VEGF in situ mRNA expression along with different histopathological parameters of colorectal adenocarcinoma

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الخلاصة

تقدم السرطان هو عمليه معقده، متعدده الخطوات؛ هنالك خطوتين مهمتين في نمو وأنتشار السرطان وهما: عمليه تحطيم المكونات البروتينيه الخارج خلويه والأخرى تكوين أوعيه دمويه جديده، وذلك لتجهيز الورم بالدم أن عامل نمو ألبطانه الوعائيه Vascular endothelial growth factor (VEGF) هو المنظم الرئيسي لتكوين أوعيه دمويه جديده في الحالات الفسلجيه والمرضيه ، وهو أيضا مسوؤل عن زيادة نفاذية تلك الأوعيه، لذلك يعتبر عامل مهم لحث تكوين الأوعيه الدمويه.أن هدف هذه ألدراسه هو لتحديد، هل أن VEGF له دور في قدرة سرطان القولون على ألانتشار.من أجل بلوغ هذه ألاهداف ٣٥ عينه نسيجيه مطموره بالشمع نظرت من مرضى سرطان القولون و المستقيم في العراق، بالاضافه الى حافه النسيج السرطاني المأخوذه من نفس المريض. وقد تم الحصول على العينات من مستشفى الكبد والجهاز الهظمي / بغداد.كان هناك فرق معنوي في التعبير الداخلي للحامض الرايبي الرسول للـ VEGF عندما تمت المقارنه ما بين النسيج السرطاني مع ما يقابله من حافه النسيج السرطاني ($p<0.001$).

عندما قسمت العينات السرطانيه بالاعتماد على التغيرات النسيجيه المرضيه، وجد بصوره عامه ان التعبير الداخلي للحامض الرايبي الرسول للـVEGF، له علاقه ايجابيه معنويه مع تقدم مرحلة الورم ($r_S=0.585$، $p<0.01$). من جانب اخر عندما تتم المقارنه مع انتشار الورم للعقد اللمفاويه نجد ان التعبير الداخلي للحامض الرايبي الرسول للـVEGF يملك علاقه ايجابيه معنويه ($r_S = 0.474$، $p<0.01$)، كانت إستنتاجاتنا من هذه الدراسة بأن التعبير الداخلي للحامض الرايبي الرسول لـ VEGF ظهر على إنه يتعلق مع زيادة قدرة السرطان على تكوين أوعية جديدة كمتطلب اساسي لانتشار الورم.

Abstract

Cancer progression is a complex multi-step process. Two critical steps in tumor growth and invasion: are the proteolytic processing of the extracellular matrix environment and the angiogenic switch enabling blood supply into the tumor.

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VEGF is a major regulator of both physiological and pathological neovascularization thus, considered as an important factor for the initiation of angiogenesis. The aims of the current study were to determine whether VEGF mRNA in-situ expression, has any significant correlation with various histopathological parameters during colorectal adenocarcinoma progression and its correlation with metastatic potency. Accordingly, 35 colorectal adenocarcinoma paraffin embedded sections prepared from Iraqi patients, in addition to their respective resection margins were retrospectively collected from (liver and gastrointestinal hospital) /Baghdad.

Based on in –situ hybridization staining, mRNA expression of VEGF demonstrated a significant increase when its level at the tumor sites versus resection margins were analyzed ($p<0.001$). moreover, when these 35 paraffin embedded sections were broken down according to their various histopathplogical variables, the current study reveals a significant up regulation of VEGF m RNA in situ expression with respect to tumor stage ($r_S = 0.585$, $p<0.01$), as well as VEGF mRNA in situ expression showed a significant positive correlation with respect to lymph node involvements ($r_S = 0.474$, $p<0.01$). In conclusion, over expression of VEGF was seem to be associated with increased invasive and metastatic potential of colorectal adenocarcinoma because it was seem to increase angiogenic potential of colon carcinoma . Thus it could be used as target for therapeutic management of patients with primary colorectal adenocarcinoma.

Introduction

Colorectal adenocarcinomas are malignant neoplasms of epithelial cells origin affecting the large bowel. They are common types of malignancies that affect gastrointestinal tract and they are of favorable prognosis provided that they are diagnosed and treated in early stage [(1)].

The development of colorectal adenomas has been directly related to the consumption of meat and animal fat, and to low physical activity. In contrast, vegetables, fruit, fiber and several micronutrients (such as calcium, folate and antioxidant vitamins) seem to have protective effect [2, 3]. Other risk factors are age, prior colorectal cancer, ulcerative colitis and Crohn disease, hereditary polyposis and colorectal cancer syndromes, and genetic factor [4, 5].

