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ARTICLE

Disease-associated mutations in conserved residues of *ALK-1* kinase domain

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Activin receptor-like kinase-1 (*ALK-1*), the gene mutated in HHT type 2 (HHT2), is a serine/threonine kinase receptor type I of the TGF-β superfamily, specifically expressed on endothelial cells. We established an HHT2 genotype in 16 families and report nine novel mutations. These include insertions and deletions of single base pairs in exons 3, 8 and 9, as well as nonsense mutations in exons 4 and 8 of *ALK-1*, which would lead to premature truncation and unstable mRNA or protein. Three novel missense mutations were identified in exons 7 and 8 of the kinase domain. Five previously reported substitutions were also observed in the families analyzed. Our results bring to 36, the number of mutations associated with HHT2, and are mostly found in exons 8 and 3 followed by exons 4 and 7. To ascertain the potential functional implications of the missense mutations in the *ALK-1* kinase domain, we generated a model based on the three-dimensional structure of the homologous *ALK-5* kinase domain. Our data reveal that the 11 missense mutations modify residues conserved among type I receptors and alter the polarity, charge, hydrophobicity and/or size of the substituted amino-acid and likely lead to misfolded and nonfunctional proteins.
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Introduction

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterized by epistaxis (recurrent nosebleeds), mucocutaneous telangiectases, and arteriovenous malformations (AVMs) in lung, brain, and liver. *Endoglin* (*ENG*) and *ALK-1* are the genes mutated in HHT type 1 (HHT1) and HHT type 2 (HHT2), respectively. Mutations in these genes lead to similar phenotypes, although HHT1 is associated with a higher incidence of pulmonary AVMs while HHT2 tends to be milder and has a later onset.[1–3]

To date, 55 distinct *ENG* mutations and 27 *ALK-1* mutations have been reported. Protein expression studies, in human umbilical vein endothelial cells (HUVECs) and peripheral blood activated monocytes, have confirmed haploinsufficiency as the prevalent model for HHT1 since mutant proteins are generally not expressed.[4–7] Reduced ALK-1 levels were also observed on HUVECs of newborns with HHT2, suggesting that mutant ALK-1 proteins might also be nonfunctional.[8]

Like other type I receptors, ALK-1 consists of an N-terminal extracellular domain with 10 conserved Cys residues, a transmembrane region, and an intracellular region consisting almost entirely of serine/threonine kinase domains.[9] ALK-1 shares a relatively high amino-acid

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identity in the kinase domain to ALK-2 (77%), ALK-5 (63%), ALK-4 (59%) and ALK-3 (58%).[10,11]

In this study we performed molecular analysis in families with a clinical diagnosis of HHT and report nine novel mutations in the *ALK-1* gene. Using homology modeling and the three-dimensional structure of *ALK-5*,[12,13] we generated a structure of the *ALK*-1 kinase domain and discuss possible structural and functional consequences of the known missense mutations on this domain.

Materials and methods

Patients and samples

A clinical diagnosis of HHT is based on the presence of three of four criteria: epistaxis, telangiectases, a visceral manifestation (AVM or gastrointestinal bleeds), and a family history of HHT.[14] Thus, clinically affected individuals with normal endoglin levels relative to age-matched controls[8] (and data not shown) are considered putative HHT2 candidates and their DNA samples are first analyzed for *ALK-1* mutations.

Blood samples and medical histories were obtained with informed consent from all individuals participating in the study. For newborns, consent was obtained from parents and umbilical cord and placenta samples prepared as described previously.[4,6] All procedures were reviewed and approved by the Research Ethics Board of the Research Institute of the Hospital for Sick Children, Toronto, Canada.

Mutation analysis

Genomic DNA was prepared from peripheral blood lymphocytes, placenta and HUVEC using Puregene® DNA Isolation Kit (Gentra Systems) according to the manufacturer's protocols.

All exons of *ALK-1* were analyzed using the Open Gene Automated DNA Sequencing System II (VGI).[6,8] Two sets of primers were used for amplification and sequencing of each of the nine coding exons and were designed to cover exon/intron boundaries allowing for detection of alternative splice sites and flanking intronic polymorphisms.

ALK-1 modeling

Alignments of human ALKs were made using PILEUP from Genetics Computer Group (GCG) package, exported as multiple sequence files (MSF) and displayed using the program BOXSHADE. The crystal structure of the kinase domain of *ALK-5*[12,13] was used to build a model of the *ALK-1* kinase domain using homology modeling techniques.[15] Using SwissPDB program, amino-acid residues of the *ALK-5* kinase domain (PDBID: 1b6c) were substituted with the respective amino-acids of *ALK-1* to generate a homology model of the (wild type) *ALK-1* kinase domain. To examine the possible effects on this model of selected missense mutations, amino-acid residue side chains were altered to correspond to the known *ALK-1* missense mutations.

