Differential Expression of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ in Angiomas and Squamous Cell Carcinoma Cell Lines of the Head and Neck

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Abstract. *Vascular endothelial growth factor A (VEGF) is one of the major regulators of angiogenesis. It plays an important role during the process of physiological and pathological neovascularization. Variant VEGF isoforms are generated as a result of alternative pre-mRNA splicing. To determine the expression of VEGF isoforms in angioma and head and neck squamous cell carcinoma (HNSCC), both being dependent on pathological neovascularization, we included 11 HNSCC cell lines, 4 hemangiomas and 5 vascular malformations (VMs) in the study. Tonsil mucosa served as normal control. Using reverse transcription polymerase chain reaction (RT-PCR) sequencing, the VEGF isoforms $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$ were regularly detected in all tested samples. $VEGF_{121}$ was the most abundant isoform in all tested tissues, whereas $VEGF_{165}$ exhibited lower levels, and $VEGF_{189}$ only very small amounts of transcript. Interestingly, VMs expressed significantly higher ($p=0.0286$) amounts of $VEGF_{121}$ compared with hemangiomas, which had levels similar to normal control mucosa. HNSCC cell lines demonstrated on-average higher levels of all three isoforms compared with the controls. Consistent with the clinical staging, a trend for VEGF overexpression was observed in tumor cells derived from N+ tumors compared to those derived from N0 tumors. One drawback of this study was the small number of specimens available, particularly since VMs and hemangiomas are relatively rare diseases. Future studies need to follow-up on these observations and further evaluate the potential role of specific VEGF isoforms in the pathogenesis of hemangioma and VM.*

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Angiogenesis is widely required for a variety of physiological and pathological proliferative processes such as tumorigenesis, inflammation and wound healing (1). Abundant evidence has indicated that vascular endothelial growth factor A (*VEGF*-A, commonly referred to as *VEGF*) is one of the most important endothelial cell-specific mitogens and angiogenesis inducers. It is involved at almost every stage of vascular development.

VEGF exists in multiple isoforms that are formed during alternative pre-mRNA splicing particularly between exons 6 and 8 of the *VEGFA* gene. Whereas exons 6 and 7 confer heparin-binding affinity, exon 8 is involved in promoting mitosis (2). $VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$ are the main isoforms of the human *VEGF* family (3). In most systems, $VEGF_{121}$ and $VEGF_{165}$ represent the major species, whereas $VEGF_{189}$ is found to be only minimally expressed (4). The different isoforms appear to have similar functions but differ in their binding affinity for extracellular matrix (ECM) molecules. The larger isoforms, $VEGF_{165}$ (lacking exon 6) and $VEGF_{189}$ (lacking exon 6b), have higher affinities for heparin and therefore usually remain tightly associated with the ECM. In contrast, $VEGF_{121}$ is freely diffusible since it lacks exons 6 and 7 (2, 3). Recently, another anti-angiogenic splice isoform, $VEGF_{165b}$, has been found in normal kidney cells (5).

VEGF is reportedly overexpressed in most of solid human and animal tumors but is also found highly expressed in several types of normal adult epithelia such as those present in kidney, lung and breast (6, 7). It is also expressed at high levels in sarcomas, hematological malignancies, multiple myeloma, and malignant vascular tumors (8-11). Increased expression of *VEGF* is a frequent observation in some premalignant lesions (12, 13). The role of *VEGF* in angiogenesis at early stages of tumor development is not fully understood.

