Quantitative gene-expression of the tumor angiogenesis markers vascular endothelial growth factor, integrin αV and integrin β3 in human neuroendocrine tumors

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Abstract. Anti-angiogenesis treatment is a promising new therapy for cancer that recently has also been suggested for patients with neuroendocrine tumors. The aim of the present study was therefore to investigate the level of tumor angiogenesis, and thereby the molecular basis for anti-angiogenesis treatment, in neuroendocrine tumors. We used quantitative real-time PCR for measuring mRNA gene-expression of vascular endothelial growth factor (VEGF), integrin αv, and integrin β3, and CD34 for a group of patients with neuroendocrine tumors (n=13). Tissue from patients with colorectal cancer liver metastases (n=14) and normal liver tissues (n=16) was used as control. We found a lower mRNA level of VEGF in neuroendocrine tumors compared to both colorectal liver metastases (p<0.001) and normal liver tissue (p<0.01). For integrin β3 there was also a borderline significant lower level of mRNA in neuroendocrine tumors compared to both colorectal liver metastases (p=0.10) and normal liver tissue (p=0.06). In neuroendocrine tumors, gene-expression was highly variable of VEGF (530-fold), integrin αv (23-fold) and integrin β3 (106-fold). Quantitative gene-expression levels of the key angiogenesis molecules VEGF and integrin β3 were lower in neuroendocrine tumors than in colorectal liver metastases and were highly variable. Therefore, individual selection of patients may be necessary if anti-angiogenesis treatment is to be successful in patients with neuroendocrine tumors.

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is critical for the growth of the primary tumor, release of tumor cells into the circulation, and growth of metastases (1). Accordingly, tumor progression depends on tumor angiogenesis. Many molecules mediate angiogenesis. The most important molecules are growth factors (e.g. vascular endothelial growth factor; (VEGF), VEGF receptors (e.g. VEGFR-1, -2, -3), cell adhesion molecules (e.g. integrins), proteinases (e.g. MMPs), extracellular matrix (ECM) proteins (e.g. Fibronectin) and transcription factors (e.g. HIFs) (2,3).

VEGF is a family of glycoproteins consisting of 6 ligands (VEGF A-F) and of three receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1) and VEGFR-3 (Flt-4). VEGF-A (commonly referred to as VEGF) has been regarded as an important molecular marker for angiogenesis. VEGF, as a pro-angiogenesis factor, stimulates endothelial cell proliferation, prevents regression of newly formed vessels, and increases micro-vascular permeability. Increase in VEGF mRNA expression has been identified in many tumors. Positive correlation between tumor VEGF expression and tumor vascularity as well as prognosis has been shown (4). VEGF is strongly related to liver metastases of colorectal cancer and its gene-expression levels are useful not only as a predictive marker for distant metastases but also as a prognostic marker in these tumors (5,6). VEGF has been targeted for cancer therapy, either alone or in combination with chemotherapy.

Integrins are a family of cell adhesion receptors binding to extracellular matrix (ECM) adhesion proteins, consisting of a heterodimer complex of two transmembrane subunits (α and β). Integrin αvβ3 is highly expressed on activated endothelial cells and tumor cells, but is not present in resting endothelial cells or other tissues and thereby specific for neo-angiogenesis. Studying αv and β3 gene-expression in tumors may therefore show whether and to what extent angiogenesis is taking place and may predict whether the tumors are likely to be susceptible to anti-angiogenesis treatment (7-11).
Table I. Descriptions of the patients in the NET group.