Results

Mutation analysis in 16 families with HHT2

A genotype of HHT2 was established in 16 families. We identified 14 *ALK-1* mutations, including nine novel ones (Figure 1 and Table 1). They were single base pair deletions, insertions and substitutions distributed in exons 3 and 4, corresponding to extracellular and transmembrane regions, respectively, and in exons 7, 8 and 9 of the intracellular kinase domain.

Mutations in the extracellular and transmembrane domains

We identified two novel mutations in exon 3. In family 38, a G86 deletion causing a frameshift at codon 29, was found in DNA from proband H118 (Figure 1a and Table 1). This

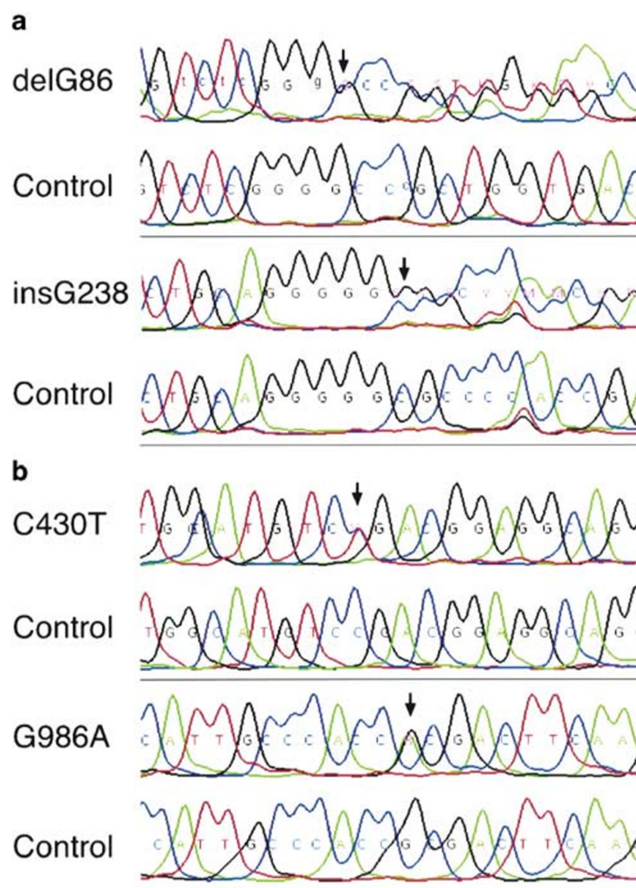

Figure 1 Nine novel mutations identified in HHT2 patients. *ALK-1* exons amplified from genomic DNA were sequenced using the Microgeneblaster® automated sequencer (VGI). (**a**) In exon 3, deletion of G86 and insertion of G238 are shown with respective controls. (**b**) C430T and G986A substitutions present in exons 4 and 7. (**c**) In exon 8, G1121A and T1123C substitutions were found in adjacent codons and a G1113 insertion was identified. (**d**) G1171T substitution and C1299 deletion were found in exons 8 and 9, respectively. (Base codes: M = A or C; S = G or C; Y = C or T; K = G or T; R = A or G).

