Evaluation of Expression Pattern of Vascular Endothelial Growth Factor (VEGF) and Interleukin-23 (IL-23) Genes in Human Colorectal Tumors

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Abstract

Background: There is a very long interval between the time of tumor initiation and diagnosis of CRCs. So, by early screening by molecular markers, individuals can be diagnosed in a much more convenient time to start the therapies. Among key factors deregulated in advanced CRC, are VEGF (vascular endothelial growth factor) and interleukin-23, which have critical roles in tumor angiogenesis and growth respectively.

Objectives: The main purpose of the study was to elucidate the patterns of VEGF-A and IL-23 (IL-23p19) molecular expression in CRC.

Material and methods: 47 human colorectal cancer tissues and 47 matched tumor-free margin tissues were gathered during surgery. The mRNA expression levels of VEGF-A and IL-23p19 were examined by real-time quantitative polymers chain reaction (qPCR).

Results: The mRNA expression levels of VEGF-A and IL-23p19 were higher in tumor tissues than in the tumor-free margin (control) (p=0.008 and p=0.002, respectively). Though, there was no important association between the mRNA expression levels of VEGF-A and IL-23p19 with clinicopathological features. There was also a positive correlation between these 2 genes expression levels.

Conclusion: The analysis of IL23p19 and VEGF-A genes expression could be considered as a biomarker for screening CRC patients and are suggested to be used for diagnosis. Thus, collectively, the high expression level of VEGF-A and IL-23p19 can be considered as a risk factor for CRC carcinogenesis, contributing to high proliferation and invasive properties of the tumor.

Keywords: Colorectal cancer; Interleukin-23 (IL-23); Vascular endothelial growth factor (VEGF); Quantitative real-time PCR (qRT-PCR)

Introduction

Colorectal cancer (CRC), colon cancer, or rectal cancer that, also known as bowel cancer is any malignancy that affects the colon and the rectum. In colorectal cancer, tumor progression is a complex phenomenon consisting many different steps from the beginning of tumor generation by genetic changes to development and metastasis. In primary steps, genetic variations are involved in tumoral over-proliferation and subsistence, while tumor development and metastasis depend on various signaling pathways for the tumor cells to acquire invasive properties and eventually colonize secondary organs [1-11].

Growing body of evidence suggests a critical role for Vascular endothelial growth factor (VEGF) as the key mediator of angiogenesis (the formation of new blood vessels) in different kinds of carcinogenesis, as well as chronic inflammatory diseases like IBD, asthma and psoriasis [12-14].

Among potent inflammatory components, Interleukin-23 (IL-23) and IL-17, stimulated by tumors lactic acid secretion [15]. Interleukin-23 is an important heterodimer molecule which is formed by linkage of IL-23-specific subunit, IL-23p19 (IL-23a), and a subunit shared with IL-12 (IL-12p40) [16]. IL-23 is one of the IL-12 (interleukin-12) superfamily’s member [17]. It has been confirmed that, separately from its immunity roles (like IL-12), IL-23 promotes T helper 17 (Th17) cells maturation and consequently the secretion of IL-17, therefore leading to the development of chronic inflammation and tumor growth [17-19]. Although the mechanisms are not absolutely obvious, the activity of regulatory T (Tregs) cells and Th17 rises by IL-23, and according to this procedure, the activity of STAT3 (Signal Transducer and Activator of Transcription 3) is affected within cancerous cells and leads to tumor progression [20,21].

It has been previously suggested that the role of chronic inflammation in tumor development is through the induction of genetic alterations [22]. However, recent findings provide evidence of the straight effect of inflammation on cancer development [23].

The aim of this study was to examine the expression level of IL-
23p19 and VEGF-A at mRNA level among individuals with colorectal cancer. Moreover, we performed the study to conclude whether these 2 genes might be helpful in differentiating the limit of the margin with the tumor of CRC.

Material and Methods

Study population

Tumor samples and matched tumor-free margin were gathered from 47 CRC patients that had undergone resection of the tumor by surgeries in Imam Reza hospital of Tabriz, Iran. All of the patients have given written consent and the protocol of the study was approved and accepted by the Ethics Committee. The age of the patients ranged from 23 to 76 years (mean age: 55.4 years), and the samples consisted of 27 males and 20 females. The subjects were chosen based on clinicopathological factors (Age, Gender, Tumor size, Histology, Depth of tumor, metastasis, Venous invasion, AJCC stage, classification, and Liver metastasis) that was gained over patient interviews and clinical documents.

