the expression of p53 and VEGF. However, concomitant expression of VEGF and p53 showed a significant correlation with the histological and nuclear grade of the carcinoma.

The data presented in our study indicate that IL-17 might act as a valuable tumor marker in patients with CRC and that combined analysis of p53 and VEGF expression might provide additional information about tumor features.

Key words: colorectal carcinoma (CRC), interleukin-17 (IL-17), p53, vascular endothelial growth factor (VEGF)

Tumor growth, expansion and metastasis are promoted by the development of vascular networks (i.e., angiogenesis), which deliver essential nutrients to the growing tumor [1, 2]. Tumor angiogenesis is a critical step in the growth and metastatic spread of colorectal cancers. The survival and growth of colorectal tumors (CRCs), and thus their metastases, depend upon the balance of endogenous angiogenic and anti-angiogenic factors such that the outcome favours increased angiogenesis [3].

Vascular Endothelial Growth Factor (VEGF) is one of the most potent angiogenic factors known, and is a key molecule in orchestrating the formation and function of vascular networks. VEGF, also known as vascular permeability factor, in conjunction with the leaky state of microvessels, may cause extravasation of tissue metalloproteinase and promote cancer cell invasion into the circulation [4, 5]. VEGF is overexpressed by the vast majority of solid human cancers and has been found to correlate with a poor prognosis [2, 6–11]. In addition to its angiogenic activity, VEGF may also act as an immunosuppressant by inhibiting dendritic cell maturation [12].

Experimental results have shown that some tumor suppressor genes are involved in the regulation of angiogenesis [13]. The protein p53 has a variety of important functions in cellular integration, including cell growth control, response to DNA damage, checkpoint mechanisms during the cell cycle, regulation of transcription and control of genomic stability. The frequency of p53 gene mutations is elevated in colon, stomach, breast, and lung cancers, as well as in leukaemia, osteosarcoma, ovarian cancer, and brain tumors [14]. Interesting data have been reported on the genetic inactivation of p53 in cancer cells, showing that the loss of wild-type p53 function contributes to the activation of the angiogenic switch in tumors [15]. In human colon cancer cell lines expressing mutant p53, transient restoration of wt p53 function by adenovirus-mediated gene transfer has been found to down-regulate VEGF expression [16].
Infiltration of inflammatory cells into colorectal cancer tissue is considered to be important for tumour progression [17]. There is evidence that proinflammatory cytokines, which can be produced by various tumor cells and tumor-associated leukocytes, play an important antitumorigenic role, but have also been shown to contribute to the growth and spread of malignancy [18]. The proinflammatory cytokine interleukin-17 (IL-17) is predominantly produced by activated CD4 T-cells. Nevertheless, studies in humans have subsequently demonstrated that CD8 T-cells can also produce IL-17 [19].

In addition to its role in the proinflammatory response, IL-17 has also been associated with tumors. IL-17 has been shown to be expressed in a considerable proportion of ovarian cancers and to promote tumor angiogenesis [20]. IL-17 had no direct effect on the in vitro proliferation of vascular endothelial cells. Therefore, IL-17 promotes angiogenesis via stimulation of vascular endothelial cell migration and cord formation. These characteristics of IL-17 appear to resemble those of the class of indirect angiogenic stimulators that mediate angiogenesis in vivo but do not stimulate proliferation of vascular endothelial cells in vitro [21].

To date, there are few reports on the role of IL-17 in relation to colorectal cancer. In the present study, we have examined the serum concentration of IL-17 in patients with CRC and compared it to that of healthy control subjects. Our aim was to investigate the relationship between the expression of p53 and VEGF in colorectal carcinomas, as well as the relationship between these markers and certain histopathological parameters, such as the histological and nuclear grade of the tumor and the infiltration of leukocytes. In addition, we examined the relationship between serum levels of IL-17 and the expression of VEGF and p53 in CRC patients.

**Materials and methods**

*Patients and tissue sampling.* This study utilized tissue samples obtained from patients with colorectal carcinoma \((n=59)\) diagnosed between November 2006 and November 2007, with the consent of the ethics board of the Medical Faculty of Krugujevac. All patients were surgically treated at the Department of General Clinical Surgery Centre Krugujevac, Serbia and Hospital "Blazo Orlandić", Bar, Montenegro. The cases included 39 males and 20 females (mean age 66.61±9.94). Sporadic tumors were collected and classified according to UICC-TNM classification. Tumors were graded according to the WHO classification criteria as well, moderately or poorly differentiated. Histological diagnosis and grading were performed on H&E-stained sections.