Head and neck squamous cell carcinomas (HNSCCs), hemangiomas and vascular malformations (VMs) are diseases implicated with angiogenesis. Much of the

Table I. *Characteristics of HNSCC cell lines.*

ID	Cell line	Patient age (years)	Gender	TNM stage	N status
T1	UM-SCC-3	73	F	T1N0M0	N0
T2	UM-SCC-27	62	M	T1N0M0	N0
T3	UM-SCC-9	71	F	T2N0M0	N0
T4	UM-SCC-1	73	M	T2N0M0	N0
T5	UT-SCC-24A	41	M	T2N0M0	N0
T6	UT-SCC-26A	60	M	T1N2M0	N+
T7	UMB-SCC-745	48	M	T4N2M0	N+
T8	UMB-SCC-969	67	M	T4N2M1	N+
T9	UM-SCC-4	47	F	T3N2M0	N+
T10	UMB-SCC-864	59	M	T2N2M0	N+
T11	UM-SCC-22B	58	F	T2N1M0	N+

Table II. *Primer sequences used.*

Primer ID	Sequence
P1199	5'-GACCCTGGTGGACATCTT-3'
P1818	5'-TTTCTGTATCGATCGTTCTGTA-3'
P1245	5'-ATCTTCAAGCCATCCTGTG-3'
P1796	5'-TCAGTCTTTCCTGGTGAGAGAT-3'
Beta-actin 3'	5'-GTGCCTCAGGGCAGCGGAACCGCTCA-3'
Beta-actin 5'	5'-GATGATGATATCGCCGCGCTCGTC-3'

angiogenesis research about *VEGF* in this field focuses on HNSCC (14), and rarely on angiomas such as hemangiomas and VMs. The aim of this study was to evaluate the presence and expression level of *VEGF* splice variants in HNSCC cells, hemangiomas, VMs and normal mucosal tissues. This information could provide the basis for further studies in helping to elucidate the role of specific *VEGF* variants in angiogenesis.

Materials and Methods

Cell lines and tissues. Eleven different HNSCC cell lines (15), were divided into two groups according to TNM stage (Table I), 5 belonged to group N0 (derived from tumors without clinically detectable lymph node metastasis, T1-T5) and 6 to group N+ (derived from tumors with lymph node metastases, T6-T11). Tissues were derived from 4 hemangiomas and 5 VMs. The number of tissues was limited since angioma cases are rare. Four normal tissues (tonsil mucosa) were used as a control. All the tissues were obtained immediately after a scheduled surgery and were flash-frozen and kept in liquid nitrogen. The Ethics Committee approved the use of tissues for research purposes. Tissues were used with informed consent of the patient.

RNA extraction and RT-PCR. Total RNA of tissues and cultured cell lines were isolated with TRIzol reagent (Invitrogen). Reverse transcription was carried out with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Basel, Switzerland) using an oligo-dT primer. Subsequent polymerase chain reaction (PCR) was performed with the REDTaq™ ReadyMix™ PCR reaction Mix (SIGMA, Sigma-Aldrich Corp. St. Louis, MO, USA). Primers to amplify either the exon 8 or 8b containing transcript (Table II) were designed by the Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA). The first primer set is complementary to exon 3 (P1199) and the 3'-UTR (P1818). Reactions were performed in a final volume of 25 μl using 1 μl cDNA as template, 0.5 μl (50 pmol/μl) of each primer, 12.5 μl PCR reaction mix and 10.5 μl water. PCR settings were as follows: 94˚C for 30 s, 63˚C for 30 s and 72˚C for 60 s; all steps were repeated 35 times. The first-round PCR products were diluted 1:10 and 1.0 μl of the dilution was used as template for the second-round PCR. The second set of primers was complementary to exon 3 (P1245) and to exon 8b (P1796). PCR settings for the second round were: 94˚C for 30 s, 53˚C for 30 s and 72˚C for 60 s; all steps were repeated 35 times. In each reaction, ß-actin was used as an internal control. PCR products were analyzed via electrophoresis on 8% polyacrylamide gels containing 0.5 μg/ml ethidium bromide, and were subsequently quantified with the Gel Doc™ 2000 Gel Documentation System using QuantityOne software (BioRad, Hercules, USA).

PCR cloning and sequencing. PCR bands were excised from 2% agarose gels using UV transillumination, and DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). In accordance with the StrataClone™ PCR Cloning Kit (Stratagene, La Jolla, CA, USA) manual, the DNA was digested and ligated into a PCR cloning vector that was used to transform competent bacterial cells. Growing on Luria-Bertani broth (LB) plates in the presence of 100 μg/ml ampicillin and X-Gal (SIGMA) overnight at 37˚C, white clones were picked and cultured overnight at 37˚C in LB media in the presence of 100 μg/ml ampicillin. The plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced with T3- or T7-specific primers (4base lab GmbH, Reutlingen, Germany).

