SPANDIDOS PUBLICATIONS

Does the genotype of HHT patients with mutations of the *ENG* and *ACVRL1* gene correlate to different expression levels of the angiogenic factor VEGF?

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Received July 10, 2008; Accepted August 29, 2008

DOI: 10.3892/ijmm_00000058

Abstract. The aim of this study was to determine in what way HHT (hereditary hemorrhagic telangiectasia) patients with mutations for the endoglin (*ENG*) or activin receptor-like kinase 1 (*ACVRL1)* gene show different expression levels of the angiogenic factor VEGF (vascular endothelial growth factor) by correlating VEGF to the HHT genotype. In 18 HHT patients, who were screened for *ENG* and *ACVRL1* gene mutations and 25 healthy controls the VEGF plasma level as well as the VEGF tissue expression were determined by ELISA technique and cryostat sections of the nasal mucosa. In general, the VEGF plasma levels as well as the VEGF tissue expression were significantly higher in HHT patients compared to healthy controls. However, the correlation of VEGF to the HHT genotype did not show any significant differences, i.e. the VEGF plasma levels as well as the VEGF tissue expression in HHT patients with *ENG* gene mutations did not differ significantly to those of HHT patients with *ACVRL1* gene mutations or mutations for both the genes. In spite of the fact that the angiogenic factor VEGF seems to play an important role in the pathogenesis of HHT, it cannot serve as a specific diagnostic screening marker. These results underline the importance and necessity of molecular analyses in HHT patients.

Introduction

Hereditary hemorrhagic telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, is an autosomal-dominant disorder which is characterized by the occurrence of multi-systemic vascular malformations with haemorrhages and bleedings due to associated vascular lesions. The prevalence of HHT is 1 in 8,000-10,000 individuals (1). The main clinical symptoms in patients with HHT include recurrent epistaxis, cutaneous and mucocutaneous telangiectases as well as arteriovenous malformations (AVM) in the pulmonary, cerebral, gastrointestinal or hepatic vasculature (2,3). These clinical features together with the inheritance of the disease have been classified, according to the Consensus Clinical Diagnostic criteria of the Scientific Advisory Board of the HHT Foundation International Inc., as the Curaçao criteria (3). These parameters define a 'definite HHT manifestation' where three out of four criteria are present, a 'suspected HHT manifestation' with two criteria, or an 'unlikely HHT manifestation' with one criterion. Clinical manifestations of HHT are highly heterogeneous between families as well as within members of the family itself (4,5).

Linkage studies revealed that mutations of primarily two genes cause HHT. The HHT type 1 locus was mapped to chromosome 9q33-34 and encodes the endoglin (*ENG*) gene (6). The HHT type 2 locus was mapped to chromosome 12q13 and encodes the activin receptor-like kinase 1 (*ACVRL1* or *ALK1*) gene (7,8). Both *ENG* and *ALK1* are expressed predominantly in endothelial cells and play distinct roles in the transforming growth factor-ß (TGF-ß) signalling pathway which is known to regulate the vascular development, homeostasis and integrity (7-10). Studies performed on a murine model have proposed that the defect of both the genes, impairing the TGF-ß signalling pathway, leads to the persistence of the activation phase of angiogenesis where angiogenic factors, such as the vascular endothelial growth factor (VEGF), are produced and play a potential role in angiodysplasia (11-14). VEGF, also known as vascular permeability factor, is a 32-45 kDa heparin-binding cytokine that induces the proliferation and migration of endothelial cells to form new vessels and increase vascular permeability (15). *In vitro*, it is produced by a number of different cells, e.g. endothelial cells, smooth muscle cells, fibroblasts, macrophages, when exposed to hypoxic conditions or stimulated with interleukin or transforming growth factor-ß (TGF-ß). VEGF as a secreted and diffusible angiogenic mitogen can be detected both in plasma and serum samples of patients (16). It has been demonstrated to be involved in normal but also pathological processes including tumor growth,