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Tumor type</th>
<th>Site of sample collection</th>
<th>Metastatic disease</th>
<th>WHO classification</th>
<th>PI-index</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE-1</td>
<td>VIPoma</td>
<td>Liver</td>
<td>Yes (liver)</td>
<td>3</td>
<td>PI&gt;15%</td>
</tr>
<tr>
<td>NE-2</td>
<td>Typical bronchial carcinoid</td>
<td>Liver</td>
<td>Yes (liver)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-3</td>
<td>Functioning pancreatic NET</td>
<td>Pancreas</td>
<td>Yes (micro-metast., lymph node)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-4</td>
<td>Pheochromocytoma-1</td>
<td>Adrenal gland</td>
<td>No</td>
<td>1</td>
<td>Pl&lt;2%</td>
</tr>
<tr>
<td>NE-5</td>
<td>Pheochromocytoma-2</td>
<td>Adrenal gland</td>
<td>No</td>
<td>1</td>
<td>Pl&lt;2%</td>
</tr>
<tr>
<td>NE-6</td>
<td>Functioning pancreatic NET</td>
<td>Retroperitoneum</td>
<td>Yes (lymph nodes)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-7</td>
<td>Functioning pancreatic NET</td>
<td>Pancreas</td>
<td>Yes (lymph nodes)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-8</td>
<td>Pheochromocytoma-4</td>
<td>Adrenal gland</td>
<td>No</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-9</td>
<td>Carcinoid of the small intestine</td>
<td>Colon</td>
<td>Yes (liver)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-10</td>
<td>Carcinoid of the small intestine</td>
<td>Retroperitoneum</td>
<td>Yes (lymph nodes)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-11</td>
<td>Functioning pancreatic NET</td>
<td>Retroperitoneum</td>
<td>Yes (lymph nodes)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-12</td>
<td>Gastronoma of the duodenum</td>
<td>Duodenum</td>
<td>Yes (lymph nodes)</td>
<td>2</td>
<td>2%&lt;PI&lt;15%</td>
</tr>
<tr>
<td>NE-13</td>
<td>Pheochromocytoma-3</td>
<td>Peritoneum</td>
<td>Yes (liver, retro- &amp; peritoneum)</td>
<td>3</td>
<td>PI=15%</td>
</tr>
</tbody>
</table>

CD34 is a cell surface antigen expressed on human hematopoietic progenitor cells. CD34 is used as an endothelial cell marker of the tumor vessels. CD34 immunohistochemical staining is the most common method to assess microvessel density (MVD). In this study we used quantitative gene-expression of CD34 as a marker for MVD (12).

The VEGF antagonist bevacizumab (Avastin®) was the first anti-angiogenic drug to be approved for clinical use. Patients with metastatic colorectal cancer have been treated with bevacizumab in combination with chemotherapy and have shown an initial anti-tumor response with an improvement of 50% on progression-free survival from 6 to 10 months. However, no significant advantage has been seen past 20-24 months (13-15). The individual responses were highly variable. Also anti-angiogenic treatment with integrin antagonists (e.g. Cilengitide®) (16) is currently being tested in clinical trials (phase I, II and III studies) for different cancer types (17-22).

Neuroendocrine tumors (NET) represent a heterogeneous group of tumors. Gastroenteropancreatic NET have their origin in the gastrointestinal tract and pancreas and constitute about 2% of all malignant tumors and has an incidence of 2.3-4.2/100,000/year (3,23,24). Surgical resection is currently the only treatment able to cure patients with NET and should always be considered as first line therapy (3.25). Medical therapy is used in patients with disseminated disease. Somatostatin analogues, based on NET up-regulation of Somatostatin-Receptor 2 (SSTR2) (26), are used for management of hormone related symptoms in these patients. Significant biochemical and symptomatic improvement have been obtained in 50-60% of the patients, but tumor reduction was only seen in 3-5% of the patients (3.25). Tumor targeted biotherapy such as interferon-α is used in low proliferating NET, e.g. most intestinal carcinoid tumors, whereas chemotherapy such as streptozotocin in combination with 5-fluorouracil or doxorubicin or etoposide and cisplatin are used in intermediate and high proliferating NET, respectively (3.25). Anti-angiogenic treatments for NET are also planned or in progress (27).

