Research Square

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In Silico Analyses Reveal the Relationship Between ROCK2 Mutations and Laterality Defects

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Abstract

Laterality defects is a class of congenital disorders resulting from abnormalities in left-right (L-R) axis patterning. Previous studies indicated that the duplication of Rho-associated coiled-coil containing protein kinase 2(ROCK2) had connection with laterality disorders in human and its knockdown led to significant L-R abnormalities in Xenopus and Zebrafish. In this study, we aimed to investigate the association between the mutations of ROCK2 and L-R development in vertebrate. We analyzed a cohort of 65 patients with laterality defects and validated the ROCK2 mutations by polymerase chain reaction (PCR) and direct sequencing. Multiple in silico tools were used to predict the pathogenicity of mutations. We screened out two novel (ROCK2-Q115H, ROCK2-Y416C) and a third rare (ROCK2-A1013T) ROCK2 mutations. The two novel mutations Q115H and Y416C may alter protein properties and impact protein functions.

Conclusions: ROCK2-Q115H and ROCK2-Y416C are predicted to be pathogenic and could in part give rise to influence the biological activities of ROCK2 with the change of physicochemical properties and spatial conformation, suggesting that the mutations of ROCK2 may play a role in the pathogenesis of laterality defects.

What Is Known

• *Laterality defects is a class of congenital disorders resulting from abnormalities in left-right body patterning.*

• *ROCK2 participates in the development of L-R body asymmetry and is a candidate gene in laterality defects.*

What is New:

• We identified two novel disease-causing variants in ROCK2: p.Q115H, p.Y416C in patients with laterality defects, contributing to the knowledge about the pathogenesis of laterality defects.

Introduction

Laterality disorders are defects of left-right (L-R) axis patterning during embryonic development. These disorders manifest as an abnormal arrangement of the thoracoabdominal organs and include disorders like situs inversus(SI) and heterotaxy(HTX)[1, 2]. Establishment of L-R axis patterning is critically important to morphogenesis, proper placement and functioning of vertebrate organs. Failure to establish proper L-R body asymmetry can lead to a spectrum of disorders such as complex congenital heart defects (CHD), splenic malformations, and intestinal malrotation[1, 3]. Consequently, the outcomes and survival rate of patients with laterality disorders are unsatisfactory.

In vertebrates, the establishment of L-R asymmetry is activated by the unidirectional rotation of monocilia in the primitive node which also known as “nodal flow” [4]. Then the asymmetry signals are transmitted to the left lateral-plate mesoderm (LPM), upregulating the expression of Nodal-responsive genes such as *Nodal*, *Lefty2*, and *Pitx2* in the LPM[2, 5]. *Pitx2* can act as a transcription factor to regulate the asymmetric morphogenesis of visceral organs[6]. Any abnormality in the development of L-R body asymmetry will lead to laterality disorders. Previously reports indicate the genes associated with ciliopathies and the Nodal signal transduction pathway may be responsible for L-R asymmetry, and mutations in these genes have been identified in human laterality defects[5, 7-9]. However, the mutations reported in genes including *ZIC3*, *CFC1*and *ACVR2B* can explain only a small number of cases[10-12]. Most cases of laterality defects do not have a monogenic etiology. It is therefore important to identify other mutations that can lead to these defects.

The gene *ROCK2* (Rho-associated coiled-coil containing protein kinase 2) codes for a serine/threonine kinase that is activated by the RhoA GTP enzyme[13, 14]. ROCK2 participates in various cellular activities including proliferation, and apoptosis, actin cytoskeleton organization, cell contraction, adhesion and movement[13-15]. Previous studies suggest *ROCK2* mutations correlate with abnormal L-R patterning[16, 17]. Fakhro *et al.* identified *ROCK2* rare genetic copy-number variations (CNV) in HTX patients[17], suggesting ROCK2 participates in the development of L-R body asymmetry and is a candidate gene in laterality defects.

In this study, we first screened *ROCK2* mutations and found three single nucleotide variations (SNV) in patients with laterality defects. Using *in silico* analyses, we estimated that these mutations were likely pathogenic and discussed the relationship between *ROCK2* mutations and the abnormal development of L-R body asymmetry in humans.

Materials And Methods

Patient Ascertainment

Patients with SI and HTX were recruited from Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and Shanghai Children’s Medical Center. Some targeted clinical examinations such as echocardiography , abdominal ultrasonography, digital subtraction

angiography (DSA), Magnetic Resonance Imaging (MRI), Computed Tomography (CT) or operation recordings should be carried out in every patient . We excluded cases with complete situs solitus or other known syndromes[18]. One hundred unrelated individuals without major birth defects were recruited as control group.

Sample Preparation

Peripheral blood samples were obtained from the participants and DNA was extracted using TIANamp Genomic DNA Kit (TIANGEN, Beijing, China).

Mutation Sequencing

With the genomic DNA sequence of *ROCK2* derived from PubMed (NM_001321643.1),the software Oligo7 was used to design the primer pairs to amplify the exon regions of *ROCK2*(Additional file 1: Table S1).We used 2μL(80-200ug/ul) of genomic DNA in a 20 μL reaction, consisting of 10 μL of Premix Taq™ DNA Polymerase (TaKaRa), 4μL of forward primer (10uM / ul), 4 μL of reverse primer (10uM / ul). PCR product were sequenced and the DNA sequences were analyzed by GenBank BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the software Chromas.

The 1000-group genetic data (http://www.1000genomes.org/) and the ExAc database (http://exac.broadinstitute.org/) and the SNP database were used to see the frequency of mutations.