Sample preparation

Confirmed histopathologically carcinoma areas used to take tumor samples, and mucosa species were obtained from unaffected area at the furthest distance from tumor place (>2 cm from tumor). The tissues were gathered instantly after resection and placed directly into RNAlater (Qiagen, Germany), then stored at -80°C. Total RNA was extracted by using RNeasyTM Mini kit (Qiagen, Germany) as suggested by the manufacturer. The quantity and quality of the RNA samples was examined by Nanodrop and gel electrophoresis, Respectively. In the Next step cDNA library was made using RT-PCR. cDNA samples were synthesized in 20 μl volume using a random hexamer primer and reverse transcriptase.

Reverse Transcriptase PCR (RT-PCR)

PCR primers were designed and blasted using GeneRunner software and NCBI blast (Table 1). A specific RT-PCR was done for GAPDH, VEGF-A, and IL-23p19 genes with all samples. For each sample, total reaction volume was 15 μl, counting 7.5 μl red master mix (Ampliqon, Denmark), 0.3 μl from each primer (forward and reverse), 2 μl cDNA, and 4.9 μl water. Then, samples were located in a thermal cycler (Eppendorf, Germany) with the following program: 4 minute at 95°C, then followed 35 cycles at 94°C for 25 second, 56°C for 25 second for VEGF-A/57°C for 25 second for IL-23p19/58°C for 25 second for GAPDH, 72°C for 25 second, and finally ended with 1 cycle for terminal extension at 72°C for 4 minute. For internal housekeeping gene, GAPDH was used. PCR products were run on a 2% agarose gel for terminal extension at 72°C for 4 minute. For internal housekeeping gene, GAPDH was used. PCR products were run on a 2% agarose gel for 3 second for GAPDH, 72°C for 25 second, and finally ended with 1 cycle for 3 minute at 95°C, then followed 35 cycles at 94°C for 30 second, 56°C for 30 second, 72°C for 30 second, and finally ended with 1 cycle for terminal extension at 72°C for 60 minute.

Expression levels of VEGF-A and IL-23p19 in tumor samples (T- tumor; M- margin).

Figure 1: Comparison of the expression level of VEGF-A in tumor and marginal samples (T- tumor; M- margin).
level in tumor tissues was upper than those in tumor-free counterparts (p ≤ 0.002). A comparison of differences in the expression levels between the IL-23p19 gene in the tumor and the tumor-free adjacent mucosa is demonstrated for each patient in Figure 3. The value of ΔCt (mean ± SD) was 5.06 ±1.49 in tumoral tissues and 5.99 ± 1.25 in their matching tumor-free tissues. The variance of IL-23p19 expression was high in more individuals with CRC, but in some of them the expression levels were similar (Figure 4). Also, no association between the mRNA expression of IL-23p19 gene and clinicopathological aspects was detected (Table 3). According to the results of the Pearson correlation coefficient, a correlation between VEGF-A and IL-23p19 gene expression was detected (r=0.44; p ≤ 0.002). This state means that a rise in mRNA level of VEGF-A in each individual was concurrent with a rise in IL-23p19 mRNA level.

**Potential of VEGF-A and IL-23p19 to be CRC tumor markers**

After constructing the ROC (Receiver Operating Characteristic) curves, AUC (the area under the curve) was considered to assess the capability of VEGF-A and IL-23p19 mRNA expression levels and specificity and sensitivity of them to separate CRC from tumor-free tissue. According to the ROC curve investigation, ROC area (AUC) were 0.67, p ≤ 0.004 for both VEGF-A and IL-23p19 mRNAs (Figure 5). Sensitivity and specificity is presented at different cut-off points in the plot. A post-test from pre-test probability of 0.5 and cost ratio of

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### Table 2: VEGF-A gene expression and clinicopathological factors in 47 CRC patients.

