VEGF-A and VEGF-R1/2 Expression: Critical Importance in Different Stages of Human Gastric Adenocarcinoma

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Abstract

A key role for gastric micro vascularisation during cancer progression is the vascular growth factor VEGF-A. In gastric cancer, serum VEGF levels were also significantly higher in patients with advanced-stage cancer, higher lymph node ratio, and peritoneal invasion. Inhibition of VEGF or blockade of the corresponding VEGF-R1 and VEGF-R2 receptors has been investigated in the treatment of gastric adenocarcinoma. Treatment of VEGF-antibodies or VEGF-receptor antibodies, however, had no significant effect on overall survival. Most recently, however, a new and fully humanized IgG1 monoclonal antibody Ramucirumab (IMC-1121B) has been introduced, which targets the extracellular domain of VEGF receptor 2 (VEGFR2). The antibody, increasing the median overall survival compared to placebo. Thus, a more detailed analysis of VEGF and VEGF receptor expression in early compared to advanced stages is needed. The current review focuses on previous findings and describes own results in early compared to advanced stages of gastric adenocarcinoma. A special focus is given on VEGF-A expression as well as the expression of the corresponding receptors VEGF-R1 and VEGF-R2. Own data reveal that VEGF-R2 may be a better target since expression levels seem to be expressed at high levels in gastric cancer tissues.

Keywords: Gastric cancer; VEGF-A; VEGFR-1; VEGFR-2; CD133 antigen; Microvessels

Introduction

Gastric adenocarcinoma is the second leading cause of cancer-related death worldwide, but the overall survival rate from the disease still remains poor due to the lack of effective treatment strategies, especially in advanced situations. Approximately 650,000 people die worldwide from gastric cancer every year [1]. Epidemiological and interventional studies in humans, as well as experiments in rodents, have strongly linked H. pylori infection to the development of both types of distal gastric cancer [2]. Surgery, i.e. gastrectomy with extended lymphadenectomy is the only curative option in early stages of gastric adenocarcinomas, and this treatment option is reasonable when the tumor has exceeded the mucosal layer [3]. Tumor prognosis is favourable only in early stages of gastric cancer, and recent studies have revealed that tumors limited to the mucosa (T1m stages) can be resected endoscopically, since lymph nodes metastasis at this stage are extremely rare [4].

A key role for gastric micro vascularisation during cancer progression is the vascular growth factor VEGF-A [5]. Vascular endothelial growth factor (VEGF) is a multifunctional cellular factor which can induce neovascularization and increase capillary permeability. VEGF-A regulates important steps in angiogenesis and physiological vascularisation and controls a number of physiological processes like wound healing, ovulation, menstruation and pregnancy [6]. There are four active isoforms of the VEGF-A molecule generated by differential splicing of which VEGF-A165 is the most common. The biological activity of VEGF-A is mediated by binding on VEGFR-1 and VEGFR-2 receptors, that are expressed on vascular endothelial cells; but also by binding on neuropilins located on vascular endothelium or neurons [7]. VEGF-A expression has been shown to be of special importance for neovascularisation in gastric cancer [8]. Also, VEGF-R1 expression rate among gastric cancer patients is supposed to indicate a high risk group for metastasis [9]. Furthermore, neovascularisation may be a new target to treat advanced stages using new antibodies [10]. Indeed, a monoclonal antibody against VEGF-A, Bevacizumab, was approved for therapy of colon cancer in 2005 [11]. Clinical studies using this and other antibodies have revealed diverging results in gastric cancer.

Interestingly, most recent studies by Ebos et al. and Paez-Ribes et al. have pointed out that drugs inhibiting the VEGF pathway promote tumour invasiveness and metastasis in mice [12,13]. Thus, the molecular mechanisms underlying vascularisation in more advanced stages of gastric cancer, including molecular signals in different stages of cancer, are of special interest.

Most recently, ramucirumab, a monoclonal antibody VEGFR-2 antagonist, prolonged survival in patients with advanced gastric cancer. This international, randomised, double-blind, placebo-controlled, phase 3 trial was conducted between 2009 and 2012 in 29 countries including North America, Central and South America, Europe, Asia, Australia, and Africa. Ramucirumab showed survival benefits in patients with advanced gastric or gastro-oesophageal junction adenocarcinoma progressing after first-line chemotherapy. The findings validate VEGF-R2 signaling as an important therapeutic target in advanced gastric cancer [14].

Therefore, we investigated VEGF-A and VEGF-R1 and -2 expressions in gastric cancer tissue. We used specimens from different stages of gastric cancer in order to evaluate the importance of this factor during further tumor invasion and lymph node metastasis. Our data reveal a decreased expression of this factor in more advanced stages, and limit the potential use of such VEGF-A targets in modern therapy of gastric adenocarcinoma. However, VEGF-R2 receptors seem to be present in different stages of gastric cancer, and could serve as a potential target.