The aim of the present study was to investigate the level of tumor angiogenesis, which is the molecular basis for anti-angiogenesis treatment in NET. To do so, we investigated gene-expression levels of tumor angiogenesis markers in patients with NET, where anti-angiogenesis treatment has been suggested, and compared the levels with that in patients with colorectal tumor (CRC) liver metastases, where anti-angiogenesis treatment is established. We used quantitative real-time PCR for measuring the gene-expression of VEGF, integrin αv, integrin β3, and CD34.

Materials and methods

Patients. Twenty-seven patients with two different types of tumors were included. Neuroendocrine tumors: 13 patients (9 men and 4 women, age 18-73 years, mean 54 year) diagnosed with NET were enrolled in the study. The histopathological diagnoses of the tumors from these 13 patients were 2 carcinoids, 1 somatostatinoma, 1 bronchial carcinoid, 1 VIPoma, 1 mixed glucagonomas/somatostatinomas, 3 gastrinomas, and 4 pheochromocytomas (Table I). Tissue was in all cases obtained during surgery.

NET patients were classified according to the WHO classification: i) well-differentiated tumors (Ki-67 proliferation index <2%); ii) well-differentiated endocrine carcinomas (Ki-67 proliferation index 2-15%); and iii) poorly differentiated carcinomas (Ki-67 proliferation index >15%) (23,24). Data are shown in Table I.

Colorectal tumor liver metastases: 14 patients (7 men and 7 women, age 33-77 years, mean 64 year) with liver metastases from previously resected colorectal cancers were enrolled as control group. The tissue examined was in all cases from resected liver metastases. In addition, normal liver tissue from patients was used as non-cancer control tissue.

Patients were enrolled in the study consecutively, when they were admitted to surgery for tumor resection. Informed consent was obtained in all cases. The study was approved by the local scientific ethics committee (reference number KF 01 313726).
RNA extraction. Following surgery tissue was transferred to RNase free tubes and RNAlater (Ambion Inc., Austin, TX, USA), a tissue storage reagent that stabilizes and protects the RNA, was added. Before adding RNAlater large tissue samples were cut to <0.5 cm in any single dimension. The tissue was stored at 4°C overnight, then the supernatant was removed and the tissue was frozen and kept at -80°C until use. Tissue (25 mg) was taken for the total RNA isolation. Following addition of 350 μl lysis buffer and 5 μl β-mercaptoethanol, tissue was homogenized with a plastic pistil rotated by a small handhold rotor. The total RNA kit used was Nucleospin RNA II kit (Stratagene). Seven μl RNA (2 μg) + 1 μl oligo-(dT) primer (0.1 μg/μl) + 1 μl random primer (0.1 μg/μl) + 10 μl First Strand cDNA Synthesis Kit was added. The total RNA concentration and the possible degradation of the RNA was measured on an Experion instrument (Bio-Rad, Hercules, CA, USA), a chip gel-based electrophoresis system to separate and quantify RNA; using RNA StdSens Rad, Hercules, CA, USA). The measured total RNA concentrations were between 0.18 and 3.0 μg RNA/mg tissue.

Reverse transcription. The First-Strand cDNA was synthesized at 42°C from 2 μg total RNA with StrataScript Reverse Transcriptase, 50 U, Stratagene. Seven μl RNA (2 μg) + 1 μl oligo-(dT) primer (0.1 μg/μl) + 1 μl random primer (0.1 μg/μl) + 10 μl First Strand Master Mix x2 + 1 μl RT-enzyme; ending up with a final volume of 20 μl cDNA. The RT was performed using a MasterCycler (Eppendorf AG, Hamburg, Germany) with the following protocol: 25°C for 5 min, 42°C for 15 min, 95°C for 5 min. The cDNA was immediately placed on ice and then frozen and kept at -20°C.