*Serum samples.* Forty of the patients were available for serum collection and venous blood was collected before surgery. In order to establish IL-17 levels among healthy individuals, 37 control subjects were selected from volunteer blood donors at The Clinical Centre of Krugujevac. All serum samples were kept at −20°C until IL-17 levels were measured by ELISA (R&D Systems Minneapolis, MN) following the manufacturer’s instructions.

**Immunohistochemical studies of VEGF and p53.** Specimens of primary tumor masses were investigated. The tissues were routinely fixed in 4% buffered formaldehyde, dehydrated through graded alcohols, cleared in xylene, and subsequently embedded in paraffin. The paraffin-embedded tissue samples were sectioned at 4–5 μm, then deparaffinized by two 10 min washes in xylene and rehydrated in a series of 100%, 96%, 70% and 50% alcohol. Immunohistochemical staining was performed by the streptavidin–biotin method. Briefly, sections were deparaffinized and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were microwaved for 20 min in 10 mmol/L sodium citrate (pH 6.0) and incubated with mouse monoclonal antibodies against VEGF (1:200 dilution; Clone 14–124; Abcam, Cambridge, UK) and p53 (1:200 dilution; Clone 3F301; Abcam, Cambridge, UK) for 60 minutes, respectively. After the primary antibody, biotinylated secondary antibodies were applied, followed by detection using the ABC (Avidin–Biotin peroxidase Complex) method. Diaminobenzidine was used as the chromogen. Light counterstaining was performed with haematoxylin. Negative controls were obtained using an irrelevant antibody instead of the primary antibody. The slides were examined by conventional light microscopy.

The staining score was evaluated as the percentage of stained cells out of the total number of evaluated cells. Based on previous experience [22–26], staining for VEGF and p53 was defined as positive when >10% of the tumour cells were stained and negative when <10% of the tumour cells were stained.

**IL-17 ELISA.** Sera were collected from patients by a single needle stick and stored at −20°C until it was thawed for the assay. Cytokine levels were measured using the highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN) specific for the human cytokines according to the manufacturer’s instruction. Briefly, premixed standards were reconstituted in PBS (pH 7.2), generating a stock concentration of 1000 pg/ml for IL-17. The standard stocks were serially diluted in Reagent Diluent to generate 7 points for the standard curves. Diluted Capture Antibody was added to a 96-well, flat-bottomed, polystyrene microtiter plate, at final volume of 100 μl. Plates were sealed and incubated overnight at room temperature, then washed with Wash Buffer using an autowasher. Premixed standards or samples (100 μl) were added to each well containing washed beads, covered with an adhesive strip and incubated for 2 hours at room temperature. After incubation and washing, 100 μl of the premixed Detection Antibody was added to each well and the plate was covered with a new adhesive strip and incubated for 2 hours at room temperature. After incubation and washing, Streptavidin–HRP was added to each well (100 μl). The incubation was terminated after 20 min at room temperature and the plates were kept away from direct light. After washing, the beads were re-suspended in 100 μl of Substrate Solution. Then, 50 μl of Stop Solution was added to each well, and the optical density of each well was immediately determined using a microplate reader set to 450 nm. The results were expressed in pg/ml.

**Statistical analysis.** Differences between the patient subgroups were analyzed by the non-parametric Mann-Whitney
test. Since this non-parametric method does not make assumptions about normality and similar variances, both the Kolmogorov-Smirnov and Shapiro-Wilk normality tests were performed. In the case of the Chi-square test, the non-parametric alternative given by the Mann-Whitney test was used, since in some instances it may offer even greater power to reject the null hypothesis than the Chi-square test. The Kruskal-Wallis method was used to look at all the data simultaneously. A p-value of less than 0.05 was considered statistically significant. All statistical calculations were performed with SPSS version 13 software.

Results

*Increased levels of IL-17 in the serum of colorectal cancer patients* Serum levels of IL-17 were determined in patients with colorectal cancer (n=40) and in healthy controls (n=37). Data were analyzed by the non-parametric Mann-Whitney test. The cytokine concentrations were significantly higher in patients than in control subjects (IL-17; (Mean±SD) 128.52±47.62 pg/ml vs. 101.91±22.46 pg/ml; \( p=0.022 \)). The results are shown in Figure 1.

*Serum levels of IL-17 negatively correlate with the expression of p53 in human colorectal carcinoma.* Positive staining for p53 was detected in 43% (25/59) of colorectal carcinomas (Table 1). Only the specimens which presented 10% or more stained malignant nuclei were scored as positive, regardless of the staining intensity. In positively stained carcinoma cells, p53 localized to the nucleus with a granular or reticular pattern. Cytoplasmic staining was not observed. Phenotypic expression patterns of p53 are shown in Fig.5.