Role of Vascular Endothelial Growth Factor in tumor Angiogenesis and metastasis

VEGF plays a central role in all the processes of angiogenesis described above. It regulates vascular endothelial cell proliferation, migration, invasion, and permeability, and also functions as an anti-apoptotic factor for endothelial cells in newly formed vessels [6, 7]. The importance of VEGF-induced signaling has been demonstrated by genetic and pharmacological inactivation of its receptors which leads to a complete lack of blood vessel development in the embryo, and dramatically impairs the growth of cancer cells in vivo [8,9]. At the moment there are six known members of the VEGF family: VEGF-A, placental growth factors, (PlGF-1 and PlGF-2), VEGF-B, VEGF-C, VEGF-D, and VEGF-E [9]. These glycoproteins belong to a structural super family of growth factors that includes platelet-derived growth factor-BB, (PDGF-BB) and transforming growth factor beta 1 (TGFβ1) [10, 11], VEGF expressed by tumor cells, macrophages, T cells, smooth muscle cells, kidney cells, keratinocytes, astrocytes and osteoblasts [9], and it has mitogenic activity in vascular endothelium of arteries, veins and lymphatic, but not to an appreciable extent in other cell types [12]. Malignant transformation of cultured cells often results in an induction of VEGF expression [13].

The most important mechanism regulating VEGF gene expression is hypoxia, Hypoxia-induced transcription of VEGF mRNA which is mediated by hypoxia-inducible factor-1 (HIF-1) binding to an element in the VEGF gene and enhanced stability of VEGF mRNA [14]. Other factors shown to up-regulate VEGF mRNA expression include cytokines such as epidermal growth factors (EGFs), PDGF-BB, TGF1, keratinocytes growth factor (KGF), interleukin-1 beta (IL-1β) and oncogenes [12]. In addition to its role in the paracrine stimulation of angiogenesis, VEGF may also have an autocrine stimulatory effect on tumor cells [15], the biological effects of VEGF are mediated by three receptor tyrosine kinases (RTKs), VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4) The expression of these receptors is largely restricted to the vascular endothelium [12,16].

Materials and Methods

Patients and Sampling:

Thirty five patients (20 male and 15 females) with colorectal adenocarcinoma, who were confirmed histopathologicaly, were included in this study. Their age were ranged from 25- 80 years.

Paraffin embedded blocks of tumor and resection margins were retrieved along with the histopathological report of each patient from histopathological laboratory. For staging of the cancer, both astler-coller and TNM staging systems were adopted in this study [17, 18]. In addition, resection margins were confirmed again to be free of malignancy. adequate thin paraffin embedded sections (5µm thick) of tumor and resection margins were prepared on positively charged slides for insitu hybridization Techniques.

In situ Hybridization for detection VEGF.

Principle

In situ hybridization makes use of the high specificity of complementary nucleic acid binding to identify infectious agents in tissue sections, to localize gene expression within individual cells [19], or to detect specific DNA sequences in the genome of cells [20]. Briefly, the method involves deproteinization of fixed tissue sections mounted on slides, hybridization of the target nucleic acid sequences with a DNA or RNA probe, and detection of the hybridized probe to permit microscopic examination. The most widely used non-radioactive technique entails labeling the probe with biotin. The hybridized probe is then detected by addition of enzyme-conjugated streptavidin followed by a suitable enzyme substrate, which produces a colored end product visible, by light microscopy [21].

Materials

1- DNA Probe: Biotinylated long DNA probe for human VEGF, alltypes, Cat. No.: IH-60038.

- Size: 294 bp. This biotin-conjugated probe was produced by PCR primers (Maxim's product Catalog # SP-10659) under PCR conditions using human cDNA and biotin-dUTPs & biotind ATPs.
- Purity: The biotin-conjugated probe was purified by cartridge and showed a single band on gel.
- Sequences: (Alignment on database: Genbank, AB021221, AF2114570)

2- The DNA Probe hybridization/Detection System – In Situ Kit (Maximbio): consist of the following:

(a) Proteinase K (4 mg).
(b) DNase and RNase free Dilution Buffer.
(c) Hybridization Solution: negative Control.
(d) Biotinylated Housekeeping Gene (GAPDH) Probe.
(e) Protein Block.
(f) Rnase A (15 ug/ml).
(g) Streptavidin-AP Conjugate.

(h) Substrate.
(i) Detergent Wash Buffer.