Determination of the best housekeeping gene. To determine the best housekeeping gene for this study we tested 12 different human housekeeping genes (primer sets from TATAA Bio Center, Uppsala, Sweden). The housekeeping genes were GAPDH (glyceraldehyde-3-phosphate dehydrogenase), TUBB (tubulin, β polypeptide), PPIA (cyclophilin A), ACTB (actin, β), YWHAZ (tyrosine 3), RNR185 (18S rRNA), B2M (β-2-microglobulin), UBC (ubiquitin C), TBP (TATAA-box binding protein), RPLP (60S acidic ribosomal protein PO), GUSB (β-glucuronidase) and HPRT1 (hypoxanthine-guanine phosphoribosyltransferase). The 12 primer sets were tested in human pancreas tumor and liver tumor tissues (n=8) and were all related to their respective normal tissues. RNA was extracted from 18 to 25 mg tissue, the total RNA amount measured (Experion, Bio-Rad), reverse transcription on 2 μg total RNA performed and gene expression quantified with real-time PCR using SYBR-Green I (Brilliant SYBR-Green QPCR Master Mix, Stratagene). The housekeeping genes were analyzed in duplicates with primer concentrations of 300 nM. A dilution curve for each primer set was carried out; used for further calculation of and correction for the PCR efficiency for each housekeeping gene. The real-time QPCR was performed on an Mx3000P instrument (Stratagene). To identify the optimal normalization gene we used NormFinder (28), an algorithm that ranks the set of candidate normalization genes according to their expression stability in a given sample set and in a given experimental design. The Ct values from the QPCR were transformed to relative values (to linear scale values) using the ΔCt method and the expression (1+E)^ΔCt, where E is the QPCR efficiency for the actual gene. The algorithm estimates not only the overall expression variation of the 12 candidate genes but also the variation between sample subgroups (normal and tumor tissues).

Real-time QPCR. Design. The primers and TaqMan dual-labeled probes were designed using the software Beacon Designer (version 5.1, Premier BioSoft. All designs had an annealing temperature of 60°C.

Table II. List of primers and TaqMan probes used for the two triplex real-time QPCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
<th>5'-Fluorophore</th>
<th>TaqMan probe 5'-3'</th>
<th>3'-Quencher</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGAV</td>
<td>ggtgcatctggaacactg</td>
<td>atctgtaataactctctct</td>
<td>FAM</td>
<td>aggaacctggccctc</td>
<td>BHQ-1</td>
<td>139 bp</td>
</tr>
<tr>
<td></td>
<td>ccttcctgccttcct</td>
<td>tccgctccctcttctct</td>
<td>CY-5</td>
<td>atggcctactgtgagc</td>
<td>BHQ-2</td>
<td>89 bp</td>
</tr>
<tr>
<td></td>
<td>cctgactctgacatctct</td>
<td>gaaagcttggtccaaaacttg</td>
<td>CY-5</td>
<td>acagcacaccaaggccagggc</td>
<td>BHQ-1</td>
<td>125 bp</td>
</tr>
<tr>
<td></td>
<td>gcttccctgccttcctcttcct</td>
<td>ccctgccttcctctctcttcctg</td>
<td>CY-5</td>
<td>aggaacctggacccttacccaagtcag</td>
<td>BHQ-2</td>
<td>150 bp</td>
</tr>
</tbody>
</table>

The primers and probes were designed using the software Beacon Designer, version 5.1, Premier BioSoft. All designs had an annealing temperature of 60°C.

Optimization. We tested the primers in SYBR-Green I and optimized primers regarding concentrations between 100 and 600 nM for both forward (FP) and reverse (RP) primers. The optimized primer concentrations (final) were: integrin αv, FR/RP 300/600 nM, integrin β3, FP/RP 300/600 nM, VEGF FP/RP 300/600 nM, CD34 FP/RP 300/300 nM and TBP FP/RP 600/300 nM. The dual-labeled TaqMan probes were optimized regarding concentrations between 100 and 400 nM in a simplex QPCR. The results were: integrin αv 300 nM, integrin β3, 400 nM, VEGF 300 nM, CD34 300 nM and TBP 300 nM. The final QPCR designs are shown in Table II.
were quantified using the 2-ΔCT method (29). The data were

Quantification of the gene expression

The four genes of interest were tested in two triplex QPCR

For integrin β3 there was a borderline difference in the

Results

Housekeeping gene data analysis. Using the above-mentioned

VEGF, integrin αv, integrin β3 and CD34 gene-expression

(Fig. 2a-d). We found a significant lower mRNA level of

VEGF in NET compared to CRC liver metastases (VEGFNET;

meanlogRQN, 0.28±0.22; VEGFCRC, 1.45±0.21; p<0.001) Fig. 2a.