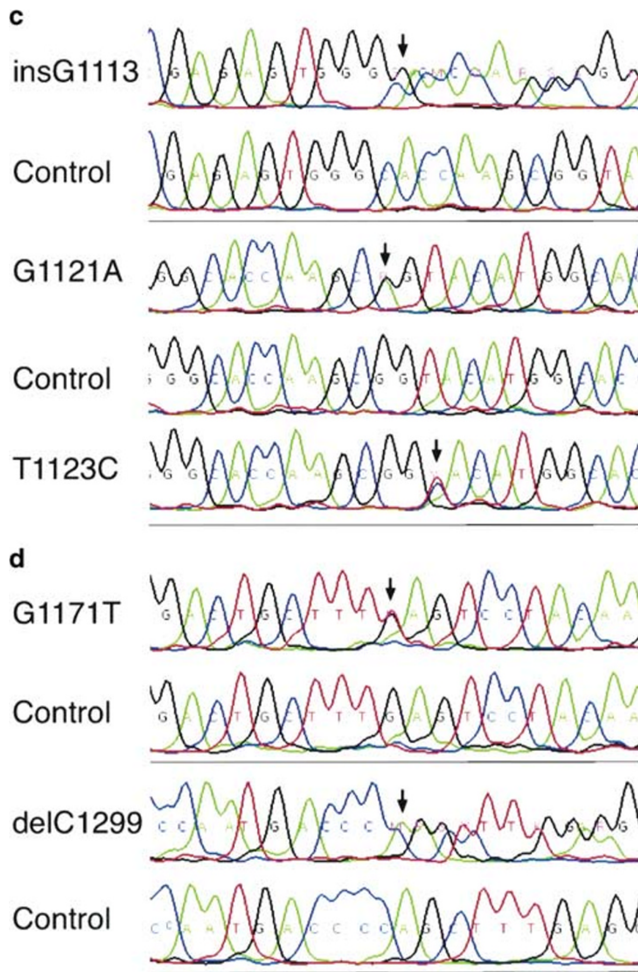

Figure 1 Continued.

patient had epistaxis, telangiectases, and a liver AVM. Four clinically affected adults and a 12-year-old boy who had not yet manifested signs of HHT, also carried the familial mutation while two nonaffected relatives did not. The second novel mutation was an insertion of G at position 238 creating a frameshift at codon 80 (Figure 1a and Table 1). It was identified in a clinically affected woman (H449) of family 133, who had a pulmonary AVM, but not in two unaffected relatives.

Two mutations were identified in exon 4. A previously reported nonsense mutation, G423A substitution,[16] converting Trp141 into a stop was detected in the proband H762 of family 198. This patient showed severe manifestations of HHT including hepatic AVM and gastrointestinal bleeds. The mutation was not detected in two nonaffected family members (Table 1). A novel C430T substitution, causing a stop at Arg144 (Figure 1b and Table 1) was found in proband H445 of family 131 and her newborn daughter. The proband's sister was also clinically affected but her newborn son did not carry the familial mutation.

Mutations in the intracellular kinase domain

In all, 10 mutations were identified in the *ALK-1* kinase domain. Two missense mutations were detected in exon 7. A novel G986A substitution that mutates Arg329 to His was found in proband H180 of family 59, who had both pulmonary and cerebral AVMs (Figure 1b and Table 1), and in one of her two young children. A G998T substitution, converting Ser333 to Ile, was found in four clinically affected individuals of family 41 (Table 1). This mutation was reported previously in a large family geographically linked to the current one, suggesting a common ancestor.[16,17]

Table 1 Summary of clinical and molecular data for the newly reported HHT2 families

Family no.	*Proband no.*	*Age*	*Clinical signs*	*Mutation type*	*Exon no.*	*Mutation*	*Effect on protein*	*Refs.*
38	H118	51	E T H	D	3	delG86	FS, Δ28 + 3aa	
133	H449	70	E T P	I	3	insG238	FS, Δ79 + 89aa	
198[a]	H762	62	E T G H	N	4	G423A	Trp141Ter	16
131	H445	42	E T	N	4	C430T	Arg144Ter	
59	H180	44	T P C	M	7	G986A	Arg329His	
41[b]	H121	51	E T	M	7	G998T	Ser333Ile	8,16,17
106	H334	50	E T	I	8	insG1113	FS, Δ371 + 51aa	
82[a]	H271	56	E T P	M	8	C1120T	Arg374Trp	16,18
167[a]	H597	43	E T					
129	H434	65	E T G	M	8	G1121A	Arg374Gln	
75	H260	36	E T P	M	8	T1123C	Tyr375His	
157	H550	57	E T	N	8	G1171T	Glu391Ter	
170[a]	H607	71	E T P	M	8	C1231T	Arg411Trp	19
20[a]	H63	36	E T	M	8	C1232A	Arg411Gln	16,20,21
185[a]	H668	37	E T					
181	H651	56	E T	D	9	delC1299	FS, Δ433 + 6aa	

[a]Previously published mutations identified here in unrelated families. [b]Mutation identified in family likely related to the original families. Clinical manifestations in the proband: E, epistaxis; T, telangiectases; P, pulmonary AVM; H, hepatic AVM; C, cerebral AVM; G, gastrointestinal bleeds. Mutation type: D, deletion; I, insertion; M, missense; N, nonsense.

Exon 8 harbored half of the mutations (7/14) identified in this study (Table 1). Four of these are novel: one insertion, two missense and one nonsense mutations. The insertion of G1113, causing a frameshift at residue 372, was identified in proband H334 of family 106 (Figure 1c and Table 1). If translated, this protein would lack part of the kinase domain and likely be nonfunctional. The mutation was present in eight of 15 family members tested and correlated with clinical manifestations.