Procedure

1. Serial tissue sections were cut 4-6 micron thick and floated in protein free water bath.
2. The sections were positioned on positively charged slides carefully.
3. Then, the slides were baked in a vertical position at 80°C overnight.
4. The tissue sections were deparaffinized by standard methods. The slides were immersed sequentially in the following solutions at room temperature for the indicated times. Xylene 5 min, 100% Ethanol 2 min, 95% Ethanol 1 min, 70% Ethanol 1
Min, and in de-ionized water 1 min.
5. After the final rinse, the slides were allowed to dry completely by incubating them at 37°C for 5 minutes.
6. To each tissue section, 2-3 drops of freshly diluted 1X Proteinase K solution were applied. Then slides were incubated at 37°C for 10-15 minutes.
7. Slides were dehydrated by immersing them sequentially in the following solutions at room temperature for the indicated times: Distilled water for 1 min, 70% Ethanol for 1 min, 95% Ethanol 1 min and 100% Ethanol for 1 min.
8. The slides were dried by incubating them at 37°C for 5 minutes.

Hybridization and detection

Tissue sections were not allowed to dry out at any time during the dehydration and staining procedures. Protein block and Detergent wash buffer need to be pre-warmed at 37°C before use.

1- One drop of the working DNA probe/hybridization solution was placed on the tissue section. Place a coverslip over each slide. Be careful to avoid trapping any air bubbles.

2- The slides with coverslips were placed in an oven or heating block at 70°C for 8-10 minutes to denature the secondary structure of RNA.
3- The slides were removed in a humid chamber and incubated at 37°C for 3-4 hours to allow hybridization of the probe with the target nucleic acid.
4- Then, slides soaked in 1X detergent wash at 37°C until the coverslips fall off. One should be careful not to tear the tissue.
5- One to two drops of RNase A (15 ug/ml) were placed on tissue section. Then slides were placed in a humid chamber and incubated at 37°C for 30 minutes.
6- Slides were washed with protein block (pre-warmed) at 37°C for 3 min. three times.
7- Excess buffer from around the tissue section was wiped off carefully. Then 1-2 drops of conjugate were added to tissue section. Then slides were kept in a humid chamber at 37°C for 20 minutes.
8- Excess reagent was tapped off then Slides were rinsed in detergent wash buffer for 5 minutes.
9- Again excess buffer was wiped from around the tissue section and 1-2 drops of substrate were placed on tissue section. Slides were incubated at room temperature for about 10 minutes, or until color development was complete.
10- Color development was monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells. Color begins to appear after 3-5 minutes, usually reaching sufficient development after 10 minutes.
11- Excess reagent was taped off; slides were rinsed in 2-3 changes of distilled water.
12- Slides then were counterstained using nuclear fast red.
13- Sections were mounted with a permanent-mounting medium (DPX). Prior to application of a permanent mounting medium, the sections were dehydrated by sequentially dipping the slides in graded alcohols, once in 95%, then twice in 100% ethanol, and then in xylene.

14- Examination and scoring done under light microscope by pathologist at power X400 and the. in situ hybridization was scored according to cut-off value. This cut-off for positivity was ≥25% positive cells for VEGF (Zheng S. et al., 2003)

Results

Histopathological Data.

Thirty five patients with colorectal adenocarcinoma were investigated. The mean age of patients was 52.34 with a range of 25-80 years. The male to female ratio was 4:3. There were only 2 cases of recurrent colorectal carcinoma. According to the histological differentiation, tumors were broken down in to three groups, well differentiated (WD, n=5), moderately differntiated (MD, n=25), and porrly differentiated (PD, n=5), moreover, patients were further grouped according to their histopathological criteria, as follow: tumor stage (B, n=11, C, n=15, and D, n=9), lymph node involvment (N0, n=11, N1, n=11, and N2, n=13).

VEGF. In situ mRNA Expression

Tumor sites versus resection margins.

Thirty-five cases of human colon carcinoma and their respective resection margins were investigated for VEGF mRNA cellular expression based on Insitu hybridization technique (typical ISH staning pattern were in tumor sample and resection margin are shown in figure 1). The mean± standard error (SE) of VEGF mRNA in situ expression in both tumor sites and their resection margins demonstrated a significant difference based on t test of statistical analysis (55.25±4.1, 19.2±2.31, and $p<0.001$, respectively, table 1). A noteworthy here is that in the periphery of necrotic foci of the colorectal cancer, labeling with the VEGF antisense probe was distinctly intensified in tumor epithelium adjacent to the necrotic foci. Moreover, the tumor stroma cells also labeled for VEGF mRNA. Labeled stromal cells included fibroblasts and smooth muscle cells.

Table (1). VEGF in situ mRNA expression in tumor sites and their resection margins, based on t.test .