When patients were categorized into groups based on negative or positive p53 staining intensity p53(+) and p53(-), there was a statistically significant correlation between the serum levels of IL-17 and the immunohistochemical staining status (\( p=0.031 \)). We also found an inverse correlation between p53 expression and the level of IL-17 in the serum of patients with CRC. In fact, the concentration of IL-17 in the serum was significantly higher in patients who did not express p53 (IL-17; (Mean±SD) 140.53±49.42 pg/ml vs. 106.24±35.83 pg/ml; \( p=0.023 \)). The results are shown in Figure 2.

<table>
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<td><strong>VEGF+</strong></td>
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![Fig. 1. The serum concentrations of IL-17 in colorectal carcinoma patients and the healthy control group.](image1)

The serum concentrations of IL-17 were significantly higher in patients than in the control subjects (IL-17; mean 128.52±47.62 pg/ml vs. mean 101.91±22.46 pg/ml; \( p=0.022 \)).

![Fig. 2. The serum concentrations of IL-17 in colorectal carcinoma patients and the p53 immunohistochemical staining status.](image2)

There was a statistically significant correlation between the IL-17 serum levels and the immunohistochemical staining status (\( p=0.031 \)). The cytokine concentrations were significantly higher in patients lacking p53 expression (IL-17; mean 140.53±49.42 pg/ml vs. mean 106.24±35.83 pg/ml; \( p=0.023 \)).
Table 2. Correlation between clinico-pathologic features and the expression patterns of p53 and VEGF

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*p values were assessed by the Kruskal-Wallis method

* p value is statistically significant

Association between VEGF expression and serum level IL-17. Of the 59 colorectal carcinomas in this study, 34 tumours (59.33%) were classified as expressing VEGF (Table 1). VEGF expression was detected primarily in the cytoplasm or on the membranes of the carcinoma cells. Vascular endothelial cells, lymphatic endothelial cells and fibroblasts were also stained, but VEGF expression was not observed in these cells. Phenotypic expression patterns of VEGF are shown in Fig.5.

The percentage of VEGF positive tumour cells was used to classify tumours as VEGF(-) (≤10% positive cells) or VEGF(+) (>10% positive cells). When the serum level of IL-17 in CRC was paired with the positive VEGF staining intensity, we were unable to find any significant association between them (p>0.05; Table 2).

Combined effect of p53 and VEGF expression and clinical-pathological parameters. Our results show no correlation between VEGF and p53 expression (p=0.092), but there was a statistically significant correlation between VEGF expression and leukocyte infiltration (p=0.049; Table 2).

The patients were categorized into 4 groups based on their immunohistochemical staining status for p53 and VEGF:
p53(-)/VEGF(-), p53(+)/VEGF(+), p53(+)/VEGF(-) and p53(-)/VEGF(+). Statistically significant correlation was not observed in all groups with possible combinations p53 and VEGF expression compared with the nuclear grade ($p=0.064$), but there was significant correlation compared to histological grade ($p=0.029$). It was noticed the most significant difference of nuclear and histological grade between groups with p53(+)/VEGF(+) and p53(-)/VEGF(+) expression ($p=0.045$ for nuclear grade; $p=0.01$ for histological grade). In fact, when considered patients with positive VEGF staining intensity only, group of patients with p53 expression had higher nuclear and histological grades than group that did not express p53. The results are shown in Figure 3 and Figure 4.

**Discussion**

The promotion of tumor growth is caused by a combination of several crucial mechanisms, including angiogenesis [27] and the abundance of cell-derived inflammatory cytokines, chemokines and growth factors [28]. The identification of molecular markers that provide insight into the potential behaviour or aggressiveness of the tumor is a necessary step toward the improvement of cancer treatment. This study investigated the role of VEGF as an angiogenic marker as well as the roles of p53 and IL-17 in human colorectal carcinomas.