Statistical analysis. Nonparametric tests were used for the limited samples tested in our studies. The Mann-Whitney test was used to evaluate statistical significance when two independent groups were compared, whereas the Kruskal-Wallis test was used to compare different expression levels between three or more groups. A p-value less than 0.05 was considered statistically significant.

Results

RT-PCR sequencing of VEGF isoforms. Total RNA was extracted from tissues and HNSCC cell lines (Table I) and was subsequently reverse-transcribed to cDNA. Variant *VEGF* isoforms were amplified by RT-PCR using primer pairs P1245/1796 and P1199/1818 (Table II). PCR products were analyzed on 2% agarose gels containing 0.5 μg/ml ethidium bromide. Three major bands were identified at 499 bp, 427 bp and 295 bp, respectively. Subsequent TA cloning and DNA sequencing confirmed the PCR bands to represent the $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$ isoforms. No anti-angiogenic *VEGF* isoforms ($VEGF\text{-}_{xxxb}$) were detected in our studies. The *VEGF* isoforms present in normal tissues, vascular malformation, hemangiomas and HNSCC cell lines

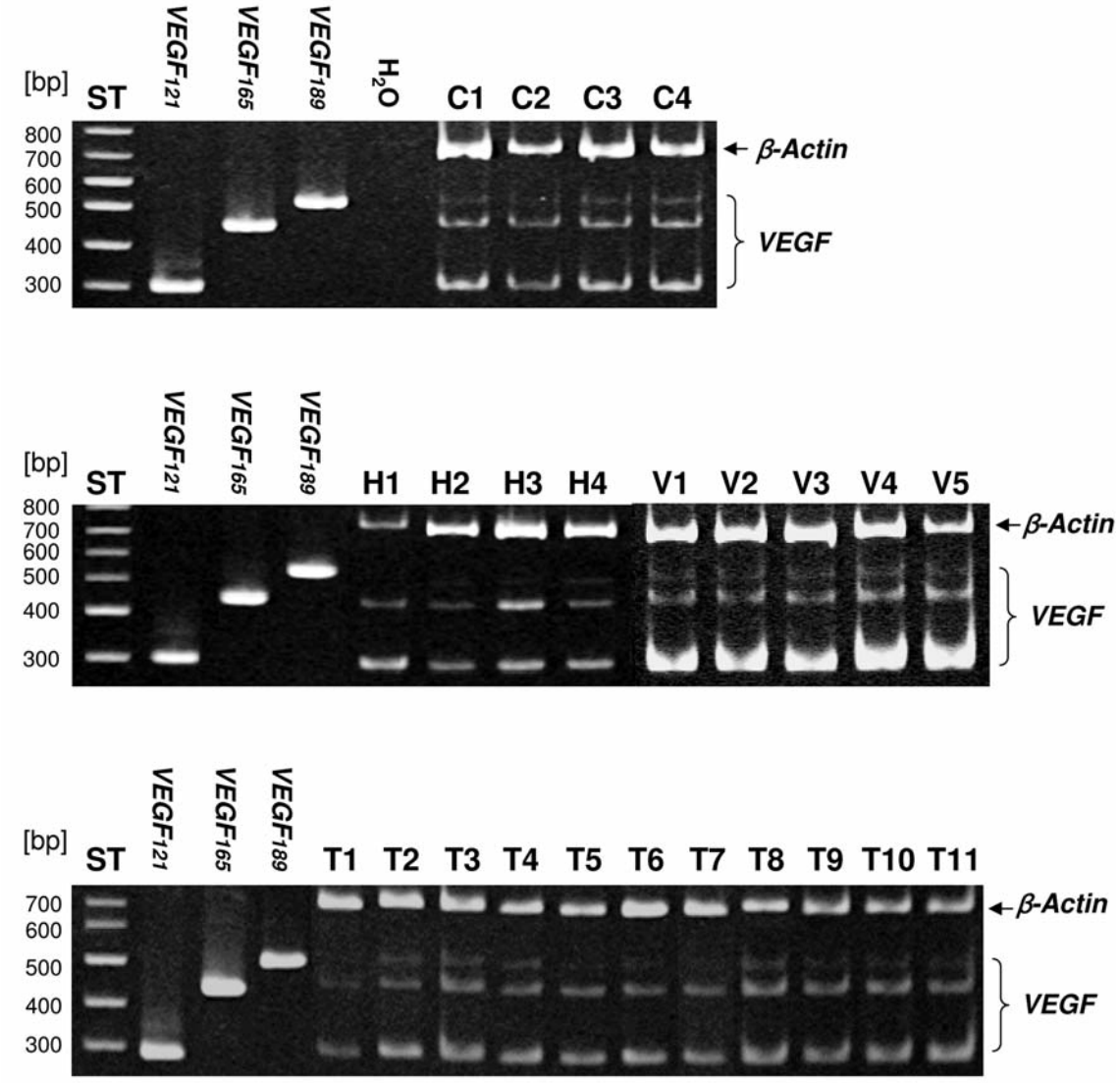

Figure 1. *Detection of VEGF isoforms by RT-PCR. PCR products were analyzed on 8% PAGE. Bands consistent with $VEGF_{189}$ (499 bp), $VEGF_{165}$ (427 bp) and $VEGF_{121}$ (295 bp) were found in all tested samples: C1-C4, normal control tissues; H1-H4, hemangiomas; V1-V5, vascular malformations and T1-T11, HNSCC cell lines. β-Actin (780 bp) was used as an internal control.*

were amplified by RT-PCR using the P1245/P1796 primer pair and analyzed on 8% PAGE. $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$ were found in all tested samples (Figure 1).