Three of the missense mutations present in exon 8 altered two adjacent amino-acids (Table 1). A previously reported C1120T substitution[16,18] was found in two unrelated families and converts Arg374 into Trp. In family 82, the mutation was found in proband H271 with pulmonary AVM and an affected sibling, but not in a nonaffected relative. In family 167, three of nine patients tested exhibited clinical signs of HHT, which correlated with the presence of the mutation. The substitution G1121A was found in family 129 (Figure 1c) and changes Arg374 to Gln. This mutation was found in proband H434 who had gastrointestinal bleeds and in an affected sibling. Another novel mutation, T1123C, that converts Tyr375 to His, was identified in family 75 (Figure 1c). It was found in proband H260 with pulmonary AVM and two clinically affected individuals as well as in a newborn.

We report the first nonsense mutation in exon 8, a G1171T substitution detected in family 157 (Figure 1d). It leads to a termination codon at Glu391, and the translated protein would likely be inactive due to partial loss of the kinase domain. This mutation was detected in three clinically affected individuals but not in a relative without signs of HHT.

Another two missense mutations affecting the same amino-acid, were identified (Table 1). A recently published substitution, C1231T, that changes Arg411 to Trp[19] was found in unrelated family 170. This mutation was present in four clinically affected individuals, including proband H607 with a pulmonary AVM, and absent from seven nonaffected relatives. The second mutation, a G1232A substitution that converts Arg411 into Gln was previously reported in three studies.[16,20,21] We found this mutation in two seemingly unrelated families. In family 20, it was detected in two clinically affected individuals, while in family 185 it was found in five individuals with clinical signs and absent from four without symptoms.

A novel mutation was identified in exon 9: deletion of C1299 in family 181 (Figure 1d) that creates a frameshift at amino-acid 434. It was present in two of four samples tested and correlated with HHT signs.

The newly described mutations bring to 36, the total number of known mutations in the *ALK-1* gene (Figure 2).

Polymorphisms in the ALK-1 gene

Four of the substitutions observed, including three intronic, were considered neutral polymorphims as they were found in normal alleles. Substitution IVS1-38C>T was found in 14 of 100 individuals tested. Substitution IVS3+11C>T was expressed as a single allele in 40% and on both alleles in 6% of 159 individuals tested. Substitution IVS9-30G>A was found in 2 of 100 individuals tested (2%). The substitution G747A was also found in 2 of 100 individuals tested; this is a silent mutation that does not alter Val 249.

Homology modeling of ALK-1

To compare ALK-1 and ALK-5 structures, we first aligned their amino-acid sequences, revealing the overall similarity of the GS and kinase domains (Figure 2a). The 11 mutated amino-acids, representing 13 known missense mutations in the *ALK-1* kinase domain all correspond to conserved residues. When compared to other TGF-β superfamily type I receptors (GenBank sequences at http://www.ncbi.nlm.nih.gov/), 10 of these residues were identical in the six mammalian type I receptors (ALK-1 to ALK-6) while the 11th (Ile398) differed only in ALK-6 where it was substituted with Met. All 11 normal amino-acids were also conserved in mouse and rat.

A structural model of the cytoplasmic domain of *ALK-1* was generated by computer modeling based on the homologous structure of *ALK-5*. The catalytic domain of *ALK-1* is predicted to contain a small N-lobe dominated by a five-stranded β-sheet and a larger, mostly helical C-lobe (Figure 3). The 11 mutated amino-acids were localized onto this molecular model and found to be concentrated in the core of the kinase C-lobe. One of the 11 mutated side chains (Cys344) was contained in a β-pleated sheet, three (Ile398, Glu407, Arg484) were within α-helical structures, while the remaining seven were in the loops between β-pleated sheets (Arg329, Ser333) and/or α-helices (Arg374, Tyr375, Met376, Arg411, Pro424) (Figure 3).

Effects of missense mutations on the structure of ALK-1

Each of the 13 missense mutations was then analyzed for its position in the kinase C-lobe. Potential alterations, if any, in the hydrogen bonding scheme, and/or presence of induced steric clashes, were considered. For example, Arg329 and Ser333 are conserved residues of the catalytic segment in subdomain VI of type I receptors and are part of one of the two consensus motifs that confer specificity in either serine/threonine or tyrosine kinases.[9,22] This motif, His328–Arg–Asp–Leu–Lys–X–X–Asn335, contains two invariant residues – Asp330 and Asn335 – that are implicated in ATP binding and phosphotransfer,[9] while Glu334 is involved in substrate binding by forming an ion pair with Arg329 in the consensus sequence. Upon mutating Arg329 to His, a basic residue is replaced with a structurally distinct residue with a side chain imidazole ring. Modeling suggests this could lead to the formation of a new hydrogen bond to the backbone of Val 353 of the β9-

