Vascular Endothelial Growth Factor serum level as a diagnostic and prognostic marker for colorectal carcinoma

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Abstract: Colorectal cancer is a major cause of cancer death and the main line of treatment is still radical surgery but this necessitates a very early diagnosis. Serum markers currently used in clinical practice for colorectal cancer are carcinoemberyonic antigen (CEA) and carbohydrate antigen (CA19-9). Both are used during surveillance and as prognostic measures for disease-free survival but not for diagnostic or screening purposes because of insufficient sensitivity and specificity. Therefore there is an urgent need for a serum marker that can help in early diagnosis. Angiogenesis plays a key role in tumor growth and progression and its targeting has been shown to be an effective anti-tumour measure. Vascular endothelial growth factor (VEGF) is one of the major factors that stimulate angiogenesis and its serum concentration could be a prognostic marker in solid tumors and has been described in a large variety of human malignancies. The aim of this work was to investigate the expression of Vascular Endothelial Growth Factor (VEGF) in colorectal cancer (CRC) patients; and correlate the findings with the patients' clinicopathologic features. Serum level of VEGF was determined in 68 patients and in 10 healthy controls and was compared to the levels of CEA and CA19-9. The results showed that serum level of VEGF were elevated in CRC patients and was significantly correlated with the levels of CEA and CA19-9. In conclusion VEGF serum level determination can be a good diagnostic marker for CRC and also open the way for VEGF inhibitors to be used as targeted therapy in CRC patients.

[Medhat Asem, Abbas AT, Mohamed Al-Hemaly, Asem Shalaby, Mohamed Sami. **Vascular Endothelial Growth Factor serum level as a diagnostic and prognostic marker for colorectal carcinoma.** *Life Sci J* 2013;10(3):1975-1981] (ISSN:1097-8135). http://www.lifesciencesite.com. 292

Keywords: Rectal carcinoma; VEGF; CEA; CA19-9

1. Introduction

Colorectal cancer (CRC) is one of the major causes of cancer death worldwide, accounting for more than 150,000 new cases and 55,000 deaths in the United States every year and 125,000 mortalities each year in Europe[1]. Worldwide, approximately one million new cases of colorectal cancer (CRC) are diagnosed each year, with nearly 500,000 deaths attributed to this disease annually[2].

The main line of therapy for patients with localized disease is radical surgery, followed by adjuvant chemotherapy[3]. However, a significant proportion of patients present get recurrence and patients with the same tumor stages may show different outcomes, indicating that the conventional staging procedures may be unable to precisely predict Cancer prognosis[4]. Therefore, it is necessary to search for new prognostic factors capable of identifying high-risk patients and of modulating therapeutic options[5].

For colorectal carcinoma (CRC), serum markers in current clinical use are carcinoemberyonic antigen (CEA) and carbohydrate antigen (CA19-9). Both markers are widely used during surveillance and as prognostic measures for disease-free survival but not for diagnostic or screening purposes because of insufficient sensitivity and specificity [6-8]

Angiogenesis, a physiological process involving the growth of new blood vessels from pre-existing vessels, plays a key role in tumor growth and progression. Targeting of tumor angiogenesis has been shown to be an effective approach to suppress tumor growth and metastasis [9,10]

Tumor development is possible due to formation of new blood vessels. Vascular endothelial growth factor (VEGF) is one of the major factors that stimulate angiogenesis and its concentration could be a prognostic marker in solid tumors [11,12]. VEGF expression has been described in a large variety of human malignancies e.g. lung, breast, colon.[13-15] Some studies suggested that expression of VEGF correlates with poor prognosis and metastasis [15-17] so, VEGF has been demonstrated to be a major contributor to angiogenesis [18,19]. Thereby, Serum VEGF levels are elevated in colorectal cancer patients[20].

The most common presented symptoms and signs of CRC (44% of cases) are rectal bleeding, persistent

change in bowel habit and anemia without other gastrointestinal symptoms. The second common presented symptom is abdominal pain (40% of cases). This pain can be caused by a partial obstruction, peritoneal dissemination, or intestinal perforation leading to generalized peritonitis.[21] on the hand, it was added that some symptoms do not become apparent until the cancer is far advanced. Approximately 55% of patient present with advanced colorectal cancer (spread to the lymph nodes, metastasized to other organs, or is so locally invasive that surgery to remove the primary tumor alone is unlikely to be sufficient for cure).

