

Mutational Analysis Mapping on The Molecular Structure of The ACVRL1 Protein and Implications For Rendu-Osler-Weber (ROW)

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Abstract—About 80% of Rendu-Osler-Weber (ROW) patients carry mutations in endoglin (*ENG*) or activin receptor-like kinase1 (*ACVRL1*; *ALK1*) genes. In order to investigate the molecular mechanisms that govern the pathogenic effect of the mutations in *ACVRL1*, we have collected and analyzed the mutational effect of over 80 different predominant mutations, as well as their location, on the 3D molecular structures of N- and C-terminal domains of *ACVRL1*. We have used macromolecular modeling on the protein structural components of *ACVRL1* and structural component interface analysis to locate position and interaction of point mutations. Specific mutations were identified using genomic DNA sequencing from blood leucocytes. Out of the 151 point mutations reported for the *ALK1* gene, the majority are located on the surface of the ARD and PK structural domains, with some on the interaction interface. New observed mutation Cys90Phe found in two Cretan ROW patients, located on loop F4 of ARD, introduces conformational steric hindrance and disruption of stability. We have mapped point mutations on the structural domains of *ACVRL1*, correlating location and severity of ROW. In addition, we report the identification and location of a novel missense mutation, Cys90Phe, which has not yet been described. It is identified in a Cretan ROW patient, and associated with severe clinical appearance according to the Curacao criteria.

Index Terms—*ACVRL1*, mutation, Rendu-Osler-Weber (ROW), 3-D model.

I. INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT), which is also known as Osler-Weber-Rendu (ROW) syndrome, leads to abnormal blood vessel formation on the skin, mucous membranes, and often internal organs such as lungs, liver, and brain [1]. Five indistinguishable genetic types of ROW have been recognized and defined by associated gene loci so far, and three of these types of the disease have been linked to particular genes, namely activin receptor-like kinase 1 (*ACVRL1* or *ALK1*), Endoglin (*ENG*) and *SMAD4* [2]. HHT1 (MIM #187300), with a high incidence of gastrointestinal bleeding, pulmonary and cerebral arteriovenous malformations, and HHT2 (MIM #600376), with a high incidence of hepatic arteriovenous malformations, which account for more than 80% of ROW, are caused by heterozygous mutations in *ENG* [3],[4] and activin *ACVRL1* [5] genes, respectively [6],[7]. Alterations in the sequence of both genes include point mutations, deletions, insertions, nonsense, and missense mutations, splice-site alterations, and intragenic rearrangements [8]. Additionally, an association of the ROW phenotype with colonic polyposis and mutations in the *SMAD4* gene, which is located in 18q21.1 and codes for SMAD4 (involved in the TGF-beta signaling pathway) has been demonstrated as well [9].

Many different mutations are so far known and it is likely that there is predominance of either type in a specific population. *ENG* mutations are more frequently found in patients from Northern Europe and the Americas, while Mediterranean populations have the majority of *ACVRL1* mutations. To date, 397 *ENG* and 332 *ACVRL1* mutations and polymorphisms have been reported (HHT Mutation Database) and the majority of *ACVRL1* (~53%) are located in exons 3, 7, 8 and 10 [7]. The number is continuously increasing, indicating that any nucleotide may be mutated, except probably those encoding for the transmembrane region (exon 13) or cytoplasmic domain (exon 14) [10]. It has been proposed that disease severity is more pronounced in HHT1 (caused by mutations in *ENG* gene) compared to HHT2 (mutations in *ACVRL1* gene). Considering all these limitations, genetic testing of ROW is valuable, especially in cases with limited symptoms, not only to confirm the clinical diagnosis but also to identify asymptomatic carriers among a

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ROW family [11]. Of note, the severity and age of onset vary considerably both between families and among members of the same family, making diagnosis very difficult [10].

The mechanisms that govern the pathogenic effect of the mutations in *ACVRL1* remain to be further elucidated. To this end, in this study we have located their position on the experimental 3D structures of the N- and C-terminal domains of ACVRL1 [12],[13]. Furthermore, we applied the mutations and constructed three-dimensional models of the mutants, as a first step before their further classification according to the clinical severity of ROW. In addition, herein we report the identification of a novel missense mutation, Cys90Phe, identified recently in a Cretan ROW patient.