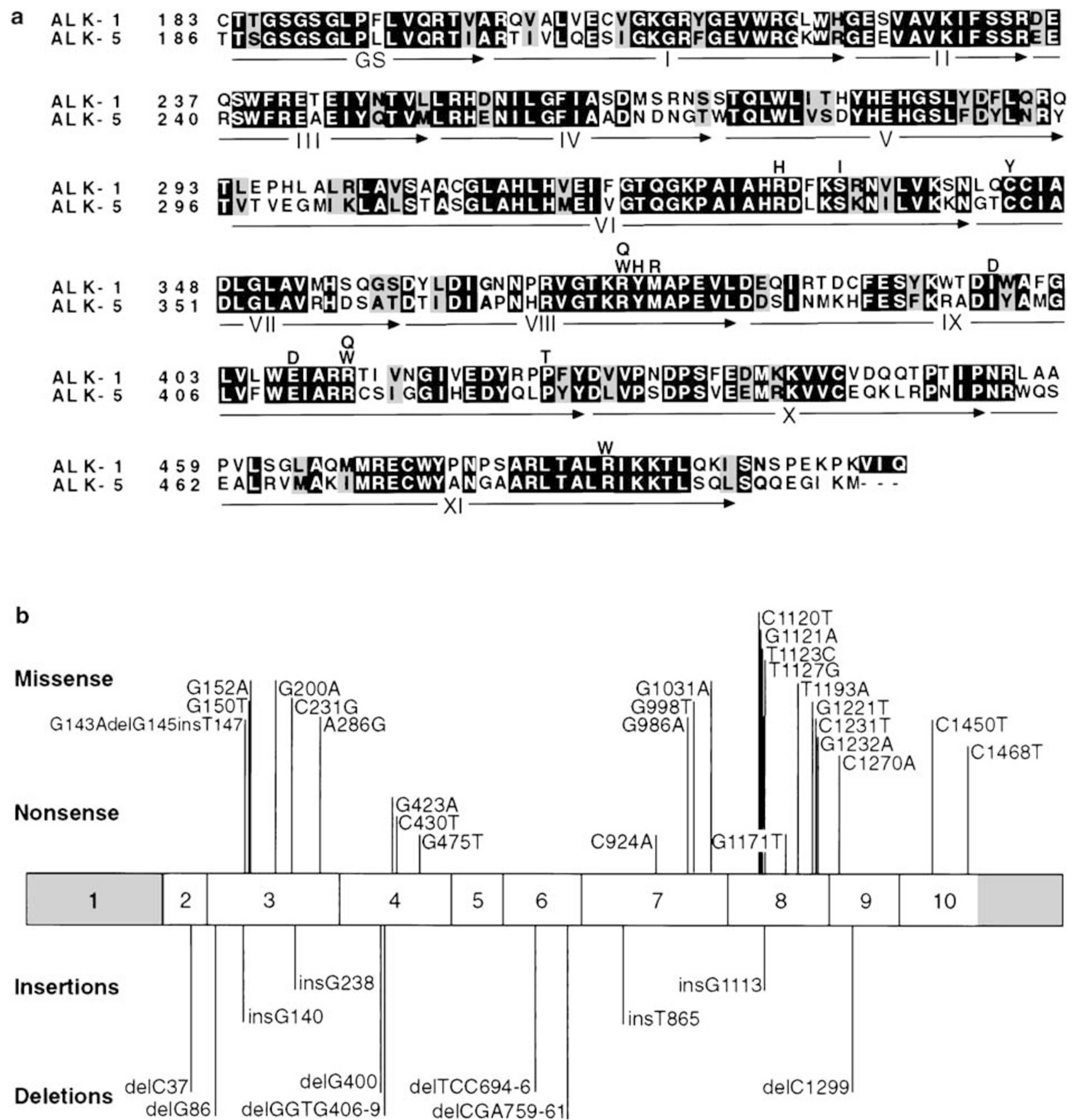

Figure 2 (**a**) An alignment of *ALK-1* (residues 183–503) and *ALK-5* (residues 186–503) cytoplasmic kinase domains. Identical amino-acids are represented by dark boxes while conservative substitutions are shown as gray boxes. The position of the 13 known *ALK-1* missense mutations is indicated above the alignment. The GS domain and the 11 kinase subdomains are shown below the alignment. (**b**) Summary of known mutations in the *ALK-1* gene. A map of the *ALK-1* gene with the 36 published mutations is illustrated approximately to scale. The coding region (exons 2–10) is represented as open boxes and noncoding exons as gray boxes.

pleated sheet (Figure 4a, c) and thus potentially interfere with substrate binding. Ser333Ile replaces a purely hydrocarbon side chain with a relatively hydrophilic side chain. Both mutated residues, located in the catalytic loop between the E-helix and the activation segment, could disrupt the activation site and interfere with catalysis.