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Key words: hereditary hemorrhagic telangiectasia, endoglin, activin receptor-like kinase 1, genotype, vascular endothelial growth factor, angiogenesis

metastasis, psoriasis, rheumatoid arthritis, retinopathies, collateral vessel formation in ischemic tissues, several vascular diseases and inflammation (17-21). The high serum VEGF levels in these diseases have proposed VEGF as a possible diagnostic marker for disease progression and severity (22,23). Already in our previous studies, we verified this phenomenon even in patients with HHT (24,25). However, the correlation of VEGF to the genotype in inherited vascular disorders such as HHT still needs to be elucidated. Therefore, the aim of this study was to determine in what way HHT patients with mutations for the *ENG* or *ACVRL1* gene show different expression levels of the angiogenic factor VEGF, in order to gain further insight into the role of VEGF in HHT pathogenesis.

Patients and methods

Patients and tissue samples. Eighteen consecutive patients with clinically manifest hereditary hemorrhagic telangiectasia (HHT) were enrolled in this prospective study at our Department of Otolaryngology and Head and Neck Surgery, University Hospital of Mannheim, Germany. Nine patients were female and 9 patients male with a mean age of 56 years (range, 21-89 years). Patients were considered to be affected with HHT if they met at least three out of the four cardinal symptoms - epistaxis, telangiectasia, family history of the disorder and organ manifestation - according to the current Curaçao criteria (3). The HHT patients were screened for *ENG* and *ACVRL1* gene mutations. Altogether, six HHT patients had mutations for the *ENG* gene with 1 deletion, 1 insertion and 4 missense mutations. Ten HHT patients showed mutations for the *ACVRL1* gene with 2 deletions, 3 insertions, 5 missense mutations. Two HHT patients revealed for both the genes *ENG* and *ACVRL1* missense mutations. The phenotype revealed in 83% of the HHT patients (5 out of 6 patients) with *ENG* gene mutations a lung manifestation, whereas 40% of the HHT patients (4 out of 10 patients) with *ACVRL1* gene mutations had liver manifestations.

A group of 25 healthy volunteers without history of a known neoplasm, without recent trauma or surgery and who were not pregnant served as controls. In both groups of patients, the VEGF plasma levels were determined by standard ELISA assay. The VEGF plasma levels were then correlated to the HHT genotype, comparing the results between HHT patients with *ENG* gene mutations, HHT patients with *ACVRL1* gene mutations and HHT patients with mutations for both the genes. For VEGF immunohistochemistry, tissue samples of the nasal mucosa from 10 HHT patients (4 HHT patients with *ENG* gene mutations, 4 HHT patients with *ACVRL1* gene mutations and 2 HHT patients with *ENG* and *ACVRL1* gene mutations) and 5 healthy controls were obtained. The tissue samples were collected in liquid nitrogen and stored at -70˚C. They were then cut in 6-μm thick sections and fixed in acetone for immunostaining. All studies were approved by the Ethics Committee of the Medical Faculty of Mannheim, University of Heidelberg, Germany. Informed consent was obtained from all subjects prior to the study.

VEGF plasma levels. Peripheral venous blood samples (10 ml) were drawn from each subject and collected in sterile test tubes, centrifuged at 3,000 rpm for 15 min. Plasma was then stored at -70˚C until assayed collectively for VEGF levels by an investigator who was blind to the patient assignment. The plasma VEGF concentrations were determined by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique (R&D System, Wiesbaden, Germany) as described previously (24,25). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against human recombinant VEGF pre-coated onto a 96-well microtiter plate. According to the manufacturer's instructions, each ELISA measured 100 μl serum. All analyses and calibrations were carried out in duplicate. Optical density was determined using a microtiter plate reader (Dynatech) at 450 nm. Wavelength correction was set at 540 nm and concentrations were given in picograms/milliliter (pg/ml). Plasma VEGF concentrations were determined without knowledge of the main clinicopathological features and the genotype of the patients studied.