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Materials and Methods

Study population and tissues

Gastric cancer tissues investigated were obtained from patients (n=52) who underwent curative gastrectomy between 2001 and 2006 at the Division of Surgery, Klinikum rechts der Isar, Technical University of Munich. Patients' characteristics are listed in Table 1. Under RNAse-free conditions, formalin-fixed paraffin-embedded (FFPE) tissue samples were sectioned at 10 µm. Sections were dewaxed with xylene, rehydrated and stained with haematoxylin. Whole tissue or separated areas (tumour or normal mucosa) of the section were microdissected to extract total RNA. Tissue amounts per slide lay between 50 mm² and 300 mm² in most cases. Small tumour areas were balanced by increased numbers of serial sections used. Quantitative TaqMan® real-time(RT)-PCR was performed using the Step One Plus sequence detection system of Munich.

Microvessel density (MVD) was determined by counting CD31 positive structures according to Weidner [17]. Staining was considered immunoreactive when the cytoplasms of endothelial cells were stained brown, a lumen was not required. Branching structures were counted as a single vessel. At least eight 500x500µm sectors at a 200x magnification were counted. The average vessel count per mm² was calculated as the MVD (details of protocol as reported previously in Besig et al., 2009) [16].

Immunohistochemistry and determination of microvessel density

Immunohistochemical staining for endothelial CD31 was performed on an automated staining system (Ventana BenchMark, Ventana Medical Systems Tucson, AZ). Antibody retrieval was performed by heating (CC1 mild, Ventana BenchMark). The primary antibody, a monoclonal mouse anti-human CD31 clone JC-70-A (Dako ChemMate, Glostrup, Denmark), was incubated at a dilution of 1:30 for 50min at RT. Antibody binding was visualized using the LSAB-POX-method and DAB, which yields a brown staining. Hematoxylin (AppliChem, Darmstadt, Germany) was used for counterstaining. Microvessel density (MVD) was determined by counting CD31 positive structures according to Weidner [17].

Results

Expression of the VEGF ligand VEGF-A and VEGF-R1/2 in gastric adenocarcinoma

Gastric cancer tissues (n=52) were obtained from patients who underwent curative gastrectomy between 2001 and 2006 at the Division of Surgery, Klinikum rechts der Isar, Technical University of Munich. Gastric carcinomas were grouped according to Lauren's classification and to the pathohistological T-stage. Initially, VEGF-A and VEGF-R1/2 mRNA levels were determined in gastric cancer tissue using TaqMan real-time PCR, and copy numbers of each gene were normalized to 10⁶ GAPDH copies. Figure 1A shows the VEGF-mRNA expression in different T-stages. VEGF-A mRNA expression was significantly lower in intestinal type carcinomas exceeding the mucosa compared to carcinomas limited to the mucosa (Figure 1B, p=0.013). Interestingly, VEGF-A expression was significantly elevated in diffuse type of gastric cancer compared to other cancer types (Figure 1C, p=0.017). A students t-test was used for statistical comparisons.

VEGFR-1 mRNA showed no significant difference between the different T-stages (Figure 1D). VEGFR-1 expression was significantly higher in the diffuse type of gastric carcinoma (Figure 1E, p=0.030). Most interestingly, VEGFR-2 expression was expressed at a 100-fold higher level compared to VEGF-R1 expression levels, but revealed no significant difference between the different T-stages (Figure 1F). VEGF-R2 mRNA expression was significantly lower in intestinal type of gastric cancer (Figure 1G, p=0.017) compared to diffuse or mixed type. In average, the expression level of VEGF-R2 ranged at 10000-50000 GAPDH copies, while VEGF-R1 was expressed at 500-1000 copies/GAPDH.

Microvessel density in different stages of gastric cancer

Microvessel density (MVD) in carcinoma tissue was determined by counting CD31 positive vessel structures, and results are shown in Figure 2. MVD in the carcinomas was decreased in more advanced stages of gastric cancer (p=0.022). MVD was also decreased in the

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### Table 1: List of Patients' characteristics in the investigation of gastric cancer from patients (n=52) who underwent curative gastrectomy between 2001 and 2006 at the Division of Surgery by Technical University of Munich.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63.45 years</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>11.55 years</td>
</tr>
<tr>
<td>Range</td>
<td>25.6 – 84.7 years</td>
</tr>
<tr>
<td>Gender</td>
<td>Male 26, Female 26</td>
</tr>
</tbody>
</table>