Relative distribution of VEGF isoforms within specific groups. The relative distribution of the three *VEGF* isoforms was similar in hemangiomas, HNSCC cell lines and normal tissues, however, the $VEGF_{121}$ isoform was found to dominate in VMs (Figure 2).

Comparison of VEGF isoform expression between the tested specimens. Quantification of PCR bands (Figure 1) was performed (see Materials and Methods). Although not reaching significance, the expression levels of the three *VEGF* isoforms were on average higher in HNSCC cells than in angiomas and normal tissues, except for the $VEGF_{121}$ isoform found in VMs that reached levels comparable to those of HNSCC cells. In all tested samples, $VEGF_{121}$ exhibited higher levels than the other two isoforms, with $VEGF_{189}$ being the lowest ($p<0.05$) (Figure 3A). $VEGF_{121}$ was found to be significantly higher ($p<0.05$) in VMs (mean expression relative to that of β-actin 0.66±0.15) compared with hemangioma (mean 0.28±0.02), which had expression levels comparable to those of normal tissues (mean 0.34±0.08). *VEGF* expression was determined from 11 HNSCC cell lines and cell lines derived from N0 stage tumors were compared

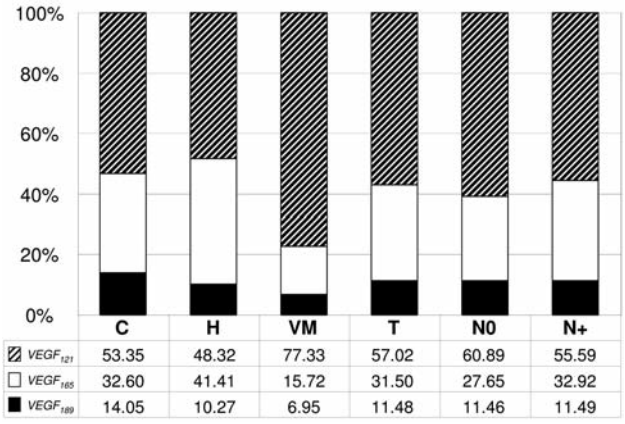

Figure 2. *Relative ratio of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ in the tested tissues. The proportion of the three VEGF isoforms is depicted for control (C), hemangioma (H), vascular malformation (VM) and HNSCC cell lines (T, N0, N+). The proportion of $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$ appears similar in the C, H, T, N0 and N+ groups, whereas $VEGF_{121}$ dominates (77.33%) in VMs.*