Survival rates in individuals with colorectal cancer have increased substantially in the past few years, possibly as a result of early diagnosis and improved treatment. Although substantial information about risk factors exists, about 75% of diagnosis is in patients with no apparent risk factors other than the old age. [22]

Proangiogenic factors include VEGFs, fibroblast growth factors, platelet-derived growth factors, insulin-like growth factor, and transforming growth factors; antiangiogenic factors include thrombospondin-1, angiostatin, and endostatin. Physiologic angiogenesis is only observed transiently, during embryogenesis, wound healing, and reproductive functions in adults.[23] Normal and pathologic angiogenic processes differ in the tightly regulated balance of proangiogenic and antiangiogenic signals.

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. Tumors angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This singling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels.[24] The aim of this study was to investigate the expression of Vascular Endothelial Growth Factor (VEGF) in colorectal cancer (CRC) patients; and correlate the findings with the patients' clinicopathologic features.

2. Material and Methods

This study was carried on 68 histologically confirmed for colorectal carcinoma patients underwent elective surgery at Gastroenterology center, Mansoura University: 39 males and 29 females with ages ranged from 24-74 years. Ten healthy individuals (6 males and 4 females) of matched age and sex were used as a control group.

Patients were subjected to clinical evaluation through history taking, clinical examination, endoscopic evaluation, radiological evaluation and laboratory investigation that included liver function tests (serum albumin, serum bilirubin, ALT and AST, and serum creatinine), complete blood picture and serum levels for CEA and CA19.9. All patients were confirmed to have CRC by histopathological examination with grading and staging according to Dukes' staging system for colorectal cancer.

Blood samples for patients and controls:

1-2 mls venous blood were withdrawn from each subject by aseptic venipuncture from an antecubital vein and were left to clot in plain polypropylene tube at 25 C for 30 minutes, then the separated serum was used for the assay before centrifugation for 15 minutes approximately 1000 × g. serum were removed and samples were stored at ≤ -20 ^{0}C for the following:

Quantitative determination of Human VEGF concentrations by enzyme linked immune sorbent assay.

Principles of the assay

The kit assay human VEGF level in the sample, use purified Human VEGF antibody to coat microtiter plate wells, make solid-phase-antibody, then added VEGF to wells, Combined antibody which with enzyme labeled goat anti-human become antibody-antigen-enzyme-antibody complex, after washing completely, Add substrate, substrate become blue color At HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of VEGF in the samples is then determined by comparing the optical density (O.D) of the samples to the standard curve.

Assay procedure:

Set 10 Standard wells on the microtiter plate coated, started with 100 µl in the first and the second well with serial dilution in every two consecutive wells of the rest 8 wells (density: 900pg/ml, 600pg/ml, 300pg/ml, 150pg/ml, 75pg/ml). Set blank sample wells separately (blank comparison wells don't add Sample and Enzyme Conjugate. Add Sample dilution 40µl to sample well, then add Sample 10µl (sample final dilution is 5-fold), add sample to wells, don't touch the well wall as far as possible, and gently mix. Incubate: After closing plate with Closure plate membrane, incubate for 30 mins at 37˚C. Prepare solution: 20-fold wash solution diluted 20-fold with distilled water and reserve. Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, fill each well completely with wash solution, then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure four more times for a total five washes. After final wash, invert plate, and blot dry by hitting plate onto absorbent paper or paper towels until no moisture appears.(note: Hold the sides of the plate

frame firmly when washing the plate to assure that all strips remain securely in frame. Add enzyme: Add 50µl Enzyme Conjugate reagent to each well, except blank well. Incubate and wash. color reaction was induced by the addition of premixed TMB substrate solution

, cover and incubate for 15 mins at 37˚C. Stop the reaction by adding 50µl Stop solution to each well. Mix well. Determine the optical density of each well within 15 mins by a microplate reader set to 450 nm.