VEGF is involved in vascular permeability, endothelial cell migration, proliferation and vessel maturation [29–31]. Conflicting studies have been published concerning VEGF expression in colorectal cancer biology. Experimental studies have provided evidence that VEGF is an important angiogenic factor in colorectal cancer progression and metastasis formation. In colorectal cancers, VEGF is associated with tumor aggressiveness, poor survival, local failure and the presence of metastatic disease [32]. Ferroni et al. [33] observed a significant correlation between advanced stage of colorectal tumors and higher VEGF expression. Conversely, according to Zheng et al. [34], Hanrahan et al. [35] and Uner et al. [36], no significant correlation was noticed between VEGF expression and the CRC stage. This is in agreement with the data presented in this work showing no significant association between advanced stage of colorectal tumors and higher VEGF expression. Additionally, there was no significant correlation between the expression of VEGF and pathohistological parameters such as nuclear and histological grade, and lymph node metastasis, but there was a highly significant association between VEGF expression and leukocyte infiltration ($p=0.049$).

Recent studies have indicated that p53 may be involved in angiogenesis. Several clinical studies have examined the relationship between p53 and VEGF expression in human cancers *in vivo* but have reported conflicting results. The direct correlation between p53 and VEGF in colon cancer has already been reported by Takahashy, who suggested that the p53 tumor suppressor gene influenced VEGF expression [37]. Some studies have also showed that aberrant p53 expression is associated with VEGF overexpression in human solid cancers, including colon cancer [38, 39], gastric cancer [40] and NSCLC [41, 42]. Famulski W. et al. also demonstrated the positive correlation between serum levels of p53 and VEGF, and it has been suggested that this is a probable sign of a poor prognosis in colorectal cancer [43]. In contrast to these results, we were unable to find a significant correlation between p53 and VEGF expression in patients with CRC in this study. Similar results were observed by other studies which found no correlation between mutated p53 and VEGF expression in brain cancer.

![Fig. 3. The correlation of nuclear grade with possible combinations of p53 and VEGF expression in CRC patients.](image)

There was no statistically significant correlation between the nuclear grade and these groups ($p=0.064$).

![Fig. 4. The correlation of histological grade with possible combinations of p53 and VEGF expression in CRC patients.](image)

There was a statistically significant correlation between the histological grade and these groups ($p=0.029$).
Fig. 5. Localization of VEGF and p53 protein in paraffin sections of the colon cancer.

A positive VEGF stain in colon cancer cells shows strong signal intensity in the cytoplasm or on the membrane of all tumour cells (A)
A negative VEGF stain (B)
A positive p53 stain shows strong nuclear staining in the tumour cells (C)
A negative p53 stain (D) (400x magnification)

[44], oral squamous cell cancer [45], and NSCLC [46]. The overexpression of p53 detected by immunohistochemistry is based on the accumulation of the protein in cells. However, not all aberrant mutations of p53 cause the protein to accumulate, which may lead to false negative results [47]. By correlating the results for the studied markers with the morphology of the cells, we concluded that p53 overexpression was associated with the nuclear and histological grades of the colorectal carcinomas ($p<0.05$).

Examining the co-expression of multiple markers could be used to better understand tumor behaviour and tumor aggressiveness [48]. We investigated the combined effect of p53 and VEGF expression and clinicopathological parameters. Patients were categorized into 4 groups based on their levels of p53 and VEGF expression, as determined by their immunohistochemical staining status: p53(-)/VEGF(-), p53(+)/VEGF(+), p53(+)/VEGF(-) and p53(-)/VEGF(+). We did not find a statistically significant association between the nuclear grade and these groups ($p=0.064$), but there was a significant correlation between the histological grade and these groups ($p=0.029$). However, we noticed the most significant difference of nuclear and histological grade between groups with
p53(+)/VEGF(+) and p53(-)/VEGF(+) expression (p=0.045 for nuclear grade; p=0.01 for histological grade). In fact, when considered patients with positive VEGF staining intensity only, group of patients with p53 expression had higher nuclear and histological grades than group that did not express p53. According to our results, concomitant expression of VEGF and p53 showed a significant correlation with pathohistological features, such as the histological and nuclear grades, suggesting that the expression of both of these markers could be used as a prognostic factor in CRCs.