Four missense mutations affect three consecutive amino-acids (Arg374, Tyr375, and Met376) found in the loop preceding the αEF helix. They fall within subdomain VIII containing an Ala–Pro–Glu motif, which is highly conserved among protein kinases. The eight amino-acids preceding this sequence are conserved and form part of a

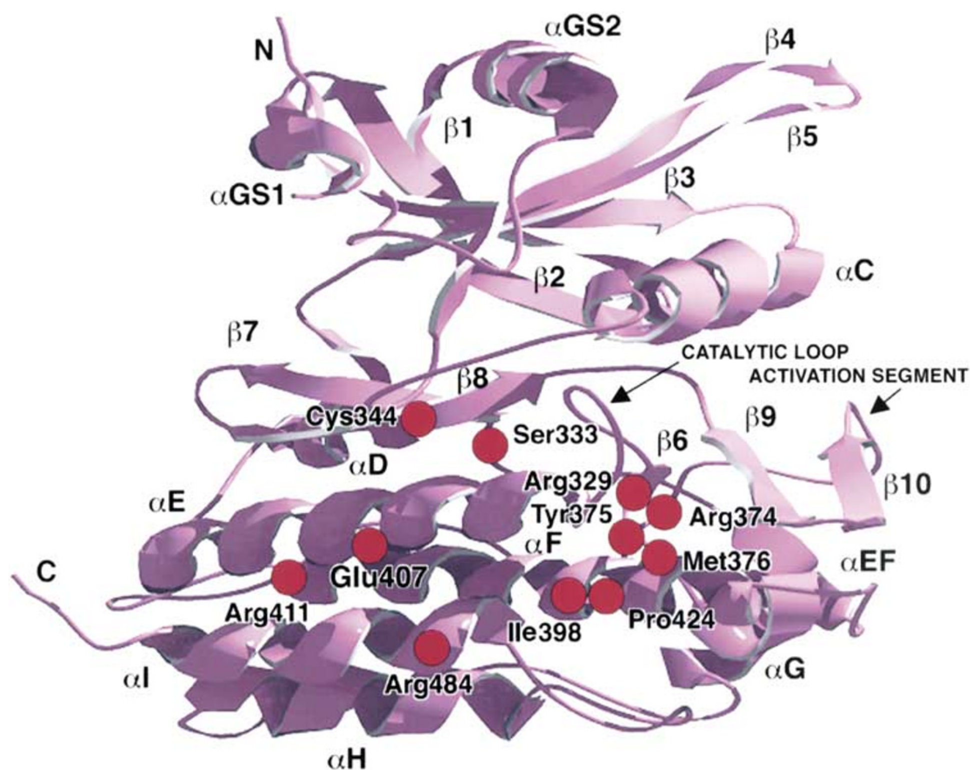

Figure 3 Structural model of *ALK-1* cytoplasmic domain, as generated by homology modeling to the corresponding domain of the crystal structure of *ALK-5*.[12,13] The position of the 11 *ALK-1* mutated residues, overlaid on the *ALK-5* structure, is indicated with red circles.

consensus (Gly371–Thr/Ser–X–X–Tyr/Phe–X–Ala–Pro–Glu) specific to serine/threonine kinases.[9,22,23] Subdomain VIII plays an important role in substrate recognition by providing a pocket to accommodate hydrophobic residues. Two mutations (Gln and Trp) affect Arg374; the possible participation of Trp374 in alternative H-bonding schemes *versus* wild-type Arg374 is illustrated in Figure 4b,d.

Arg484, located in the αI helix, is part of a highly conserved COOH-terminal sequence between residues 479 and 489 of ALK-1. This region, referred to as NANDOR BOX (for non-activating non-down-regulating), is highly conserved among type I receptors in mammals, *Drosophila* and *Xenopus* and was shown to be critical for ligand-induced TGF*β*-receptor signaling and downregulation.[24] *ALK-5* missense mutations in NANDOR BOX resulted in the inability of TGF-*β* to stimulate expression of PAI-1, fibronectin, and Smad2 phosphorylation.[25,26] An Arg484Trp mutation that falls within this motif would clearly be implicated in a loss of function.