| Clinicopathologic Variables | Number | VEGF-A, ΔCt | P value |
|-----------------------------|--------|-------------|---------|
| **Age**                     |        |             |         |
| <55                         | 20     | 4.82 ± 1.50 | 0.503   |
| >55                         | 20     | 5.52 ± 3.18 |         |
| **Gender**                  |        |             |         |
| Male                        | 27     | 5.18 ± 2.73 | 0.868   |
| Female                      | 20     | 4.93 ± 1.80 |         |
| **Tumor size (cm)**         |        |             |         |
| <3                          | 3      | 5.09 ± 1.91 | 0.714   |
| ≥3                          | 37     | 5.18 ± 2.54 |         |
| **Histology**               |        |             |         |
| Well                        | 23     | 4.72 ± 1.58 | 0.752   |
| moderate                    | 13     | 5.74 ± 3.65 |         |
| poor                        | 4      | 5.89 ± 2.13 |         |
| **Depth**                   |        |             |         |
| T2, T3                      | 12     | 4.55 ± 1.10 | 0.796   |
| T4                          | 28     | 5.43 ± 2.85 |         |
| **Lymph node metastasis**   |        |             |         |
| Absent                      | 16     | 4.79 ± 1.75 | 0.705   |
| Present                     | 24     | 5.42 ± 2.87 |         |
| **Venous invasion**         |        |             |         |
| Absent                      | 11     | 4.66 ± .74  | 0.405   |
| Present                     | 29     | 5.36 ± 2.87 |         |
| **AJCC stage classification** |     |             | 0.823   |
| I, II                       | 14     | 4.82 ± 1.88 |         |
| III, IV                     | 26     | 5.36 ± 2.76 |         |
| **Liver metastasis**        |        |             |         |
| Absent                      | 35     | 5.16 ± 2.63 | 0.416   |
| Present                     | 5      | 5.25 ± 1.08 |         |

Note: Data presented as mean ± SD; P-values obtained using T-Test and One-Way ANOVA test.

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### Table 3: IL-23p19 gene expression and clinicopathological factors in 47 CRC patients.

| Clinicopathologic Variables | Number | IL-23p19, ΔCt | P value |
|-----------------------------|--------|---------------|---------|
| **Age**                     |        |               |         |
| <55                         | 20     | 5.19 ± 1.45   | 0.808   |
| >55                         | 20     | 4.93 ± .33    |         |
| **Gender**                  |        |               |         |
| Male                        | 27     | 5.06 ± 1.56   | 0.617   |
| Female                      | 20     | 5.05 ± 1.43   |         |
| **Tumor size (cm)**         |        |               | 0.817   |
| <3                          | 3      | 4.98 ± 2.42   |         |
| ≥3                          | 37     | 5.07 ± 1.41   |         |
| **Histology**               |        |               | 0.359   |
| Well                        | 23     | 5.21 ± 1.32   |         |
| moderate                    | 13     | 4.83 ± 1.75   |         |
| poor                        | 4      | 4.96 ± 1.55   |         |
| **Depth**                   |        |               |         |
| T2, T3                      | 12     | 4.90 ± 1.58   | 0.540   |
| T4                          | 28     | 5.13 ± 1.43   |         |
| **Lymph node metastasis**   |        |               |         |
| Absent                      | 16     | 5.26 ± 1.47   | 0.371   |
| Present                     | 24     | 4.92 ± 1.47   |         |
| **Venous invasion**         |        |               | 0.550   |
| Absent                      | 11     | 5.16 ± 1.41   |         |
| Present                     | 29     | 5.02 ± 1.50   |         |
| **AJCC stage classification** |     |               | 0.107   |
| I, II                       | 14     | 5.47 ± 1.42   |         |
| III, IV                     | 26     | 4.84 ± 1.46   |         |
| **Liver metastasis**        |        |               | 0.996   |
| Absent                      | 35     | 5.09 ± 1.49   |         |
| Present                     | 5      | 4.85 ± 1.33   |         |

Note: Data presented as mean ± SD; P-values obtained using T-Test and One-Way ANOVA test.

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Table 2: VEGF-A gene expression and clinicopathological factors in 47 CRC patients.

Figure 2: Expression level of VEGF-A in marginal samples (T-tumor; M-margin).