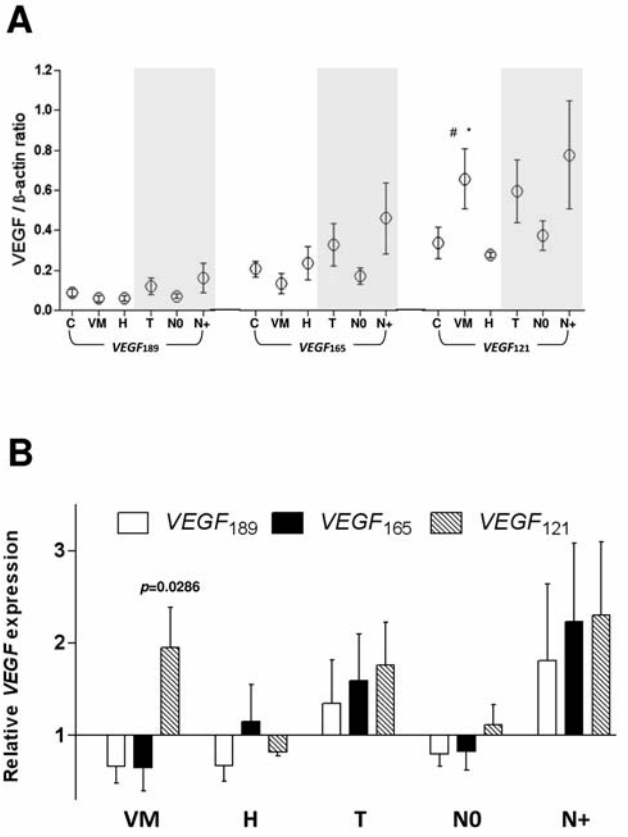

Figure 3. *A: Expression levels of VEGF isoforms in angiomas and HNSCC cell lines. Expression of the major VEGF isoforms ($VEGF_{189}$, $VEGF_{165}$, $VEGF_{121}$) were compared with normal controls (C), vascular malformation (VMs), hemangiomas (H) and HNSCC (T, N0, N+). $VEGF_{121}$ was expressed at higher levels in all tested samples, significantly in VMs when compared with the other two isoforms $VEGF_{165}$ and $VEGF_{189}$ (*$p<0.05$). Significance was also reached when the $VEGF_{121}$ levels found in VMs were compared with that in hemangiomas ($p=0.0286$). $VEGF_{189}$ was regularly found at lower levels than the other two isoforms ($p<0.05$) and HNSCC cell lines (shaded area) showed a tendency to up-regulate all three isoforms compared with control tissues and angiomas. This difference was even more pronounced in the higher stage N+ group, whereas the levels in the lower stage N0 group did not differ from those of normal control mucosa. B: Comparison of VEGF expression levels in the different tested groups. The values of VEGF expression relative to normal control mucosa (set as 1) are shown. Note the up-regulation of all three transcripts in the HNSCC cell (T and N+) population and the single and significant ($p<0.05$) up-regulation of $VEGF_{121}$ in VMs.*

with those derived from N+ tumors (Figure 3A, grey area). Consistent with clinical staging N+ cell lines exhibited higher average levels of *VEGF* transcripts than N0 cell lines, which had levels comparable to those of controls.

Comparison of relative VEGF expression levels in the different tested groups. To be able to better compare changes in the *VEGF* expression levels of the different tested specimens, expression values were related to the values for normal control tissue. Figure 3B shows the ratios of *VEGF* expression levels, with 1 marking the expression level of normal tissue. It becomes clear that particularly the N+ HNSCC cell lines overexpress all three isoforms, whereas expressions of the N0 HNSCC cell lines were no different from those of control cells. Interestingly, $VEGF_{121}$ was significantly elevated in VMs, whereas the other two isoforms did not show any difference from normal values. No clear changes in *VEGF* expression was observed in hemangioma tissues compared to normal controls (Figure 3B).