PARAMETER	RESECTION MARGINE (NO=35)	TUMOR TISSUE (NO=35)
VEGF Mean ±SE† 99%C.I ‡ t-test p-value	 19.2±2.31 ------ ------	 55.25±4.1 26.55-45.55 P<0.001

† Standard error . C.I ‡ confidence interval

Correlation between mRNA in situ expressions of VEGF. With different histopathological variables

VEGF mRNA expression in colorectal adenocarcinoma was analyzed against the different histopathological features of the tumors based on Spearman's correlation. According to Table 2 unlike with tumor grade, that demonstrated no significant correlation between VEGF mRNA ISH expression and the tumor grade catagorize, however, on the contrary, there is highly significant positive correlation between VEGF mRNA ISH expression and both tumor stage (r_S = 0.585, p<0.01) as well as with lymph node involvment (r_S = 0.474, p<0.01).

Table (2). Correlation between VEGF in situ mRNA expressions along with different histopaphological variables of CRC, based on spearman's correlation (r_S)

HISTOPATHOLOGICAL VARIABLES	VEGF
Stage	0.585**
Grade	0.222
L.N involvment	0.474**

**highly significant correlation, p<0.01

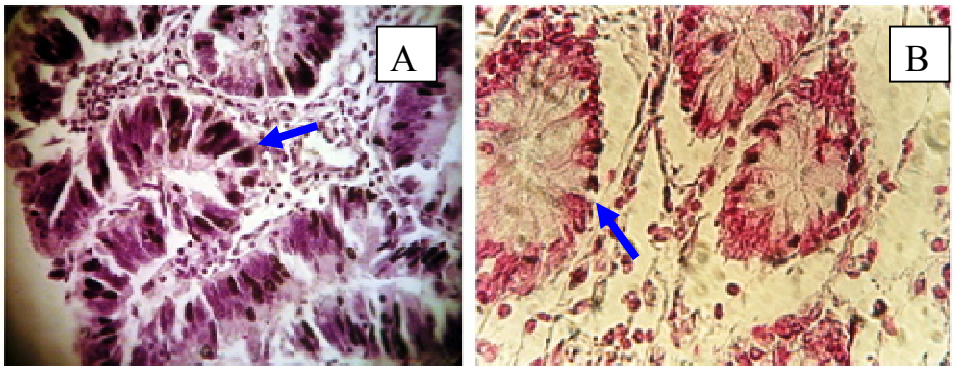

Figure 1. (A) VEGF in situ mRNA expression, of stained cells (92%). (B) Resection margin stained with VEGF in situ mRNA expression, magnification power from A to F (400X), except B(100X).

Discussion

Solid tumors require angiogenesis to grow beyond 1 to 2 mm^3 in size and to facilitate metastasis [22]. VEGF plays a central role in tumor angiogenesis; it is expressed in most tumors, often at substantially increased levels [23].

Regarding to the current study had demonstrated a significant over expression of VEGF mRNA in tumor tissue in compares with their resection margins ($p<0.001$, table 1), with a highly significant positive correlation between mRNA in situ expression with tumor stage and lymph node ivolvement ($r_S= 585$, $p<0.01$; and $r_S= 474$, $p<0.01$, respectively, table(2).This observation came in compatible with previous studies that focused on VEGF in colorectal and other tumors since, they illustrated a significant increase in VEGF protein expression with respect to tumor status, nodal metastasis, and with Astler Coller classification [24,25,26]. Similarly, Hou-Quan et al., 1998, analyze VEGF mRNA expression in gastric carcinoma and they found that VEGF mRNA expression significantly correlated with serosal invasion and L.N metastasis, although no correlation was found between its expression and

histological type. Consequently, the results of current study confirm previously published data about the role of VEGF in the neovascularization and metastasis in colon cancer, since they have stated VEGF levels were elevated and correlated with a poor clinical outcome [26, 28, 29]. Recently, it has been shown that VEGF expression in benign colonic adenomas is significantly up regulated compared to normal colonic mucosa with a further increase during the development of adenocarcinomas. Furthermore, it has been shown that within the tumor the tumor cells had the highest expression of VEGF, to establish invasive phenotype [30,31, 32].

Since, hypoxia is an important stimuli for mediating VEGF mRNA cellular expression during angiogenic response and since the current data demonstrated an increase of VEGF mRNA expression around necrotic tissue thus, it would be acceptable to anticipate that hypoxia could has the upper hand behind this current outcome. Molecular studies have determined that hypoxia may leads to an increase in the transcription of VEGF as well as an increase in the stability of this angiogenic message [33, 34].

Finally we conclude that VEGF is a guide marker in tumor angiogenesis and metastasis, and have prognostic importance, and provide rational basis for the development of molecular therapeutic approaches, however other studies will be necessary to better clarify if possible at which stage during the development of colorecal carcinoma, angiogenic may switch occur.

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