Variable Somatic *TIE2* Mutations in Half of Sporadic Venous Malformations

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Key Words

Hyperphosphorylation · Receptor tyrosine kinase · Somatic mutation · Sporadic · *TIE2*/TEK · Vascular malformation · Venous malformation

Abstract

Venous malformations (VMs) are the most frequent vascular malformations referred to specialized vascular anomaly centers. A rare (1–2%) familial form, termed cutaneomucosal venous malformation (VMCM), is caused by gain-of-function mutations in *TIE2*. More recently, sporadic VMs, characterized by the presence of large unifocal lesions, were shown to be caused by somatic mutations in *TIE2*. These include a frequent L914F change, and a series of double mutations in cis. All of which cause ligand-independent receptor hyperphosphorylation in vitro. Here, we expanded our study to assess the range of mutations that cause sporadic VM. To test for somatic changes, we screened the entire coding region of *TIE2* in cDNA from resected VMs by direct sequencing. We detected *TIE2* mutations in 17/30 (56.7%) of the samples. In addition to previously detected mutations, we identified 7 novel somatic intracellular *TIE2* mutations in sporadic VMs, including 3 that cause premature protein truncation.

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Venous anomalies are the most frequent vascular malformations referred to specialized vascular anomaly centers [Boon and Vikkula, 2012]. They are classified into venous malformations (VMs, 95%) and glomuvenous malformations (GVMs, 5%) [Brouillard and Vikkula, 2007; Limaye et al., 2009a, Brouillard et al., in press]. VMs are commonly present at birth and grow proportionally with the child. They usually affect skin and mucosa, but can also involve deeper structures (i.e. organs, bones or muscle) [Boon et al., 2004; Domp Martin et al., 2009, 2010]. Lesions vary from small blebs to large lesions, from light to dark blue (fig. 1). Histologically, VMs are characterized by enlarged channels lined with endothelium and an irregular layer of surrounding smooth muscle cells [Vikkula et al., 1996]. Large lesions with phleboliths are associated with localized intravascular coagulopathy (LIC) [Domp Martin et al., 2008, 2009].

Most VMs are sporadic. A rare (1–2%) familial form, cutaneomucosal venous malformation (VMCM), is caused by activating mutations in the endothelial cell-specific tyrosine kinase receptor *TIE2*/TEK [Vikkula et al., 1996; Calvert et al., 1999; Boon et al., 2004; Wouters et al., 2009]. In one surgically resected VMCM, a somatic second-hit mutation was identified in the wild type al-

Fig. 1. Sporadic VM on **A** the neck, **B** genitals, **C** chest, and **D** thigh.

lele of *TIE2* [Limaye et al., 2009b]. Following this finding, sporadic VMs, typified by the presence of large unifocal lesions, were also shown to be caused by somatic mutations in *TIE2*. Indeed, mutations were identified in about 50% of lesions [Limaye et al., 2009b], absent in the blood. The mutations detected were exclusively in exon 17 and lay within the first tyrosine kinase and kinase insert domains of the receptor [Limaye et al., 2009b]. They included a frequent L914F change (85%) and a series of double mutations on the same allele, all of which caused ligand-independent receptor hyperphosphorylation in vitro. Double mutations caused greater hyperphosphorylation than their constituent single mutations [Limaye et al., 2009b]. Here, we expanded our study to assess the range of mutations that cause VMs. Our previous data showed that RNA-based screens are far more sensitive and, therefore, more likely than DNA-screens to detect somatic mutations in heterogeneous lesion-derived tissue. We therefore systematically screened the whole *TIE2* gene in cDNA derived from resected VMs by direct sequencing.

Material and Methods

Patients

Thirty patients with sporadic unifocal VMs were recruited. Informed consent was obtained for each patient participating in the study before enrolment, as approved by the ethical committee of the Medical Faculty at the Université catholique de Louvain, Brussels, Belgium. The clinician responsible for the patients (L.M.B.) collected clinical information.

Samples

Tissues were snap-frozen in liquid nitrogen directly after surgical resection. DNA was extracted from frozen tissues and blood samples using Wizard® Genomic DNA Purification Kit (Promega). RNA from frozen tissues was extracted using TriPure (Roche), according to the manufacturer's instructions.

Mutational Screen of Tissue cDNA

cDNA synthesis was processed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) with the recommended protocol. Five amplicons that overlap to cover the entire 3.3-kb *TIE2* transcript were PCR-amplified from tissue cDNAs and sequenced on an ABI 3130XL genetic analyzer (Applied Biosystems).

Fig. 2. A Somatic changes identified in patients with sporadic VM. Mutations identified in original (DNA) tissue screen on the left; additional mutations identified in this (cDNA) tissue screen on the right. **B** Five individuals had TIE2 double substitutions. T1105N + T1106P occurred in 2 patients. **C** Ligand-independent phosphorylation of the double mutants T1105N + T1106P and T1105N + G1115* compared with constituent single mutants, WT, common germline mutant R849W and common somatic mutant L914F. NT = Non-transfected controls.

Bioinformatic Analysis

All identified nucleotide changes were subjected to bioinformatic analyses. First, we assessed whether they are known polymorphisms, present in the dbSNP and dbEST databases (<http://www.ncbi.nlm.nih.gov/SNP/> and <http://www.ncbi.nlm.nih.gov/dbEST/>). Changes modifying amino acids were submitted to POLYPHEN (<http://genetics.bwh.harvard.edu/pph2/>) and PANTHER (<http://www.pantherdb.org>) to estimate the likelihood of a functional impact on the protein.

