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Expression of VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, in colorectal cancer

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Abstrac: **Colorectal cancer is the third most common cancer and the third leading cause of cancer-related death. VEGF165b has been described as exerting anti-angiogenic activity. The aim was to compare the expression of serum VEGF165b level in CRC patients with paired normal serum samples and explore the association between VEGF165b expression status and poor pathological parameters. Pre-treatment serum samples were available from 55 patients. The expression serum VEGF-165b levels were analyzed by an ELISA. Group comparisons were made using the independent samples t test. Serum samples were analyzed from 55 colorectal cancer patients. Median serum levels of VEGF-165b were significantly higher in patients with lower stage (p=0.03), no lymph node metastases (p=0.045) or Vascular invasion (p=0.026). Our data support the role of VEGF165 b as a tumor suppressor factor in colorectal carcinogenesis and its possible prognosis value.**

Keywords: Vascular Endothelial Growth Factor-165b; VEFG165b; Colorectal Cancer; ELISA

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1. Introduction

Colorectal cancer (CRC) is reported to be the third most common cancer and the second leading cause of cancer-related death [1]. One million people are diagnosed with CRC worldwide each year [2], and about half of them will eventually develop metastatic disease and become candidates for palliative therapy. Solid tumour growth is dependent on the induction of their own blood supply by inducing a proangiogenic state in the tissue environment, regulating this balance between proangiogenic growth factors and antiangiogenic inhibitors (Folkman, 1985 1995; Boehm et al, 1997). One growth factor that has been shown to be an effective target for antiangiogenic therapy (AAT) is vascular endothelial growth factor-A (VEGF-A) [3]，and it have been studied extensively in tumours.

VEGF-A is generated by alternative splicing from 8 exons within the VEGF-A gene. All isoforms contain exons 1– 5 and the terminal exon, exon 8. Exons 6 and 7, which encode heparin-binding domains, can be included or excluded. This gives rise to a family of proteins termed according to their amino-acid number, VEGF165, VEGF121, VEGF189 and so on [4]. Recent evidence indicates that 2 families of VEGF proteins are formed by alternative splice acceptor-site selection in the 3' untranslated region within the terminal exon 8 to give 2 different C-terminal sequences that differ in only 6 amino acids [4-5]. VEGFxxx, the classic proangiogenic family of isoforms, is generated by proximal splice-site (PSS) selection in exon 8 (resulting from inclusion of exon 8a). The more recently described VEGFxxxb isoforms are formed by distal splice-site (DSS) choice, 66 bp further along the gene from the proximal splice site. This results in splicing out of exon 8a and the production of mRNA sequences that encode the VEGFxxxb family [4]. The two resultant families of proteins are of the same length, but with different carboxyl termini.

VEGF165b was the first of these exon 8b-encoded isoforms identified and subsequent studies demonstrated the existence of VEGF121b, VEGF183b, VEGF145b and VEGF189b [3]. But the only one of these isoforms for which there is any functional information is VEGF165b. VEGF165b has been described as acting as an endogenous inhibitory form of VEGF and, therefore, has a putative anti-angiogenesis role. Further experiments showed that VEGF165b inhibited VEGF165-induced endothelial proliferation, migration, vasodilatation and angiogenesis in the rabbit cornea and the rat mesentery [6]. Furthermore, VEGF165b was found down-regulated in melanoma [7], renal [8], prostate [4] or colorectal carcinoma [9], and its overexpression inhibits the growth of a variety of human tumour xenografts in mice [3-4, 8, 10-11]. Its absence has been recently described to predict metastatic spread in patients with primary melanoma [7]. An imbalance of the expression of the two VEGF families of isoforms has also been observed in the pediatric cancer neuroblastoma [12].

As the amino-acid structure of antiangiogenic VEGF165b is 95–96% identical to that of VEGF165 [5, 8], the majority of studies that investigated VEGF expression in CRC could not distinguish between the proangiogenic and antiangiogenic VEGF isoforms. We therefore undertook the present study to compare the expression of serum VEGF165b level in CRC patients with paired normal serum samples and explore the association between VEGF165b expression status and poor pathological parameters to support its possible tumor suppressor function and its potential as a prognostic marker in clinic.

2. Methods

2.1 Patients

The serum samples were obtained from 55 patients who treated at the Affiliated Hospital of Qingdao University Medical College from Jan 2013 to May 2013. All of the patients had histologically confirmed colorectal cancer, age <75 years, ECOG performance status of 0 or 1 and adequate hematological, hepatic, and renal functions, previously untreated. The study was approved by the local Ethics Committee and Institutional Review Board and, therefore, performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.2. Serum samples collection

Patient consent was obtained before sample collection. Serum samples were obtained before bevacizumab-based chemotherapy regimens. Serum samples were kept between 2℃ and 8℃, centrifuged at 10,000 rpm for 10 min, and then frozen at -81℃ until assayed.