### Table 2: Analysis of Quantitative TaqMan® real-time(RT)-PCR.

<table>
<thead>
<tr>
<th>VEGF-A</th>
<th>Forward primer 5'-TAC CTC CAC CAT GAC AAG TG-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse primer</td>
<td>5'-GAT GAT TCT GCT CGC TCT CTT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'FAM-TCC CAG GCT GCA CCC ATG GC-3'TAMRA</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>VEGFR-1</th>
<th>Forward primer 5'-CTC CTG CCA CTC TAA TTG TCA ATG T-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse primer</td>
<td>5'-AAA CGA TGA CAC GGC CTT TT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'FAM-AAA CCC CAG ATT TAC-3'TAMRA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VEGFR-2</th>
<th>Forward primer 5'-CAA GCC AGG AAG ACC AAG AAG AAG C-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse primer</td>
<td>5'-GTT GCC ACA CGC TCT AGG A-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'FAM-TTG CGT GTG CAG GCA CCT CAC A-3'TAMRA</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>CD-133</th>
<th>Forward primer 5'-GCC AAA CCA CGA CTG TCG TA-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse primer</td>
<td>5'-CGA TAT CTG AAC CAA TGG AAT TCA-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'FAM-CAG GTA TCA AAA GGG TC-3'TAMRA</td>
</tr>
</tbody>
</table>
diffuse type according to Lauren’s classification (p=0.012). Interestingly, the actual density of the vessels in tumor tissue was lower, but the vessels themselves were often larger. Figure 3A shows an immunohistochemical staining against the blood vessel endothelial marker CD31 and gives an illustration of the microvessel density in an intestinal type of early gastric cancer. Figure 3B is an image of an early gastric cancer stained with an antibody against the VEGF-R2. The receptor is detected on vessels.

Expression of CD133 mRNA and correlation with VEGF-R1

Since VEGF-A expression did not correlate with microvessel density, we sought to determine another parameter indicating presence of potential endothelial precursor that would be upregulated by this ligand. CD133 has been shown to be present on endothelial precursor cells [18]. We therefore investigated the relative CD133 mRNA expression in the present tumour specimens using TaqMan-
PCR. CD133 was significantly reduced (p=0.008) in tumours with submucosal invasion (pT1sm, T2, T3), compared to those limited to the mucosa (pT1m, data not shown). VEGFR-1 (corr.coeff.: 0.515, p<0.001) but not VEGF-R2 (p=0.103) expression was significantly associated with CD133 expression; indicating a possible co-localisation on endothelial precursor cells (Figure 4A).

Discussion

The current study investigated the expression of VEGF-A and the corresponding VEGFR-1 and -2 receptors in gastric adenocarcinomas. In parallel to other observations, VEGF-A expression was decreased in more advanced stages; limiting the potential use of VEGF-A antibodies.
in treatment of gastric cancer. In contrast, throughout the different
tumor types and tumor stages, VEGF-R2 receptors were expressed at
high levels. The new data thus suggest new implications of high clinical
importance.

VEGF-A mRNA expression was significantly lower in carcinomas
exceeding the mucosa compared to carcinomas limited to the mucosa,
and was significantly elevated in diffuse type of gastric cancer. One
explanation for this finding is the hypothesis that VEGF-A is mainly
produced by the tumor cells themselves and although there is a
progress of tumor cell mass, there is reduced expression or production
of this growth factor, for example due to a loss of differentiation and
chromosomal instability, leading to a generally reduced gene expression
profile. It has been shown that VEGF-A is expressed in almost every
kind of solid tumour and has been associated with progression and
survival in different types of cancer [19-23]. However, it appears
unlikely that especially gastric cancer plays a special role and resembles
an exceptional tumor entity. Gene expression profiles of the divergent
tumor cells were not available here; however, other authors have
presented data in which a decreased VEGF-A expression in advanced
stages was observed [24,25]. Our observation is in line with these last
data, and may be explained by a more clear differentiation within the
T-stages and by the accurate use of TaqMan PCR. In contrast,
immunohistochemistry using antibodies against VEGF-A may be quite
difficult to quantitate.