Immunohistochemistry for VEGF. Immunohistochemistry for VEGF expression was performed for light microscopy (Zeiss Axiophot) by using a streptavidin-biotin complex procedure as described previously. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were then incubated with 10% normal sheep serum in phosphate-buffered saline (PBS) solution for 30 min, followed by an overnight incubation at 4˚C with rabbit anti-human VEGF polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany; dilution 1:50). Next, each slide was treated with biotinylated anti-rabbit immunoglobulin for 10 min and then incubated with streptavidin-peroxidase complex for 45 min. Aminoethyl-norbazole (AEC) was used as a chromogen and nuclear counterstaining was performed with Mayer's hematoxylin solution. Negative controls were performed using PBS instead of primary antibody, resulting in no detectable staining. Immunoreactivity was evaluated semi-quantitatively using following grading range: samples with weak staining <25% were graded '+', samples with medium staining between 25-50% were graded '+ +' and samples with strong staining >50% were graded as '+ + +'. Immunohistochemical staining was assessed independently by two investigators without knowledge of the clinicopathological findings and the genotype of the patients studied.

Statistical analyses. Data were expressed as mean ± standard deviation (SD). For statistical analyses, VEGF plasma levels of HHT patients (carrying mutations for the *ENG* or *ACVRL1* gene) and healthy controls were compared using the Student's t-test for two samples. Differences with p-values <0.05 were considered statistically significant. The SPSS software package for Windows was used to perform all statistical analyses.

Results

VEGF plasma levels in HHT patients and healthy controls. The VEGF plasma levels in all the HHT patients (n=18) and the healthy controls (n=25) were determined by an ELISA assay. The assay revealed that in general, the majority of

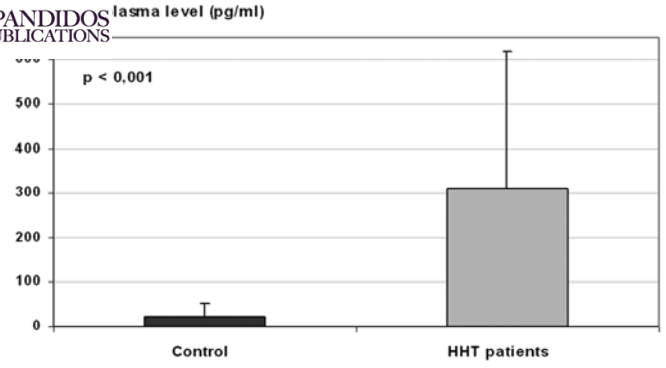

Figure 1. VEGF plasma concentration levels in 25 healthy controls and 18 HHT patients. The mean values and the standard deviation are given for each group (p<0.001).

patients with HHT had significantly higher plasma VEGF concentrations than the healthy control group (p-<0.001, t-test). The VEGF levels of all the HHT patients ranged from 10 to 1480 pg/ml with a mean value of 321 pg/ml. In contrast, the VEGF plasma levels in the healthy control group ranged from below the detection limit to 172 pg/ml with a mean value of 25 pg/ml (Fig. 1).

VEGF plasma levels amongst HHT patients with ENG and ACVRL1 gene mutations. Within the group of HHT patients, the VEGF plasma levels were determined in 6 patients with *ENG* gene mutations, 10 patients with *ACVRL1* gene mutations and 2 patients with mutations of both the genes. Other than the comparison to the healthy control group, the correlation of the VEGF plasma concentration to the HHT genotype did not reveal any significant differences (p=0.53, t-test). In HHT patients with mutations of the *ENG* gene, the VEGF plasma level ranged from 88 to 578 pg/ml (mean value 237 pg/ml), in patients with *ACVRL1* mutations the VEGF plasma level ranged from 58 to 715 pg/ml (mean value 339 pg/ml) and in patients with mutations of both the genes the VEGF plasma level ranged from 390 to 582 pg/ml (mean value 487 pg/ml), respectively (Fig. 2).