There is evidence that proinflammatory cytokines play an important antitumorigenic role. Nevertheless, it has also been shown that these cytokines contribute to the growth and spread of malignancy [17]. Secretion of the proinflammatory cytokine IL-17 is limited in T lymphocytes. Recently, it has been reported that IL-17F, a member of the IL-17 family, is secreted by monocyte/macrophage lineages [49]. In contrast, the IL-17 receptor is widely distributed among various cell types [50, 51]. There is increasing evidence that IL-17 is a potent mediator of inflammatory responses in various tissues. For example, IL-17 induces several genes associated with inflammation, including IL-6, granulocyte/macrophage-colony stimulating factor, leukaemia inhibitory factor, and intercellular adhesion molecule-1 [52]. In addition, IL-17 enhances the proinflammatory responses induced by IL-1β and tumor necrosis factor α (TNF-α) [53–55]. Moreover, IL-17 not only serves as a marker of the inflammatory process, it has also been implicated in the tumours development. In the current study, an ELISA was used to determine the serum level of IL-17 in CRC compared to the healthy control group. Many studies demonstrated that IL-17 serum value from the healthy controls was not higher than 40 pg/ml [56, 57, 58]. We found higher serum level of IL-17 in healthy controls (around 100 pg/ml) in comparison to those studies. But, nevertheless, we reported that the level of IL-17 in serum from patients with CRC was higher than in the healthy control group (128.52 vs. 101.91), as shown in figure 1 (p=0.022). One possible explanation for this result is that the highly significant difference between mean IL-17 serum concentrations of tumor patients and controls reflects an increased production of this cytokine in the tumor and its entry into systemic circulation. These circumstances may imply a role for IL-17 in colorectal carcinogenesis. Elevation of proinflammatory cytokines, such as IL-1β and IL-6, is considered to be associated with colorectal cancer [59, 60]. In an in vitro assay, IL-17 induced secretion of IL-1β from macrophages and has been shown to stimulate epithelial and fibroblastic cells to secrete IL-6 [52, 61]. Recently, IL-17 has been demonstrated to increase the production of the active metalloproteinase MMP-9 [62]. The role of metalloproteinases in the progression of human malignancies has been well documented in numerous reports [63, 64]. Likewise, some authors have reported new aspects of the biological role of IL-17 family cytokines, as well as indicators of leukocyte activity in IL-17A and IL-17E-dependent reactions in patients with oral epithelial squamous cell carcinoma [65].

Recent evidence suggests that IL-17 belongs to a class of indirect angiogenic factors which stimulate angiogenesis in vivo but have no mitogenic activity for endothelial cells in vitro. IL-17 plays a crucial role in T-cell mediated angiogenesis [66] and it has recently been suggested that IL-17 may promote angiogenesis by enhancing VEGF [67]. Moreover, IL-17 has been shown to be expressed in a considerable proportion of ovarian cancers and its expression significantly correlates with increased vascularity [68]. In contrast to the results published by Alexandrakis et al. [57], who found increasing serum levels of IL-17 in multiple myeloma (MM) patients, as well as a correlation of IL-17 with the angiogenic factor VEGF, we failed to find any significant associations between them in this study. On the other hand, other studies have reported that the serum level of IL-17 is not elevated in patients with colorectal cancer [69], acute myeloid leukaemia [58], or breast cancer [70].

Despite the clear increase in IL-17 serum levels in CRC patients found in this study, we also noticed the statistically significant association between p53 expression and the IL-17 serum levels in patients with CRC. When patients were categorized into groups based on negative and positive p53 staining intensity there was a statistically significant association between the IL-17 serum level and the immunohistochemical staining status (p=0.031). We found a significant inverse correlation between IL-17 serum levels and p53 expression (p=0.023). The cytokine concentrations were significantly higher in patients who were not expressing p53. One possible explanation for this result is that IL-17 increased the levels of proinflammatory cytokines such as IL-17 and IL-6. Expression of IL-6 and DNMT-1 (DNA maintenance methylation enzyme), which inversely correlate with p53 expression levels, suggests that the ability of IL-6 to induce methylation of the p53 promoter through up-regulation of DNMT-1 is a common mechanism of p53 inactivation in multiple myeloma cells [71]. Clearly, the loss of p53 expression is a critical event in these cells. The combined action of IL-6 to mediate both promoter methylation and escape from apoptosis [72] provides an attractive model as to how chronic inflammation could lead to cancer. This data suggests that the inflammatory cytokine IL-6 is crucial for the establishment and maintenance of p53 promoter methylation, providing a model that demonstrates how mediators of inflammation contribute to epigenetic silencing of tumor suppressor genes and tumor cell survival [73].

Until today, there has been little information available about IL-17 in cancer, especially in human colorectal carcinomas. Our results suggest that IL-17 plays an important role in colorectal carcinomas and that further studies are necessary to clarify the role of IL-17 in colorectal carcinogenesis and its clinical relevance. We therefore propose that IL-17 may be a putative target for the development of novel treatment concepts which could be used in the therapy of colorectal carcinomas.

Acknowledgments. This work was supported by grants from the Ministry of Science and Technological Development (project...
D/F 145065) Belgrade, and from the Medical Faculty, University of Kragujevac (project JP 2/06) Serbia. We thank Dragana Markovic for excellent technical assistance.

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