Figure 3: Comparison of the expression level of IL-23p19 in tumor and marginal samples (T-tumor; M-margin).

Figure 4: Expression level of IL-23p19 in marginal samples (T-tumor; M-margin).

Figure 5: ROC curve of VEGF-A and IL-23p19 on CRC patients.
sensitivity and specificity, respectively. p19, <6.01 with 0.79 and 0.49 specificity, respectively and for the optimum cut-off point was <5.60 with 0.74 and 0.64 sensitivity and specificity, respectively. 1.00 was prepared to determine the optimal cut-off value. For VEGF-A, the optimum cut-off point was <5.60 with 0.74 and 0.64 sensitivity and specificity, respectively and for IL-23p19, <6.01 with 0.79 and 0.49 sensitivity and specificity, respectively.

Discussion

In the course of inflammation, the abundantly found angiogenic and inflammatory components may contribute to progression of the disease [24,25]. This process especially observed in IBD patients with possibility for CRC and suggests a critical connection between chronic inflammation and tumor growth [1,26].

The main goal of this study was to measure the mRNA expression levels of VEGF-A and IL-23p19 genes and assess their ability in differentiating the limit of the margin with the tumor of CRC. We accomplished a real-time PCR to quantify the relative expression of VEGF-A and IL-23p19 genes in total of 47 CRC patients. Our outcomes confirmed that the levels of VEGF-A and IL-23p19 mRNA expression increased considerably in cancer tumor tissues comparing to adjacent normal tissues. Also, we were unable to discover an important link between clinicopathological information and the mRNA expression levels of both genes. Colorectal carcinoid tumors have classified by the American Joint Council on Cancer (AJCC) [27] and the clinical characteristics of them (consist of depth, size, age, histology, gender, and metastasis of liver, venous and lymph node) have assessed in this investigation.

Although in colorectal cancer, the function of interleukin-23 is not absolutely obvious, its decisive function in inflammatory bowel diseases is pointed in several previous investigations [28-30]. Some of papers informed that IL-23 has anti-tumor action in murine tumor models [31-33]. Whereas in a study by Langowski et al. was shown has anti-tumor action in murine tumor diseases is pointed in several previous investigations [28-30]. Some environmental variants such as facility of test performing, effect on the CEA sensitivity and specificity [44-46]. In early stages of colon cancer, the sensitivity of CEA is low, and it rises with rising tumor stages [47]. In stage I and II of the disease, for a CEA >2.5 ng/ML, the sensitivity and specificity were 36% and 87% respectively. Nevertheless, the amount of them are 74% and 83%, respectively in stage III and IV of the CRC [48]. Due to the weakness of the test for detecting the colorectal cancer in early stages, it is dedicated that the test is inappropriate for early tumor screening [49].

In current investigations for colorectal cancer screening, microarray analysis has been suggested [50-52]. But because of some complications for using this technique in routine diagnosis in laboratory, using molecular tests such as this study, could be helpful. Development of new clinical protocols, in which inhibitors of angiogenesis are used alongside conventional therapies such as chemotherapy/radiotherapy and also immunotherapy, are suggested. These inhibitors could be beneficial to be used in early stages to prevent early invasion and dissemination of tumor cells, as well as late stages to maintain the effect of therapy and prevent tumor recurrence [53].

Conclusion

In conclusion, this investigation indicated that VEGF-A and IL-23p19 are meaningfully upregulated in CRC tumoral tissues, suggesting that their high expression is elaborated in colorectal carcinogenesis. There is a very long interval between the time of tumor initiation and diagnosis of CRCs [54]. Therefore, by early screening taking advantage of newly developed molecular markers, individuals can be diagnosed in a much more convenient time to start the therapies that can eventually be more effective. VEGF-A and IL-23p19 are suggested to be such molecular markers with high sensitivity and specificity, to be used for the diagnosis of CRC. The high expression level of VEGF-A and IL 23p19 can be considered as a risk factor for CRC carcinogenesis, contributing to high proliferation and invasive properties of the tumor. Finally, deeper functional understanding of the role of VEGF-A and IL-23p19 seems to be a promising path to the early diagnosis and eventually more effective therapy of CRC.

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