Discussion

The human *VEGF*A gene is located on the short arm of chromosome 6. Several different *VEGF* isoforms are generated due to alternative pre-mRNA splicing. The major isoforms regularly found in tissues are $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. Other isoforms such as *VEGF*183, $VEGF_{145}$, $VEGF_{148}$, and $VEGF_{111}$ have been reported (16), but their physiological significance remains unclear. Organ specificity was reported for different *VEGF* isoforms. For example in rodent animal models, the lung, heart and liver express high levels of $VEGF_{188}$ which is equivalent to the human $VEGF_{189}$ (17, 18), whereas brain, eyes, muscles, kidneys had highest expression levels of $VEGF_{164}$ and $VEGF_{120}$ (equivalent to $VEGF_{165}$ and $VEGF_{121}$) (19). Similarly, $VEGF_{145}$ was preferentially observed in carcinomas of the female reproductive system and $VEGF_{206}$ expression was found to be restricted to embryonic tissues. Increased ratio of $VEGF_{121}$ relative to $VEGF_{165}$ or $VEGF_{189}$ was reported to be essential

for the angiogenic phenotype of prostate cancers (20). Anti-angiogenic *VEGF* isoforms were previously reported to be expressed in normal tissues and to a lesser extent in tumor tissues. Our studies regularly detected pro-angiogenic *VEGF* isoforms whereas anti-angiogenic isoforms were never detected in our settings. $VEGF_{165}$ and $VEGF_{121}$ were the major isoforms that were found expressed in all tested samples, whereas $VEGF_{189}$ appeared to be expressed at much lower levels. $VEGF_{165}$ is considered one of the most potent pro-angiogenic isoforms involved in pathological angiogenesis of malignant tumors, which is also implicated in wound healing, myocardial infarction, strokes and some chronic inflammatory diseases. $VEGF_{189}$ is usually present in low amounts; it binds strongly to heparin and therefore is completely sequestered in the ECM. $VEGF_{121}$ is a smaller isoform secreted as a freely diffusible non-heparin-binding protein. It has been used successfully as a highly selective carrier delivering therapeutic agents such as toxins to tumor vascular endothelial cell resulting in inhibition of tumor growth and metastasis (21). Interestingly, in our study, $VEGF_{121}$ was found to represent the major isoform in VMs. Hemangiomas and VMs are common vascular disease entities found in the head and neck region. Frequently, it is difficult to diagnose, evaluate and treat these two diseases (22) and these disease entities are not always diagnosed correctly. It is necessary to use a common nomenclature since VMs frequently were just classified as hemangiomas. Although the underlying mechanism is not yet fully understood, the observed differences in the expression of *VEGF* isoforms between hemangiomas and VMs further underscore these two forms as representing different disease entities.

VEGF clearly plays a critical role in tumor angiogenesis. The relevance of selective *VEGF* isoform expression by tumors has been demonstrated: $VEGF_{121}$ is associated with increased dilated and peripheral blood vessels while $VEGF_{189}$ is found expressed in a highly branched neovasculature (23-25). We observed no obvious differences in the *VEGF* isoform composition in tumor samples, however, there was a trend for a proportional up-regulation of all three *VEGF* isoforms ($VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$). This general up-regulation appeared to correlate more with tumors of higher stages (with lymph node or distant metastasis) and less with lower stage disease (no lymph node or distant metastasis). This suggests that higher *VEGF* expression correlates with tumor progression.

In summary, our study indicates that an overexpression of *VEGF* isoforms correlates with advanced tumor stage in head and neck cancer cells. Furthermore, it suggests that $VEGF_{121}$ could play a pathophysiological role in the formation of VMs. However, since our study investigated only a limited number of tissues, more studies are required to clarify the role of the diverse *VEGF* isoforms in the pathogenesis of HNSCC and particular vascular tumors such as hemangiomas and VMs.

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