Calculations:

The standard density as the horizontal, the O.D value for the vertical, the standard curve was drawn on grave paper, It find out the corresponding density according to the sample O.D value by the sample curve, multiplied by dilution factor, calculate the sample density, multiplied by the dilution factor, the result is the sample actual density. A standard curve were constructed by plotting the mean optical density for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The best fit line were determined by regression analysis.

Statistical analysis:

Data entry and analysis were performed using SPSS statistical package version 10 (SPSS, Inc., Chicago, IL, USA). The quantitative data were presented as a mean and standard deviation, and the qualitative data were presented as number and percentage. The chi-square (X2) was used to find the association between row and column variables of qualitative data.

The threshold of significance is fixed at 5% level (*P* value). *P* value of > 0.05 indicates non-significant results; *P* value of < 0.05 indicates a significant results while, *P* value of < 0.005 indicates a high significance result.

3. Results

This study included 68 patients with colorectal carcinoma, 39 males and 29 females, male to female ratio 1.34/1 (57% and 43%). The age ranges from (74 –24) years with median age 53 ± 12.26 years. The clinical analysis of the cases included in the study revealed that the mostly affected site is rectum (n=33) followed by distal colon (n=24) and lastly the proximal colon (n=11). Most of the patients presented with bleeding per rectum (82%) and disturbed bowl habit (70%). The analysis of VEGF, CA 19-9 and CEA in relation to age and gender revealed no significant difference. The pathologic examination of the tumors resected from the patients revealed 49 (72.1%) classic adenocarcinoma, 18 mucoid adenocarcinoma (26.5%) and 1 squamous cell carcinoma. According to the pathological grade of differentiation, grade I (12, 17.6%), grade II (47, 69%) and grade III (8, 11.8%). The lymph node status of the resected specimens revealed 19 (28%) cases with secondary nodal metastasis. The correlation between the serum levels of CEA, CA19.9 and VEGF revealed significant positive correlation in the studied cases as shown in figure 1. Correlation between the lymph node status and the levels of CEA, CA19-9 and VEGF revealed no significant correlation as shown in figure 2. There was no significant correlation between the type of carcinoma and the levels of CEA, CA19-9 and VEGF as shown in figure 3. The correlation between the pathologic grade of carcinoma and the levels of CEA, CA19-9 and VEGF revealed no significant correlation as shown in figure 4.

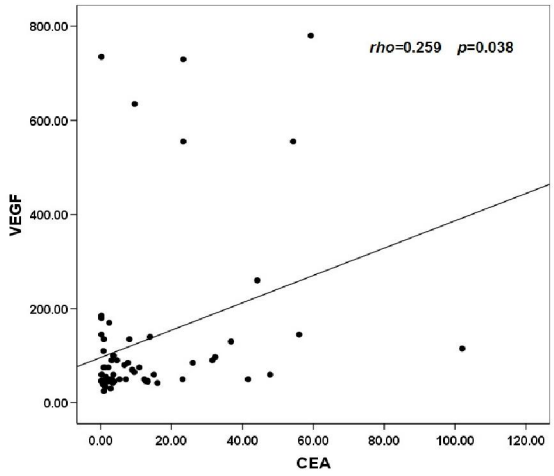

Figure 1: Positive correlation between VEGF serum level and serum level of CEA (p<0.05).

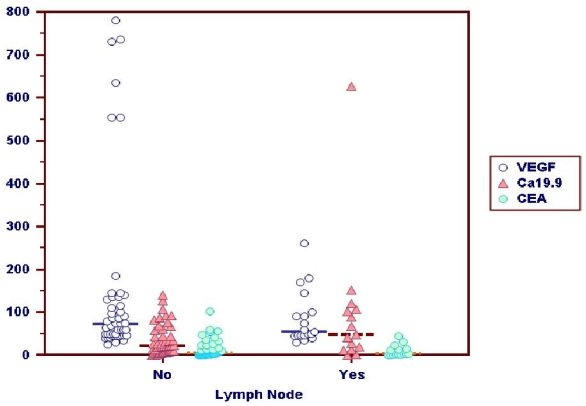

Figure 2: correlation between VEGF, CA 19-9 and CEA serum levels and the lymph node status.