Compared to VEGF gene-expression level in normal liver
tissue (VEGFliver-N, 1.23±0.20), VEGF was significantly lower

in the NET than in the liver normal group (p<0.01) but not in

cRC metastases group (p=0.48).

For CD34 we found no difference in the mRNA levels in

NET compared to CRC metastases (CD34NET, -1.39±0.29;

CD34CRC, -0.78±0.35; p=0.19) Fig. 2b. Compared to CD34
gene-expression level in normal liver tissue (CD34liver-N,

-1.56±0.32), there was no significant difference between the

CD34 level in neither the NET (p=0.68) nor in the CRC

metastases group (p=0.11).

For integrin αv, there was no significant difference in the

mRNA level in NET compared to CRC metastases (integ-
in αvNET, -0.40±0.14; integrin αvCRC, -0.11±0.14; p=0.17)

Fig. 2c. Compared to integrin αv gene-expression level in

normal liver tissue (integrin αv liver-N, -0.52±0.19) there was

no significant difference between the level in either the

NET (p=0.58) or in the CRC metastases group (p=0.08).

For integrin β3 there was a borderline difference in the

mRNA level in NET compared to CRC metastases (integ-
in β3NET, -0.96±0.16; integrin β3CRC, -0.52±0.20; p=0.10) Fig. 2d.

Compared to integrin β3 gene-expression level in normal liver
tissue (integrin β3 liver-N, -0.58±0.11) there was a borderline

significant difference to the level in NET (p=0.06) but not to

the level in the CRC metastases group (p=0.79).

In NET the gene-expression of VEGF, integrin αv and

integrin β3, relative to the housekeeping gene (normalizer)

were highly variable. VEGF, minRQN, 0.15; maxRQN, 79.6;

530-fold; integrin αv, minRQN, 0.10; maxRQN, 2.25; 23-fold;

integrin β3, minRQN, 0.01; maxRQN, 1.06; 106-fold. The

calculated relative values (RQN) are not log10 transformed

Fig. 3. There were no significant correlations between VEGF,

integrin αv, integrin β3 or CD34 gene-expression either in

NET or in the CRC metastases group.
Discussion

We found a much lower gene-expression of VEGF in NET compared to CRC metastases. The high level of VEGF gene-expression in CRC metastases supports the use of VEGF targeting anti-angiogenic treatment in many clinical trials over the last decade (30,31). In 2004, Bevacizumab was FDA (Food and Drug Administration, USA) approved for the treatment of advanced colorectal cancer and treatment is usually in combination with Fluoururacil-based chemotherapy (32).

As NET generally are highly vascularized and believed to be characterized by high levels of VEGF expression, they are potentially susceptible to therapeutic strategies targeting pathways involved in angiogenesis. A phase I/II study of Fluoururacil, Leucovorin Calcium, and Oxaliplatin (Folfox®) with Bevacizumab in patients with advanced neuroendocrine tumors is currently in progress (http://www.cancer.gov/clinicaltrials/UCSF-04458). However, the suggested use of VEGF targeted therapy with e.g. Bevacizumab, also in patients with NET may be challenged by the substantially lower gene-expressions of VEGF in these patients observed in the present study. Both in NET and CRC there are large variations in VEGF gene-expression (>100-fold) indicating that selected cases of NET may still be suitable for VEGF targeted treatment. However, final outcome is determined by several additional factors like aggressiveness of tumor and therefore can only be tested through controlled clinical trials.