Discussion

We described nine novel *ALK-1* mutations, bringing to 36 the total number of known mutations in HHT2 (Figure 2b). Nine (25%) of these are present in exon 3, ten (28%) in exon 8, five in each of exons 4 and 7, two in each of exons 6, 9, 10 and one in exon 2. These results suggest that sequencing exons 8, 3, 4 and 7 of *ALK-1* should be performed first to accelerate the process of mutation identification in HHT2.

The majority (70%) of HHT2-associated *ALK-1* mutations are single base-pair substitutions leading to missense and nonsense mutations. In contrast, most (65%) HHT1-associated *ENG* mutations cause frameshifts and premature stop codons. Furthermore, large insertions, deletions and splice site mutations are quite common in HHT1 but so far have not been identified in HHT2 (Cymerman *et al*[6] and Paquet *et al*[7] and data not shown**)**. This might be partially due to the almost exclusive use of direct sequencing in *ALK-1* mutation analysis. In our own series, we have now resolved 59 HHT1 families using quantitative multiplex PCR (QMPCR) and sequencing and 22 HHT2 families using mostly sequencing. We will now perform *ALK-1* QMPCR on probands with a clinical diagnosis of HHT and normal levels of endoglin protein, assumed to be potential HHT2 candidates. Although AVMs are presumed more frequent in HHT1 than HHT2,[1–3] it is not possible to determine the HHT type clinically. As seen in Table 1, seven of the 16 probands had an AVM: two – hepatic, four – pulmonary, and one – both pulmonary and cerebral. This represents