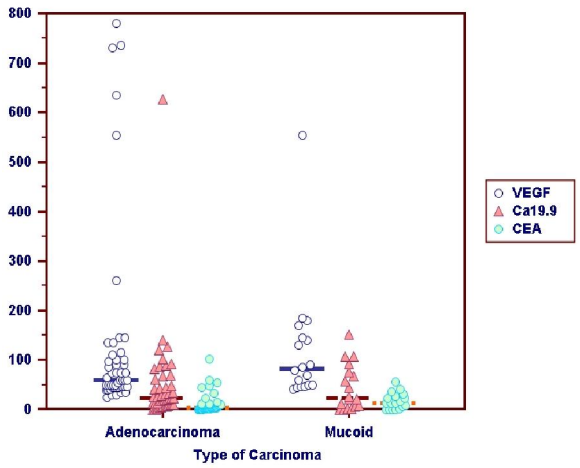

Figure 3: correlation between VEGF, CA 19-9 and CEA serum levels and the pathologic type of carcinoma.

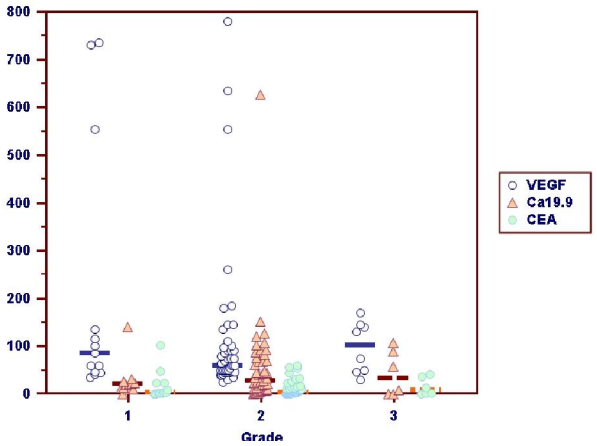

Figure 4: correlation between VEGF, CA 19-9 and CEA serum levels and the pathologic grade of carcinoma.

4. Discussion

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death in the Western World. The cause of CRC is likely to be multi-factorial, and associations have been reported with family history, diet and alcohol. Failure to define a primary cause of CRC prevents the primary prevention programs, thus, attention has therefore focused on screening programs [25]. Angiogenesis is essential for tumor growth and metastasis. Cancer is a multistep process resulting in aggressive growth potential [26]. An elevated level of a tumor marker is a non specific indicator of cancer; however, it can be useful in the follow up of treated patients [27].

The aim of this study was to investigate the expression of Vascular Endothelial Growth Factor (VEGF) in colorectal cancer (CRC) patients; and correlate the findings with the patients' clinicopathologic features.

We found that range of age of the studied patients was from 24 to 74 years and the mean age was 50 ± 12.26 years. This result coincided with Khafagy *et al.* [28] who found that the median age of the Egyptian patients with colorectal cancer was 45 years with range 17 to 78 years; on the other hand, It was founded that the mean age of CRC patients was 59.5 ± 12.6 years.[29] Another large study (607 patients) revealed that the mean age for colorectal cancer patients was 43.1 ± 9.3 years.[30]

The present study showed slight increase in incidence of CRC in males than females. This was in agreement with studies which stated that CRC is more common in males. [31,32]. On the other hand, there was a study stated that CRC is more common in females.[32] Controversy between results could be explained by large number in cases in Jover (754 patients) and Aljebreen (113 patients) studies.