II. MATERIALS AND METHODS

A. Construction of ACVRL1 domains' three-dimensional (3D) model

The 3-D experimental molecular structures of the N- (PDB code 3FA0) and C- (PDB code 3MY0) terminal domains of ACVRL1 have been used to create molecular models and generate specific mutants using Schrödinger LLC PRIME [14]. The derived models of mutants were checked for folding and packing differences against the wild type protein domains, as well as for their interaction with the TGF- β family ligands BMP9 and BMP10 and LDN-193189, respectively, *in silico* using Schrödinger Piper [15] and Schrödinger LLC GLIDE docking software [16]. PYMOL (The PyMOL Molecular Graphics System, Version 1.7.0.4 Schrödinger, LLC) was used for structural representations. Both structural domains were analysed for aminoacid residue accessibility, interface and interaction with neighboring subunits using PISA [17].

B. Genetic analysis

Blood sample of ROW patients of Greek origin with the tentative diagnosis of HHT2 was referred to our laboratory for molecular genetic analysis. Mutational analysis and examination of medical records were carried out with informed consent and under research protocols according to the declaration of Helsinki. Whole blood was collected in EDTA-containing tubes and genomic DNA was isolated from blood leucocytes by using the commercial kit Qiamp DNA Blood Mini kit (QIAGEN Inc, CA, USA). The coding exons and exon-introns boundaries of the two genes, *ENG* and *ACVRL1*, were amplified by polymerase chain reaction (PCR) using genomic DNA as template and sequenced (Genetic Diagnostic Laboratory, Department of Genetics, School of Medicine, University of Pennsylvania, USA).

III. RESULTS

A. Mapping of ROW associated mutations

We have mapped 151 ROW associated mutations on the ACVRL1 domains. 31 are located on the N-terminal Activin Receptor domain (ARD) and 120 on the C-terminal Kinase domain (PK) (Fig. 1 and 2). The two domains are linked through a transmembrane Leucine rich helical domain consisting of twenty two aminoacid residues (Fig. 1a). Moreover, we have classified all these mutations according to

severity and position, based on the existing literature

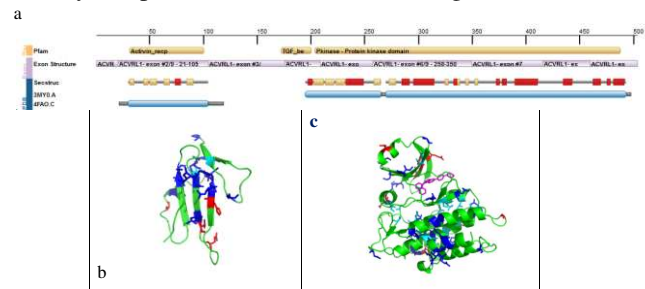


Figure 1. Representation of the ACVRL1 (ALK1) structure (gene and protein) (from Protein Data Bank). (a) Gene structure and protein domain secondary structure. (b) 3D structure of N-terminal (Activin Receptor) and C-terminal (Protein Kinase) domains of ALK1 with distribution of occurring mutations colored according to position and interaction (red for interface, blue for external and cyan for internal residues).

[5]-[8],[11],[12],[18]-[25] and the HTT mutation database (<http://hhtmmutation.org/>) or <http://arup.utah.edu/database/HHT/>) dealing with the clinical consequences and manifestations of each of them (Fig. 2).

From this study, it is apparent that mutations on *ACVRL1* fall into three categories according to their location in the three-dimensional molecular domains:

- those located at the interacting interface that may alter interaction or recognition properties between the N-terminal domain (ARD) and the interacting TGF- β family ligands or at the cytoplasmic protein kinase domain active site,
- those located on the non-interacting surface but playing an important role in the molecule's folding process and finally,
- those located in the core of the protein domains that may alter charge distribution or introduce conformational instability.

Each mutation has been examined individually with respect to its location in the two molecular domains and the intra- and inter-molecular interactions formed. On the C-terminal domain (PK) 120 effective mutations are distributed mainly in the core of the domain [26] with some on the interacting surface [27]. On the N-terminal domain (ARD), 7 mutations and one insertion are on the interacting interface while most mutations (14) are located in the core of the domain contributing to conformational instability.

	Activin Receptor domain				Kinase domain			
	INTERACTION INTERFACE	BINDING SITE	SURFACE	INTERNAL	INTERACTION INTERFACE	BINDING SITE	SURFACE	INTERNAL
SEVERE	4	0	11	2	5	4	27	12
MILD	0	0	0	0	1	0	5	0
UNKNOWN	3	0	9	2	10	4	37	15

Figure 2. Distribution of observed mutations according to position and clinical significance (severity). Mutations found in the different structural domains of *ALK1* according to location and severity.

B. Structural and functional implications of the new Cys90Phe mutation

All mutations have been characterized according to severity and location on the ACVRL1 extracellular and cytoplasmic domains (Fig. 3a,b and 3c,d, respectively).