Another potential explanation for the reduced expression of
VEGF-A in advanced stages is the idea that VEGF-A is mainly produced
in endothelial cells and the vessels themselves. This idea, however, is in
contrast to our own findings in diffuse type cancer, there is a reduced
presence of microvessel density. It has been proposed that hyposia is a
main reason for increased vessel formation in gastric cancer [26]. Other
works have shown too that in advanced stages of cancer, a decrease in
MVD can be observed [27]. This may be due to the fact that an
increasing tumor volume is attended by an augmentation of the tumor
matrix volume. Necrotic and desmoplastic reactions can be observed as
well. Thus, the decline in vessel formation and VEGF-A expression
may in fact reflect the increase in necrotic or desmoplastic tissue. At
present there is only one phase-II study that shows a little benefit for
patients with advanced gastric carcinoma receiving a combination
therapy including Bevacizumab [10], which is in clear contrast to
our own findings in diffuse type cancer; once the cells differentiate into
different types, CD133 expression is lost and thus VEGF-A
expression is high for neovascularisation in gastric cancer that
indeed be important for neo-vascularization of gastric cancer, especially
in early T1m stages. One possibility for this finding may be that VEGF-A
and the corresponding receptors are expressed by the same type of cells
– tumor cells and/or endothelial cells. Another possibility is that high
VEGF-A secretion from tumor cells, – induced for example in hypoxic
areas of the tumor – may lead to higher VEGFR-1 and -2 expression in
tumor vessel endothelial cells. In other works, VEGF-2 receptors have
been detected on vessels [30].

Here, our data observed in tumor specimens indicate that
VEGFR-2 receptors are present on vessels present in the tumor
issues, but not the tumor cells themselves, as depicted in the Figure
3B. The findings are in line with current clinical reports which point
out the targeting VEGF-R2 can be an relevant target. Ramucirumab,
a monoclonal antibody VEGFR-2 antagonist, prolonged survival in
patients with advanced gastric cancer. Ramucirumab showed survival
benefits in patients with advanced gastric or gastro-oesophageal
junction adenocarcinoma progressing after first-line chemotherapy.
The findings validate VEGFR-2 signalling as an important therapeutic
target in advanced gastric cancer [14].

No significant correlation was observable between VEGF-R2
mRNA levels and the MVD. This observation may appear divergent to
the above data; however, it may be explained by the fact that the MVD
only refers to the number of vessels in the visual field, but does not take
into account the vessel size or quality. Small normal vessels are counted
equally to abnormal tumor vessels. One must therefore mention that the
different growth patterns may also affect on the MVD count: in diffuse
type of gastric cancer there are often clusters of cells with a low cell
and vessel density. The determination method of the MVD according to
Weidner [17] does not compare the cell density to the vessel density, it
only focuses on the absolute number of vessels. In our study we did not
count vessels in exulcerated parts of the tumors, because in these cases
granulation tissue could not be distinguished from vessels.

In order to further delineate the complex interaction between
tumor cells and vessels, we used a novel marker of endothelial
precursor cells (EPC) that may be of special interest in this context,
CD133 is a pentaspan transmembrane glycoprotein of currently
unknown function [31]. First reports describe that CD133 receptors are
highly expressed on hematopoietic stem cells and related malignancies
including myelogenous leukaemia, acute lymphocytic leukaemia, and
chronic lymphatic leukaemia and myelodysplastic syndromes [32].
Recent studies by Asahara et al. [33] have shown that after bone marrow
transplantation in mice, donor endothelial precursor cells (EPCs)
were frequently found in sites of neovascularisation. These EPCs were
characterized by the expression of CD133, CD34 and VEGF-2 on
their surface. CD133 expression is lost once these cells differentiate into
more mature endothelial cells, and this differentiation is mediated by
VEGF [18]. Thus, CD133 may also be related to angiogenesis processes
since it is expressed on endothelial progenitor cells (EPCs). These cells
derive from the bone marrow and have the potential to proliferate,
migrate and to initialize endothelial growth.

When comparing CD133 expression with the above ligands
and receptors, we found close correlation between CD133 and
VEGF-R1. This finding suggests that EPCs are of special importance
for neovascularisation in early stages of gastric cancer; once the cells
differentiate into vessels, CD133 expression is lost and thus VEGF-R1
is decreased simultaneously [34,35]. Thus, endothelial precursor cells
appear to play a crucial role for neovascularisation in gastric cancer that
deserves further investigation.

VEGF-A on the one hand and VEGF-R1 and -2 on the other hand
showed a close correlation, underlining the finding that VEGF-A may
indeed be important for neo-vascularization of gastric cancer, especially
in early T1m stages. One possibility for this finding may be that VEGF-A
and the corresponding receptors are expressed by the same type of cells
– tumor cells and/or endothelial cells. Another possibility is that high
VEGF-A secretion from tumor cells, – induced for example in hypoxic
areas of the tumor – may lead to higher VEGFR-1 and -2 expression in
tumor vessel endothelial cells. In other works, VEGF-2 receptors have
been detected on vessels [30].
In summary, our findings clearly delineate the role of VEGF-A and its corresponding receptors in early stages of gastric stages. Advanced stages of gastric carcinoma however, seem to lose the expression, maybe due to a desmoplastic reaction, and are thus unsuitable for such directed molecular therapy. Further studies will investigate the role of other vascular growth patterns, such as lymphangiogenic factors, neuropilins, and HIF-1a/pha.

References