In one study integrin \( \alpha_v \beta_3 \) level (using immunohistochemistry) was found to be almost twice as high in colorectal cancer with liver metastases as in colorectal cancers without metastases. The integrin \( \alpha_v \beta_3 \) level has also been correlated with overall survival; a high vascular expression of integrin \( \alpha_v \beta_3 \) predicted a reduced relapse-free interval and a reduced...
overall survival (33). An αvβ3-antagonist, cyclic RGD peptide, Cilengitide (EMD121974, Merck KGaA, Darmstadt, Germany) has also been used as an agent in both animal studies and in clinical trials for anti-angiogenic cancer treatment (17-22). Cilengitide is currently in phase I and phase II clinical trials for the treatment of various cancers (34). However, also in the colorectal cancer group the results of anti-angiogenesis treatment have shown that in a substantial group of patients there was no or minor effect (3,13-15,35). In our study, there was a borderline significant lower level of integrin β3 in NET compared to CRC liver metastases and normal liver tissue (p=0.10; p=0.06). This could indicate that neo-angiogenesis has a low level in NET. However, it should be kept in mind that we did only include metastasizing CRC which have a high level of integrin αvβ3 (33). Furthermore, inter-individual variations of integrin αv and integrin β3 in the NET group were high (up to 100-fold). A Phase II study with a combination of the two chemotherapeutics Thalodamide (Thalomid®) and Temozolomide (Temodar®) has been executed with treatment of patients with metastatic neuroendocrine tumors. Thalodamide has anti-angiogenic activity through its ability to interfere with the VEGF and basic fibroblast growth factor (bFGF). The study showed an overall objective radiologic response of 25%, a biochemical response of 40% and a 2-year survival rate of 70% (36). Taken together these studies show that anti-angiogenesis treatment may be a possible treatment for patients with NET in the future but seems not to be very effective.

It should be noted that our sample size was not very large and thereby small differences, e.g. in the integrins, could have been overlooked. However, due to the limited availability of NET patients receiving surgery, we had to carefully calculate the sample size on basis of ability to demonstrate substantial differences of angiogenesis markers. In accordance of this we did indeed demonstrate a highly significant difference of VEGF between NET and CRC.

The variable results of anti-angiogenesis treatment combined with large variations in VEGF and integrin αvβ3 gene-expression points to the necessity of individual selection of patients suited for such treatment. We measured gene-expression in tumor tissue, but if this should be done in every-day routine a non-invasive technique would be preferable. As the ligand cyclic RGD binds to integrin αvβ3, this peptide can be used as an integrin αvβ3-antagonist inhibiting the tumor angiogenesis. Therefore, a non-invasive technique could be imaging of integrin αvβ3 using PET (positron emission tomography)-technique and the radiotracer [18F]Galacto-RGD (arginine-glycine-aspartic acid) (11). Imaging of integrin αvβ3 expression has already been validated in both animals and humans (35,37-40). These studies show that intensity of [18F]Galacto-RGD uptake correlates with αvβ3 expression (immunohistochemistry using αvβ3-specific antibody) in various tumors. Many previous studies have verified the content of integrins using immunohistochemistry, but to our knowledge no report exists on the quantitative gene-expression level of integrin αvβ3 in neuroendocrine tumors. As QPCR is a robust and reliable technique, this gives a great opportunity to quantify both the integrins and VEGF and CD34 and to relate the gene-expression level to tumor angiogenesis. The possibility to quantify integrin αvβ3 will be of great importance in future animal studies developing integrin-targeted radio-tracers (RGD-tracers) and testing drugs for anti-angiogenic treatment.

In conclusion, the main finding of our study was that the quantitative gene-expression levels of VEGF and integrin β3 were lower in NET than in CRC metastases and were highly variable. Therefore, individual selection of patients may be necessary if anti-angiogenesis treatment is to be successful in patients with NET.

Acknowledgements

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References