Table 1. Characteristics of the Colorectal Patients Series

Colorectal series Characteristics	Total (%)
Patients	55
Median age	61.5 ± 10.9
Sex	
Male	26(47)
Female	29(53)
Localization	
Colon	30(55)
Rectum	25(45)
Vascular invasion	
No	27(49)
Yes	28(51)
Tumor Stage	
I	4 (7)
II	18 (33)
III	22(40)
IV	11 (20)
Lymph Node Metastases	
Negative	26 (47)
Positive	29 (53)

2.3. ELISA for VEGF-165b

Quantitative determination of the human VEGF165b concentration in the serum samples was done by quantitative solid phase ELISA. ELISA was done using the VEGF165b ELISA kit (R&D Systems) according to the manufacturer's instructions. VEGF-165b concentration was done according to manufacturer's instructions. Each sample was analyzed in duplicate and the mean values were used as the final concentration. The intraassay coefficient of variation was 4.6%, 5%, 4%, and 4.3%, respectively.

2.4. Statistical analysis

Statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, USA). Group comparisons were made using the independent samples t test. $P<0.05$ was set as the level of statistical significance.

3. Result

The following parameters were obtained from the medical records of the 55 colorectal cancer patients: age, gender, localization, lymph node metastases (LNM), pathological stage, vascular invasion (VI). Pathological stage was assessed using the tumor-node-metastases (TNM) classification. Presence of lymph node metastases was evaluated by optical microscopy (Table 1).

Analysis of the relationship between the expression levels of VEGF165b and the pathological data revealed significant associations. One was between expression levels of VEGF165b and tumor stage when cases were classified in 2 groups, those harboring tumor Stage I or II (I + II) and those harboring Stage III or IV (III + IV). VEGF165b expression was significantly lower in those cases in stages III + IV ($p = 0.03$), with averages expression of for 95.3pg/ml Stage III + IV and 175.3pg/ml for Stage I + II (Fig. 1a).

Low levels of VEGF165b were significantly associated with vascular invasion ($p=0.026$). The average VEGF165b level of the 27 out of 55 patients (49%) who did not show vascular invasion was 167.9pg/ml; the remaining 51% with vascular invasion had an average expression of 93.35pg/ml (Fig. 1b).

Presence of lymph node metastases was associated with down regulation of VEGF165b ($p=0.045$). The average for the expression of this variant in 29 out of 55 patients (53%) harboring lymph node metastases was 95.3pg/ml; and in those without lymph node metastases (26 out of 55, 47%), it was 154.35pg/ml (Fig. 1c).

4. Discussion

A characteristic feature of CRC is increased vascularity, which correlates with increasing stage. Overexpression of VEGF, a key player in angiogenesis, has been reported in CRC cell lines and tumour samples [13]. Vasculature not only provides tumors with an adequate blood supply, but it offers a route for tumor cells to metastasize [14]. However, there are few studies to distinguish pro- and anti-angiogenic VEGF isoforms. Studies by Bates' group have shown that there are two families of isoforms generated by proximal or distal site selection of exon 8 resulting in pro-angiogenic (VEGFxxx) and anti-angiogenic (VEGFxxxb) isoforms [4,8,15-16]. An upregulation of the proangiogenic VEGFxxx variants has been widely reported in human tumors. This up regulation brings about a loss in the balance of, which causes a drop in the proportion of VEGFxxxb levels [17].

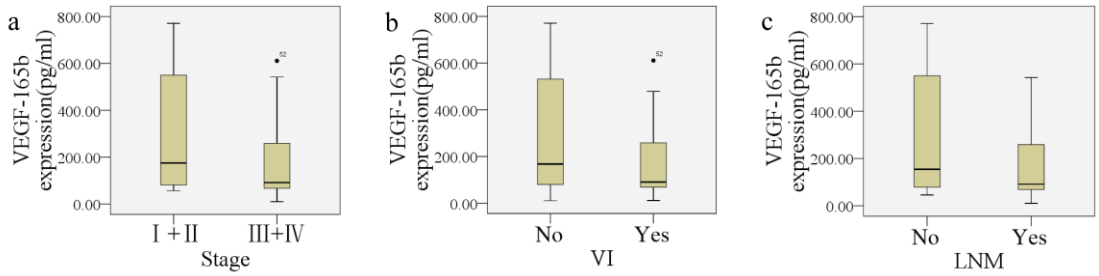

Figure 1. Association between expression levels of VEGF165b and tumor stage(a), vascular invasion (b) and lymph node metastases (c). The graphs show the 25th, 50th and 75th percentiles. VI=vascular invasion, LNM= lymph node metastases.