VEGF immunostaining in HHT patients and healthy controls. The nasal tissue samples from 10 HHT patients and 5 healthy controls were also analysed by immunohistochemistry for the expression of VEGF. Altogether, the HHT tissue samples clearly demonstrated a stronger presence of VEGF within the investigated HHT tissue samples than in those of healthy controls. Under light microscopy, VEGF staining appeared to be mainly localised within regions of multiple angiodysplasia in the endothelial layers of the vessels but also in the stroma. Thus, the distribution pattern of the VEGF staining reaction in HHT tissue samples was more heterogeneous. Taken together, 6 HHT cryostat sections (60%) with medium staining between 25-50% were graded '+ +' and 4 HHT cryostat sections (40%) with strong staining >50% were graded '+ + +'. All sections of the healthy control group revealed a weak staining <25% and were graded '+'. Representative photomicrographs of VEGF staining are shown in Fig. 3a and b.

VEGF immunostaining amongst HHT patients with ENG and ACVRL1 gene mutations. VEGF staining was determined in nasal tissue samples from 4 HHT patients with *ENG* gene mutations, 4 HHT patients with *ACVRL1* gene mutations, 2 HHT patients with mutations of both the genes and 5 healthy controls. Similar to the results of the VEGF-ELISA assay, the analysis of tissue samples of patients with different HHT genotype did not show any significant difference in the tissue staining for VEGF. Thus, the HHT tissue samples of patients with either *ENG* or *ACVRL1* mutation could not be correlated to a differentiating expression pattern of VEGF. All cryostat sections revealed a medium ('+ +') to strong ('+ + +') VEGF

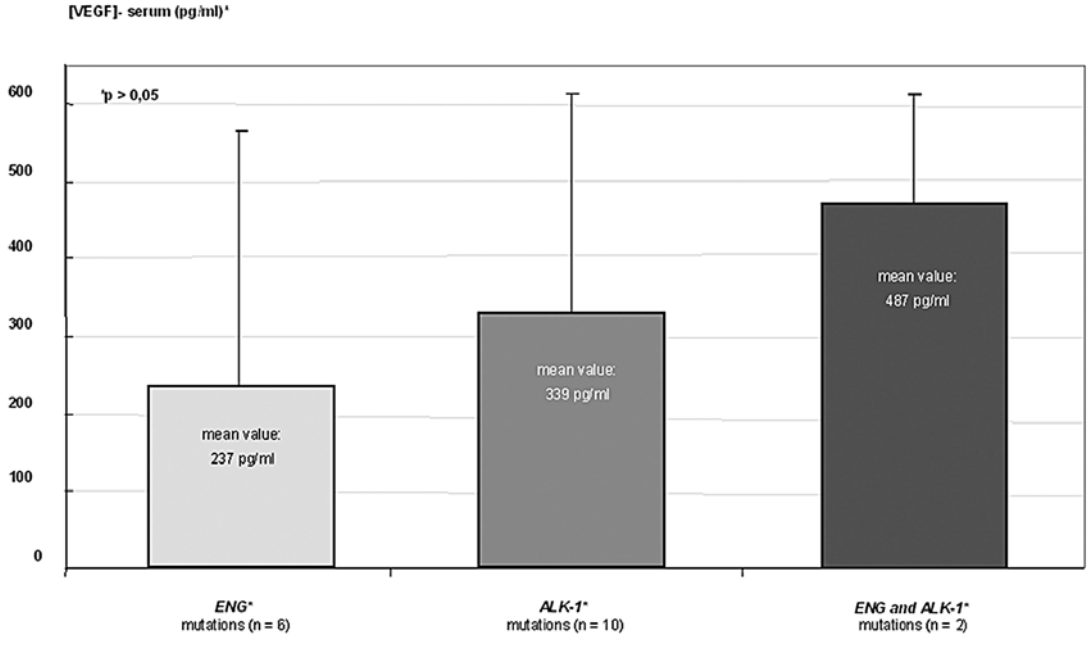

Figure 2. VEGF plasma concentration levels in HHT patients with known mutations for the *ENG* gene (n=6), the *ACVRL1* gene (n=10) and both *ENG* and *ACVRL1* genes (n=2). The mean values and the standard deviation are given for each group (p>0.05).