One family out of seventeen ROW families, which have been reported in Crete, has been analyzed by sequencing analysis so far. Two related cases (father and daughter) were found to be heterozygous for a germline mutation, that is a

single base substitution, c.269G>T, in exon 3 of the genomic

IV. DISCUSSION

Osler-Weber-Rendu (ROW) syndrome or HHT, is an autosomal dominant genetic disorder, appearing a wide ethnic and geographic distribution and occurring in one in 5000 to 8000 people, depending on the geographic or ethnic region [28]-[30].

In recent years, our scientific interest has focused on a clinical type of ROW characterized by severe recurrent bleeding tendency. This preferentially involves the nose, with the most common symptom being recurrent massive epistaxis [1]. The diagnosis of ROW is made clinically on the basis of the Curacao criteria established in June 1999 by the Scientific Advisory Board of the ROW Foundation International Inc. The four diagnostic criteria include epistaxis, telangiectasias, occurring in more than 95% of patients, visceral lesions and family history (first-degree relative with ROW). Genetic linkage studies managed to map two variants of the disease, to mutations in at least two genes, *ENG* on chromosome 9q33-34 [4] and *ACVRL1* on 12q13 (13) [18], respectively. The corresponding products of both genes are specific endothelial transmembrane receptors for the transforming growth factor b (TGFb) superfamily of ligands that are involved in controlling cell proliferation, differentiation, migration, and adhesion in the vascular system [8],[31],[32]. *ACVRL1* and *ENG* are single-pass transmembrane receptors [27]. TGF-superfamily consists of more than 30 ligands including bone morphogenic protein (BMP), growth and differentiation factors (GDF), activins, seven type I receptors and five type II receptors, as well as, several co-receptors and accessory proteins [33]. Biochemical studies have suggested that BMP9 is the plausible physiological ligand of ALK1 and, therefore, may represent a significant step in understanding HHT as it allows us to reconsider the molecular mechanisms behind the disease pathogenesis [34]. A kinetic and thermodynamic analysis shows that BMP9 displays a significant discrimination in type II receptor binding [12]. This data provides insights into *ACVRL1* signaling that could serve as a basis for further angiogenic therapies [12].

Activin receptor-like kinase 1 (ALK1) or *ACVRL1*, an endothelial cell-specific type I receptor of the TGF- β superfamily, is an important regulator of normal blood vessel development and is primarily implicated in the development of ROW. It regulates pathological tumor angiogenesis and represents an important therapeutic target [12]. Considering the multiple genetic, pharmacological and histopathological evidence that supports a critical role for *ACVRL1* signaling in the regulation of blood vessel formation, either in tumors or in normal tissues [27],[33],[34], the growing interest in *ACVRL1* as a therapeutic target that complements existing anti-angiogenesis treatments seems justified. Indeed, a soluble *ACVRL1* ligand trap (Acceleron Pharma), and an anti-*ACVRL1* antibody (Pfizer) are presently in clinical trials for treatment of advanced solid tumors [35]. Thus, *ACVRL1* has been considered to be an important therapeutic target and several agents, directed to *ACVRL1*, are currently in clinical trials as anti-angiogenic cancer therapeutics. Considering the biological and clinical importance of the *ACVRL1* signaling pathway, we sought to construct a 3-D model of various domains of *ACVRL1* protein and localize known mutations

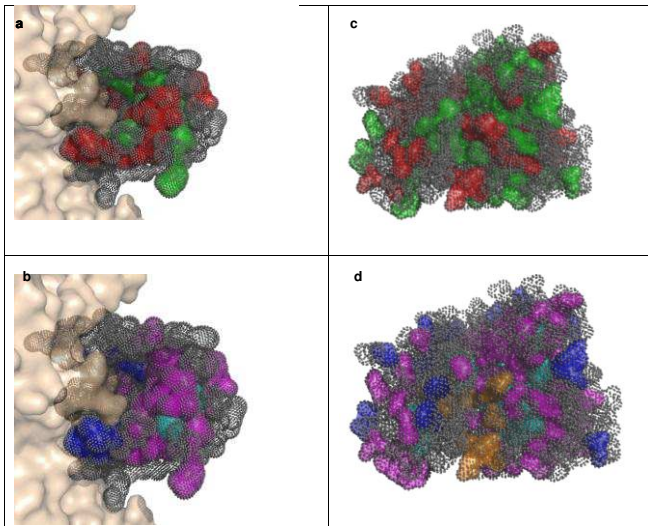


Figure 3. Distribution of observed mutations on the structural domains of ALK1: in the Activin Receptor (N-terminal) domain (PDB code 4FAO) according to clinical significance (a) and position in structure (b); in the Kinase (C-terminal) domain (PDB code 3MY0) according to clinical significance (c) and position (d). Mutations in red are those that create severe clinical effects, in yellow those with mild effects and in green those that have not been linked to clinical effects. Mutations in dark blue are located in the interaction interface, in orange in the ligand binding site, in magenta on the protein's surface while in light blue are mutations located in the internal of the protein domains.