Moderately differentiated tumor (grade II) represents the commonest histopathologic type (69.1%) found in this study. This was in accordance with Gryfe *et al.* [30] who found moderately differentiated tumor in about 69%. Also, there was a study reported the same which stated that histopathology of CRC showed that 56% of tumors were moderately differentiated.[32]. In the present study, Adenocarcinoma was the commonest histopathologic type (72.1%). This result coincided with Weitz *et al.* [34] who found that adenocarcinoma represents (85%) and Fenoglio [35] who found that adenocarcinoma represents (90%) of all studied patients. In this study, most of cases presented at stage C. This results in accordance with Abou-zeid *et al.* [36] incidence who found that Dukes' C represent 58% among CRC patients. This result also was matching with the studies that stated that 68% of CRC lesions were stage C. [32]

Serum CEA levels can be determined accurately and reproducibly, and for this reason, this marker was believed to have a potential as a serological screening tool for early detection. [37]. The present study showed that serum CEA increases with Dukes' stages and grades. These results were in agreement with studies that stated that the level of CEA increased with stages and grades of differentiation of tumor. [37-39] and there were significant high CEA levels in CRC patients with L.N metastasis which matched with the studies that reported that high preoperative serum CEA levels, and was significantly correlated with the depth of tumor invasion, and the status of lymph nodes metastasis.[37,39]

On the other hand, CA19.9 cannot be recommended for early diagnosis of CRC. As regards sensitivity, although elevated levels of CA19.9 have been reported in as many as 75% of patients with

advanced CRC, most studies suggested that it is a less sensitive marker than CEA for CRC.[40] There was a significant increase in serum CA19.9 level in CRC patients. This result was matched with studies that report that both CA19.9 level and sensitivity increased with increasing Dukes' stage of disease. [41] and also, with that stated that serum CA19.9 level is increased with stage of tumor. [39]

This study demonstrated a highly significant expression of VEGF in peripheral blood of CRC patient stages versus controls. This result is in agreement with studies that reported that serum VEGF levels in patients with colorectal cancer were higher than in control subjects.[42] Serum VEGF showed highly significant elevated levels in CRC patient groups. This result indicates that VEGF expression is increased with advancing Dukes' stages in CRC patients, which is coincided with the studies that reported that VEGF expression was significantly positively correlated with Dukes' stages.[43] VEGF expression increased with the progression of colorectal carcinogenesis classified by Dukes' stages and patients with the highest VEGF expression had a significantly poorer prognosis with earlier recurrence and death than those with intermediate or low expression levels. [44] This result was in consistent with another study which stated that VEGF expression at the deepest site of tumor invasion can be a useful predictor of poor prognosis in advanced CRC and show a close relation to angiogenesis.[45]. The high VEGF expression, which was correlated with Dukes' stages and the presence of distant metastasis could be explained by high molecular alterations that occur in advanced colon cancer. Moreover, this result was in agreement with studies that stated that VEGF plays an important role in progression, invasion and spread of colorectal cancer by influencing the proliferation and migration of endothelial cells and there have been attempts to inhibit VEGF synthesis in patients with advanced colorectal cancer with anti-VEGF therapy. [46]

VEGF was significantly positively correlated with CEA levels and this result was in agreement with studies that performed that quantitative evaluation of VEGF and CEA content on protein extracts obtained from tissue biopsies from 69 CRC patients and found that VEGF was significantly correlated with CEA content of either tumor tissue or corresponding normal mucosa.[47] the diagnostic sensitivity of VEGF for colorectal carcinoma was higher than the sensitivity of CEA, and in combination both markers the sensitivity to predict colorectal carcinoma was higher than each marker alone. [48]

It was found a significant positive correlation between the preoperative levels of VEGF and CEA and stated that VEGF was a better predictive factor for the response of cancer patients to chemotherapy. [49]. Serum VEGF and CA19.9 in CRC patients showed a highly significant correlation. This result was in a agreement with the studies that assessed plasma VEGF in patients with CRC before surgery and found an increase in VEGF with a strong positive correlation with metastatic spread and serum CA19.9.[46]

Also, there was positive correlation between CEA and CA19.9 levels in studied patients and this was in agreement with the studies which stated that there was a significant positive correlation between the plasma CEA, and CA19.9 levels and the CRC stage (Dukes' classification).[50,51]. It was postulated that at time of diagnosis of CRC 50.6% of the patients had elevated serum of CEA and 29.6% of CA19.9. In combination of both antigens this elevation was in 54.3% of CRC patients. The common use of CEA and CA19.9 was efficacious in identification of patients at high risk. The combination assay of CEA and CA19.9 did not cause a significant increase of sensitivity in diagnosis CRC.[25]

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9/2/2013