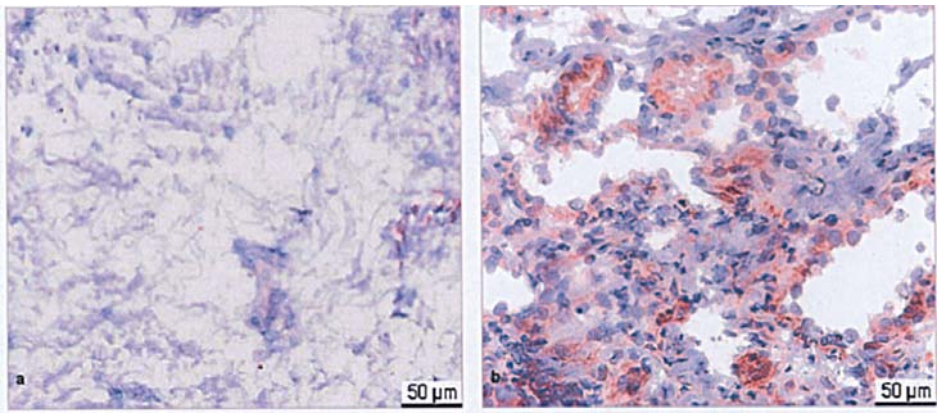

Figure 3. (a and b) Immunohistostaining for VEGF in cryostat sections of nasal mucosa tissue samples of 5 healthy controls and 10 HHT patients. (a) Representative VEGF expression in cryostat sections of healthy controls with weak staining graded as '+'. (b) VEGF expression in HHT cryostat sections with medium to strong staining graded as '+ +' to '+ + +', (magnification, x 200).

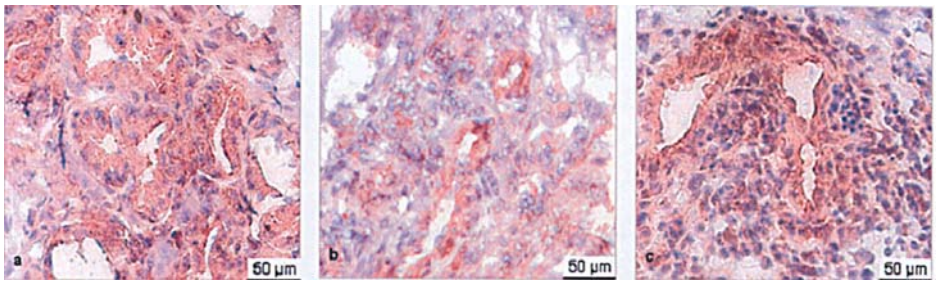

Figure 4. (a-c) Immunohistostaining for VEGF in cryostat sections of nasal mucosa tissue samples of HHT patients. All cryostat sections revealed a medium ('+ +') to strong ('+ + +') VGEF staining. (a) Representative VEGF expression in cryostat sections of HHT patients with *ENG* gene mutations, (b) with *ACVRL1* gene mutations and (c) with *ENG* and *ACVRL1* gene mutations, (magnification, x 200).

staining. Representative photomicrographs of VEGF staining are shown in Fig. 4a-c.

Discussion

The vascular endothelial growth factor, VEGF, is one of the best characterized pro-angiogenic factors with an important role in angiogenesis. VEGF is said to induce the migration and proliferation of endothelial cells, to increase the vascular permeability and to modulate chemotaxis of granulocytes and macrophages (26). Previous studies were able to demonstrate that VEGF is needed to maintain vascular integrity but that it also promotes the survival of new vessels formed in tumors and increases the capillary differentiation and formation of a vascular network (15). As already described in the literature, especially *in vitro* studies, VEGF is produced as a secreted protein by different cell lines such as endothelial cells, smooth muscle cells or macrophages, when exposed to hypoxic conditions or simulated with interleukin (15). The overexpression of VEGF has been identified as a major factor underlying pathological angiogenesis in conditions such as psoriasis, macular degeneration and tumor proliferation. Many studies have evaluated high levels of VEGF in patients with tumor diseases, rheumatic disorders, retinopathia collateral vessel formation in ischemic tissues, several vascular diseases and inflammation, thus considering this growth factor as a possible marker of disease progression and severity (17-21).