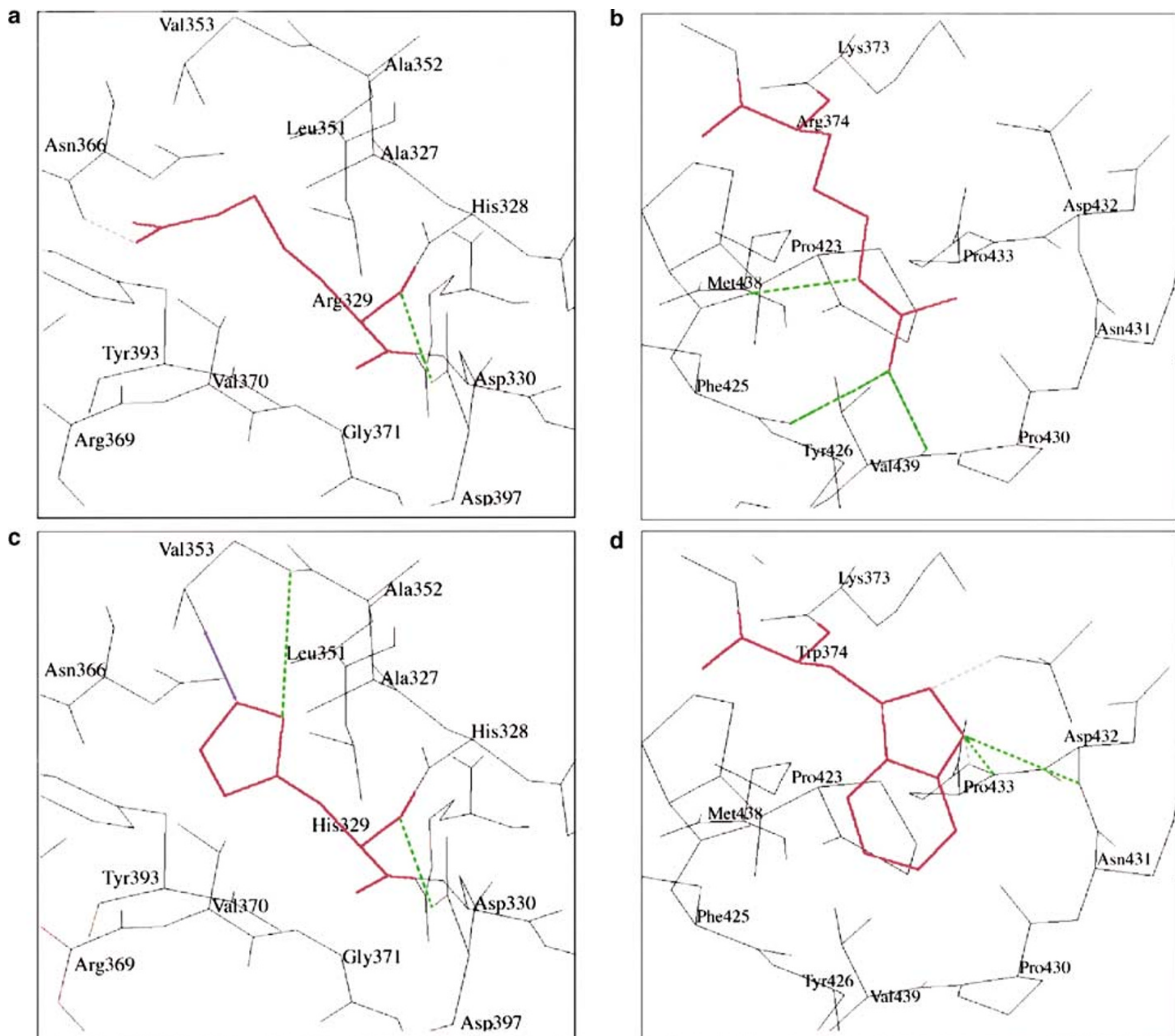

Figure 4 Examples of potential alterations in *ALK-1* structure resulting from missense mutations, as suggested by molecular modeling. (**a, c**) An Arg329His substitution removes a weak H-bond between Arg329 and Asn366 and can produce instead a strong H-bond between His329 and Val353 backbone. (**b, d**) With Arg374Trp substitution, the structural stability gained from wild-type Arg374 side chain H-bonding scheme to backbone substituents of Phe425, Met438 and Val439, is lost. The Trp374 indole ring may interact instead with substituents on Asp432 and Pro433. Wild type and corresponding mutated residues are shown in red. Strong H-bonding is shown in green, weak H-bonding in gray, and possible steric clashes induced by mutations are rendered as purple arrows. Hydrogen-bonding schemes (wild type and mutants) are as calculated by the SwissPDB program.

the highest incidence of AVMs reported in a series of HHT2 families although previous studies have reported AVMs in two large HHT2 kindreds.[17,18]

Mutations are distributed throughout the *ALK-1* gene. Frameshift and nonsense mutations predict nonsense mediated mRNA decay[27] and failure to produce functional protein. The missense mutations reported generally alter conserved amino-acids and likely result in structural changes. Six of the 19 *ALK-1* missense mutations were found in exon 3 of the extracellular domain. We have previously demonstrated that HUVECs with Gly48Glu/Ala49Pro and Trp50Cys mutations expressed reduced levels of ALK-1.[8] In addition, introduction of the Trp50Cys mutation into the extracellular domain of the ALK-1/ALK-5 chimera led to the abrogation of signaling activity due to low levels of expression of the unstable chimeric receptor.[28] The structural alterations caused by these missense mutations likely result in protein misfolding and intracellular degradation explaining the lack of surface expression of mutant proteins. A site for possible interaction between R-I and R-II receptors was identified in the extracellular domain of ALK-5.[29] Several ALK-1 residues

affected by missense mutations align with a structurally conserved region important for ALK-5 signaling and ligand internalization, but not ligand binding. Since ALK-1 can function as a receptor for TGF-β in endothelial cells,[30] we can speculate that certain mutations in this extracellular region might impair R-I/R-II interactions, ligand-dependent signaling and/or ligand internalization.

The missense mutations found in the kinase domain occur at residues that are conserved among type I receptors. Homology modeling and analysis of the missense mutations within the *ALK-1* kinase domain shows that they alter the polarity, charge, hydrophobicity, and/or size of the substituted amino-acid and will likely have structural effects creating misfolded and unstable proteins. Such nonfunctional mutant proteins would support the haploinsufficiency model. However, we cannot rule out the possibility that some of the missense mutations lead to dominant-negative effects; engineering of selected mutants should clarify this point.

TGF-β is thought to regulate endothelial function via a balance between *ALK-1* and *ALK-5* signaling.[30] ALK-5 effects mediated by Smad2/3 lead to stimulation of extracellular matrix production and vessel maturation, while signaling through ALK-1 and Smad1/5 stimulates proliferation and migration of endothelial cells.[31] Endoglin is thought to play a key role in the regulation of the ALK-1/ALK-5 balance, which is critical in vascular remodeling. This might explain why *ENG* and *ALK-1* mutations lead to a similar vascular pathology.

Mutations in *BMPRII* or *ALK-1* genes can lead to a distinct disorder, primary pulmonary hypertension.[19,32,33] Since both receptors act through Smad1/5, one can speculate that this pathway is particularly important for endothelial function. Our analysis of missense mutations in the kinase domain of *ALK-1* using homology modeling, further emphasizes the importance of the TGF-β receptor family in sustaining normal vascular structure and function. Determining the mode of action of ALK-1 is essential to our understanding of the mechanisms responsible for maintenance of vascular integrity, as disruption of its function generally leads to HHT but can also cause pulmonary hypertension.

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