sequencing of *ACVRL1* gene (GENBANK Accession number 4557242), thus resulting in a missense mutation Cys90Phe of *ACVRL1* domain. This is a novel mutation that has not been previously reported in the ROW (HHT) Mutation database (www.hhtmution.org). Of note, no mutations of the *ENG* gene were detected. However, this mutation was not detected on two cases (daughters) of this family. Based on the clinical manifestations of the patients, the novel mutation is characterized as pathogenic. Considering that this mutation has not previously been detected in patients from other studies, this codon seems to not be frequently affected.

Cys90 is located on the *ACVRL1* extracellular domain (res 30-104) that interacts with Activin (BM9 in the available crystal structure [12] through the amphipathic helix $\alpha 1$ (Fig. 4a). This helix is held in position by the disulphide bond Cys77-Cys89 and through β -strand $\beta 5$ and the disulphide Cys90-Cys95 to the core of the protein domain (Fig. 4b). The mutation Cys90Phe not only disrupts the disulphide bridge but also introduces steric hindrance between strands $\beta 5$ and $\beta 4$ and loop F2 (Fig. 4c).

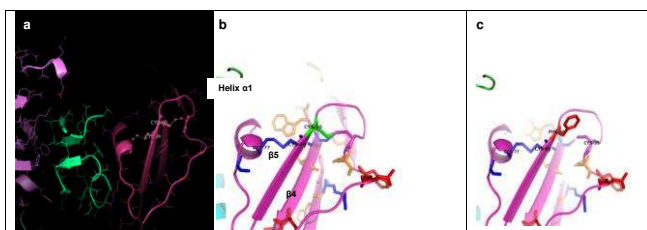


Figure 4. The Cys90Phe mutation on *ACVRL1*.

a. The location of the mutation on the *ACVRL1* extracellular domain (pink) with respect to the activin (BM9) receptor (in green). b. The network of disulphide bridges in *ACVRL1* that holds interaction helix $\alpha 1$ stable on the core β -sheet. c. Mutation Cys90Phe located in loop F4 introduces steric hindrance in the protein's conformation and disruption of stability due to the breaking of the Cys90-Cys77 disulphide bridge.

as well as a new one on it. The protein is rendered nonfunctional through missfolding or aggregation, caused by specific mutations directly associated with position on the 3D structure or functionality. These mutations were found in the extracellular, transmembrane and kinase domains of ACVRL1 including missense mutations as well as nonsense mutations, small deletions or insertions.

The majority of ROW causing mutations affect amino acid residues integral to the core structure while the minority may have an effect in BMP9 binding. In addition, it has been suggested that mutations in exon 5 lead to a more severe phenotype [6].

Presently, we are in a process of identifying this mutation of the *ACVRL1* gene of the members of 17 families from Eastern Crete, in an attempt to confirm the new Cys90Phe mutation and not only to validate its role regarding the geographic distribution of the disease in Crete, but to disclose the correlation of the severity of clinical appearance as well.

Molecular diagnosis of the disease causing mutation(s) in either one of the two ROW associated genes confirms the clinical diagnosis and, as a consequence, may be of benefit for patients and their close relatives [26]. Although a confirmed molecular diagnosis may provide an opportunity for early detection of the disease and appropriate management, it has been commented that ROW patients should be examined for mutations only after detailed clinical examination [22]. Screening for mutations in *ACVRL1* and *ENG* is justified especially in order to identify younger family members at risk of carrying a mutation. Further studies are under way to correlate mutations to amino acid residue functionality and clinical significance. Thus, correlation of position/type of mutation with clinical significance may elucidate our understanding of the contribution of particular mutations to the clinical pathology and severity of the disease in ROW patients and contribute greatly in terms of genetic counseling and prediction of ROW symptoms.

As far as treatment is concerned, the most promising intervention seems to be surgical closure of the nasal cavities [36], while other treatment modalities have been used so far with very results of success; namely, laser photocoagulation [37], and intranasal submucosal bevacizumab [38]. Given that ROW cannot be cured until now, the treatment of ROW patients remains symptomatic or has preventive character.

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