Hereditary hemorrhagic telangiectasia (HHT) provides an example of a genetic disorder of angiogenesis in which a multi-systemic angiodysplasia is probably induced by an impaired balance between several growth factors (2,14,27-29). In their studies published in 2000, Jacobson as well as Bourdeau *et al* have described angiogenesis to be the key event in the persistence of HHT (12,13). It is a complex multi-step process which is tightly controlled by a large number of pro-angiogenic and anti-angiogenic factors and their receptors. The genes responsible for these alterations are endoglin (*ENG*) and *ACVRL1*, both signaling elements of the TGF-ß1 receptor system (12-14). Transforming growth factor (TGF-ß1) itself is a multifunctional protein that is thought to play an important role in vascular remodelling. TGF-ß1 inhibits the activities of angiogenic factors in endothelial cell proliferation and migration and can stimulate the production of extracellular matrix proteins. It displays a biphasic effect on angiogenesis (30). TGF-ß1 is a known angiogenic factor that can stimulate the VEGF production in endothelial cells. However, up till now only few studies have focused on the role and function of VEGF in patients with HHT, which represents a genetic disorder of angiogenesis with its characteristic features of

temic angiodysplasia. A possible interaction of s best characterized pro-angiogenic factor, in the pathogenesis of HHT was published in a study by Sabba *et al* in 2001 (14). One of the main reasons for their hypothesis was the fact that immunohistochemical studies in pediatric and adult cases showed a strong VEGF expression in cerebral AVMs whereas endoglin was equally expressed in cerebral and pulmonary AVMs as well as in phenotypically normal endothelium (15). The same Italian research group also determined significantly higher VEGF serum levels in HHT patients than in healthy controls (14,31).

To our knowledge, this is the first study which analyses whether HHT patients with known mutations for the *ENG* and *ACVRL1* gene show different expression levels of the angiogenic factor VEGF. In order to correlate VEGF to the HHT genotype, the VEGF plasma levels were determined in both, HHT patients with *ENG* and *ACVRL1* mutations and healthy controls by ELISA technique. As it has been recommended in the literature (32) and already been described in one of our previous studies, we deliberately measured the VEGF concentrations in plasma instead of sera of all patients. The main reason is that serum concentrations need to be correlated with the platelet count (33) and most of serum VEGF is released from activated platelets during blood clotting after sample collection which can falsify the outcome measure. These reasons make plasma levels a more accurate indicator for the actual VEGF concentration. In a second step, the VEGF tissue expression was determined by immunohistochemistry in HHT patients and healthy controls. Similar to our previous study, we demonstrated that VEGF seems to play a major role in HHT pathogenesis. Again we confirmed previous observations revealing increased VEGF plasma levels and a strong VEGF tissue expression in HHT patients which conforms with the study of Cirulli *et al* who determined the VEGF serum levels in HHT. They found significantly higher VEGF serum levels in HHT patients than in healthy controls, proposing VEGF as a possible diagnostic marker for HHT screening (31). The idea whether VEGF could serve as a diagnostic screening marker prompted us to determine in a further step whether VEGF can be correlated to the HHT genotype. However, we did not reveal any significant differences in the expression levels of VEGF and the *ENG* or *ACVRL1* gene mutations in HHT patients. So at present, it is not possible to differentially diagnose the type of HHT accurately on the basis of VEGF serum levels or tissue expression, although knowing that HHT patients in general show significantly higher VEGF expression levels than healthy controls. The fact that the genotype in HHT patients cannot be correlated to the VEGF serum levels and VEGF tissue expression, indicates the importance and necessity of molecular analysis and genotyping to identify the presence of mutations within the *ENG* or *ACVRL1* gene in HHT patients.

This is a further study, elucidating the role of the angiogenic factor VEGF in patients with HHT. Although the pro-angiogenic factor VEGF seems to play a greater pivotal role in HHT pathogenesis, this study showed that it cannot serve as a specific diagnostic screening marker to determine the HHT genotype.

Acknowledgements

We would like to thank Petra Prohaska for her excellent technical assistance.

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