Construction and Expression of Full-Length Mutant TIE2

Full-length TIE2 mutants in pcDNA3.1/Zeo(-) expression vector were generated by PCR-amplification from tissue cDNAs as previously described [Limaye et al., 2009b]. Constructs were verified by sequencing and transformed into Cos7 cells using jetPEI (Polyplus-transfections SA). Phosphorylation of the receptor was evaluated by Western blotting with anti-phosphotyrosine antibody (PY-99; Santa Cruz Biotechnology) followed by stripping and reprobing with an antibody against total TIE2 (ab24859; Abcam). Proteins were visualized using goat anti-mouse-HRP (Bio-source) with a femto-range-sensitive ECL system (Pierce).

Results

By screening cDNA from tissues, we identified TIE2 mutations in 17/30 (56.7%) of the samples. In addition to previously detected mutations c.2690A>G (Y897C), c.2690A>T (Y897F) and c.2740C>T (L914F) in exon 17 [Limaye et al., 2009b], we detected 7 novel somatic changes, absent in dbSNP and dbEST, including 3 that cause premature protein truncation. The new missense mutations are located in exons 15, 17 and 23: c.2545C>T (R849W) and c.2752C>T (R918C), previously identified as germline changes in VMCM, c.3314C>A (T1105N) and c.3316A>C (T1106P). The truncating mutations are exclusively located in the C-terminal tail of the receptor (exons 22 and 23): the nonsense substitutions c.3295C>T (R1099*) and c.3343G>T (G1115*), and the small frame-shifting deletion c.3324_3334del (E1109Lfs*5) (fig. 2A). All mutations have been submitted to the Vascular Anomaly and Lymphedema Mutation Database (<http://www.icp.ucl.ac.be/vikkula/VAdb/>). The mutations were absent

in blood DNA and were predicted to have deleterious effects on receptor function by POLYPHEN and PANTHER analysis. R849W, L914F and E1109Lfs*5 occurred alone, while in 5 lesions, we detected 2 mutations in cis (on the same allele): Y897C + R918C, Y897F + R1099*, T1105N + G1115*, and T1105N + T1106P (fig. 2B).

Ligand-independent hyperphosphorylation of the receptor was evaluated by Western blotting for a selection of mutant forms, including both the most proximal (5') and most distal (3') C-terminal tail truncations. The increased phosphorylation of the T1106P, R1099* and G1115* mutants was higher than that of the most common change L914F (fig. 2C) [Limaye et al., 2009b]. T1105N induced a weaker level of hyperphosphorylation. Double-mutant forms were not consistently stronger than each of their single constituents. Finally, we could not observe any genotype-phenotype correlation.

Discussion

In this study, we increased sensitivity of mutation-detection by reducing tissue heterogeneity. By using cDNA, we obtained information exclusively from normal/abnormal endothelial cells, the only TIE2-expressing cells in lesions (instead of all cell types present). The previous molecular analysis performed on tissues from patients with unifocal VM identified a frequent L914F mutation in >92% of mutation-positive lesions [Limaye et al., 2009b]. In the current study, this mutation was present in 9 tissues, bringing the total to 33/43 unifocal VM tissues positive for L914F (77%). This drop in its prevalence (relative to the other mutations) can be attributed to the improved sensitivity of our technique, which allowed us to detect several additional changes distributed along the length of the intracellular part of TIE2.

While L914F remains the most common somatic change, the presence of double mutations is higher than that observed previously [Limaye et al., 2009b]. These pairs include truncating mutations within the C-terminal tail of TIE2, which nevertheless cause the hyperphosphorylation characteristic of VM-causative mutations. This is consistent with a study by Niu et al. [2002], which showed that the deletion of the carboxy terminus of Tie2 enhances kinase activity, likely by abolishing the inhibitory fold of this domain [Shewchuk et al., 2000].

How these substitutions cause altered TIE2 phosphorylation is not clear. It has been hypothesized that R849W may increase phosphorylation by permitting closer packing of residues at the dimerization interface [Shewchuk et

Fig. 3. TIE2 intracellular protein structure showing the nucleotide binding loop (red), the catalytic loop (green), the activation loop (blue), the kinase insert domain (pink), the C-terminal tail (yellow), and positions of somatic mutations (orange). This figure was prepared using Cn3D (<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>).

al., 2000]. The tyrosine at position 897 has been shown to inhibit TIE2 activation when phosphorylated. This may be because it prevents kinase domain dimerization or because it recruits phosphatases [Shewchuk et al., 2000]. Mutations of this residue would, therefore, be expected to cause an overall hyperphosphorylation of the receptor, known to have at least 4 other tyrosine phosphorylation sites. It is unknown how the other substitutions lead to TIE2 activation. Structural analysis shows they tend to cluster around the kinase insert domain, whose role is unclear (fig. 3).

The mechanism by which mutant TIE2 causes VMs is unknown. The lack of any correlation between phosphorylation strength and severity of patient phenotype suggests that qualitative, and not just quantitative, abnormalities in TIE2 signaling play a pathogenic role. R849W has been shown to have an ShcA and Akt-dependent anti-apoptotic effect on ECs [Morris et al., 2005, 2006]. It also causes aberrant activation of the inflammatory mediator STAT1 [Korpelainen et al., 1999; Hu et al., 2008]. R849W and L914F have both been shown to affect TIE2 subcellular

localization and translocation in response to ligand [Limaye et al., 2009b]. Further studies are required to clarify the implications of these observations for VM pathogenesis.

Conclusion

The use of cDNA-based screens has provided us with a simple yet effective means by which to reduce the level of tissue heterogeneity, thereby increasing the sensitivity of *TIE2* mutation-detection. This has allowed us to identify a much wider range of somatic mutations that produce the VM phenotype, including, for the first time, mutations that generate premature STOP codons.

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