VEGF165b, the major anti-angiogenic isoform, was the first member of the VEGFxxxb family to be described [4] and is the most studied member so far. VEGF165b mRNA was first isolated and cloned from human renal cortex tissue, and subsequently identified in other human tissues [17]. Woolard et al [18] showed that a monoclonal antibody raised against the terminal 9–amino acid sequence of human VEGF165b could particularly detect antiangiogenic VEGF165b but not proangiogenic VEGF165 in 2004. With the VEGF165b-specific antibody, it could be demonstrated that VEGF165b is widely expressed in most healthy human tissues, as well as in human plasma, with levels consistent with known circulating levels of VEGF [5, 8]. Except that VEGF165b is upregulated in intraductal breast carcinoma [19], it has been reported to be down regulated in all cancers investigated thus far, as well as in other angiogenic conditions such as Denys-Drash syndrome and proliferative diabetic retinopathy [3,5,7-8,20-23].

Remarkably, although downregulation of VEGF165b has been previously observed in human cancers [4,8], its association with poor prognosis is badly documented in the literature. Only recent data described the association between VEGF165b downregulation and tumor spread in patients with primary melanoma and colorectal cancer [24]. Therefore, studies evaluating this association could highlight the role of VEGF165b as a tumor suppressor protein and a tumor prognosis marker. In our colon cancer series we also observed an association between downregulation of VEGF165b and advanced tumor stages, vascular invasion and lymph node metastases. Classically, these 3 parameters are the most robustly associated with a poor outcome in colorectal cancer patients and consequently VEGF165b could be a sensitive marker of tumor spread and metastasis.

The downregulation of the VEGFxxxb isoforms identified here, if confirmed in a larger study, would greatly simplify the procedure for identifying patients at risk. Furthermore, it could be combined with other indices, such as the Shields' index, to further refine

These findings also show that the metastatic process appears to be associated with a splicing switch. Recently, the role of splicing in cancer has received a good deal of attention [34], but it is clear that alternative splicing eventsalso play a role in the metastatic prediction[25]. However, the mechanisms underlying downregulation of VEGFxxxb isoforms remain largely unknown. Possible explanations of this finding include the following: (1) VEGFxxxb expression inhibits tumour metastasis directly by interfering with tumour cell migration or tumour cell adhesion via a currently unknown mechanism; (2) inhibition of angiogenesis by VEGF165b expression [8] is responsible for preventing metastasis by limiting tumour size independently of thickness; (3) VEGFxxxb expression inhibits the main route of metastatic spread via the lymphatics by inhibition of lymphangiogenesis; (4) increased vascular permeability induced by VEGFxxx increases the likelihood of metastasis; or (5) a combination of any or all of the above factors [7].

VEGFxxxb has been shown to be anti-angiogenic in physiological models, and inhibits melanoma xenograft growth in vivo [8,26], but results in a very transient increase in vascular permeability to water[27], whereas VEGF165 is angiogenic and results in a chronic and sustained increase in water permeability [20-30], leading to oedema in many tumours. Upregulation of VEGF165 with respect to VEGF165b will therefore result in angiogenic, leaky tumours, and it is likely that this would provide a more facilitative environment for metastasis for a number of reasons. These include a more hydrated tissue, which would be easier for cell and molecules to move through [31]. This would result in tumour cells having a greater likelihood of detecting lymphatic-secreted chemokines to identify the lymphatics [32], and being able to secrete heparin-binding growth factors a further distance to stimulate lymphatic growth into the tumours. Recent studies have also shown that lymphatic cells can migrate along patterns of interstitial fluid flows [33], and presumably this would be enhanced in more permeable tumours. Thus, the expression characteristics of these tumours indicate that upregulation of pro-angiogenic, pro-permeability VEGF165 and its sister isoforms is associated with metastasis in melanoma.

metastatic process [35-36]. The regulation of VEGF splicing may therefore also be part of a metastatic splicing phenotype that is regulated by specific splice factors, such as SF/ASF2, described recently for the macrophage-stimulating promoter receptor

tyrosinekinase Ron [37], and for which we also have evidence that it is involved in the regulation of VEGF:VEGFb splicing [38].

5. Conclusion

Evidence is presented here on the role of VEGF165b as a tumor suppressor factor and its prognosis value in colorectal carcinogenesis, but the mechanism underlying this is not known. We suggest therefore that the functional capacity of this new VEGF isoform requires further investigation.

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