Homology analysis

With protein sequence obtained from Uniprot (http://www.uniprot.org/), ClustalX software was applied to estimate the conservation of the amino acid site by aligning the human protein to *Chimpanzee, Pig, Rat, Mouse,* and *Danio rerio*

In Silico Analyses of Candidate Sites

The potential effect of the mutations were characterized by the software Sift (http://sift.bii.a-star.edu.sg/), PolyPhen 2(http://genetics.bwh.harvard.edu/pph2/) , PROVEAN (http://provean.jcvi.org) and Mutation Taster (http://www.mutationtaster.org/). A fully automatic program Have (y)Our Protein Explained (HOPE http://www.cmbi.ru.nl/hope/) was used to analyzes the structural and functional effects of point mutations[19]. The SWISS-Pdb viewer was used to construct a three dimension (3D) conformation of wild type and mutants, with the protein sequence obtained from SWISS MODEL (https://www.swissmodel.expasy.org).

Results

Clinical Data

65 Patients with laterality defects and complex CHD were recruited from Shanghai Xinhua Hospital and Shanghai Children's Medical Center. The patients' ages ranged from 12 days to 11 years; 44 patients were male (67.7%) and 21 were female (32.3%). One hundred unrelated individuals without major birth defects were used as controls. The controls were 66 male (66%) and 34 Female (34%) with the age range from 10 months to 11 years.

Basic Information of Candidate Sites

We found three non-synonymous mutations of *ROCK2* in patients with laterality defects and these mutations were not detected in the control group Fig 1. Basic Information of these mutations are listed in Table 1. Clinically, the patient 1 and the patient 3 presented situs inversus, while the patient 2 presented HTX. The other cardiac clinical phenotypes and extracardiac abnormalities discovered in patients with ROCK2 mutations re listed in Table 1.

Table 1 Basic Information of Candidate Sites

Patient	Basic information			MAF (minor allele frequency)			Cardiac abnormalities	Extracardiac abnormalities
ID	CDS AA	PROT AA	dp SNP	1000G	ExAC	ESP		
1	c.345G>C	p.Q115H	NF	NF	NF	NF	D,ASS, TGA,VSD,PS,ASD	RSS,LSL
2	c.1247A>G	p.Y416C	NF	NF	NF	NF	L,IRAA,SV,TAPVC,PA,PDA,CAVC	Asplenia, RSS, BRB
3	c.3037G>A	p.A1013T	rs372763637	NF	0.000042	0.000085	L,ASS,SV, TGA,PS	RSS, LSL

ASD Atrial septum defect, ASS Atrial situs inversus, BRB bilateral right bronchi (short), CAVC common atrioventricular canal, D dextrocardia, IRAA isomerism of right atrial appendages, L levocardia, LI Lack of relevant information, LSL left-sided liver ,NF not found, PA pulmonary atresia, PDA patent ductus arteriosus PS pulmonary stenosis, TGA translocation of great arteries, RSS right-sided stomach, SV single ventricle , TAPVC total anomalous pulmonary venous connection, VSD ventricle septum defect.

The 1000G, the EXAC and the SNP database were applied to see the mutation frequency. The mutations c.345G>C in patient 1 and c.A1247A>G in patient 2 were newly discovered. The mutation rs372763637(c.3037G>A) found in patient 3 was described in ExAC database with an allele frequency of 0.000042 in all populations

In Silico Analysis of ROCK2 Mutations

Cross-species sequence alignment of RCOK2 suggested that the amino acid Q115 was highly conserved in vertebrates and the amino acid A1013 were conserved in mammals（Fig 1）, which suggested that the sites may have irreplaceable functions in mammals. The amino acid Y416 was only conserved in *Chimpanzee* and *Pig*（Fig 1）

The software Sift, Polyphen-2, PROVEAN and Mutation Taster were applied to predict the pathogenicity of the mutants（Table 2）. The results predicted that the ROCK2 p.Q115H variant would be damaging and disease causing in Sift and Mutation Taster. The ROCK2 p.Y416C variant was predicted as “dangerous” among all programs, while the ROCK2 p.A1013T variant may have benign effect on the RCOK2 protein. According to the analysis, p.Q115H and p.Y416C are more likely to be pathogenic mutations.

Table 2 Results of software predictions

Mutations	Sift (score)	PolyPhen-2 (score)	PROVEAN	MutationTaster	HOPE	
					Amino acid propertie	Domain functions
p.Q115H	Damaging (0.04)	Benign (0.097)	Neutral(-1.933)	disease causing	bigger	Yes
p.Y416C	Damaging (0.01)	possibly damaging (0.751)	Deleterious(-3.099)	disease causing	smaller, more hydrophobic	Yes
p.A1013T	Tolerated (0.58)	Benign (0.006)	Neutral(0.061)	polymorphism	Bigger, more hydrophobic	Yes

Sift predicts whether the mutations would be tolerated（>0.05）or damaging. (≤0.05). PolyPhen-2 shows that if the mutation is possibly damaging (score> 0.5) or benign (score < 0.5). polymorphism. Mutation Taster provides predictions whether the mutation is disease causing or polymorphism. Yes means that the mutation might impact the domain functions.

Then HOPE software was used to estimate the effect of mutations on physicochemical properties and domain functions. The mutation p.Q115H is located within a domain annotated as ‘Protein kinase’. The mutation introduces a bigger residue at this position and disturb this domain functions. Moreover, the residue is located on the surface of the protein, so that the mutation p.Q115H might disturb interactions with other molecules or other parts of the protein. The mutation p.Y416C is located within a domain annotated in UniProt as “AGC-kinase “. The mutant residue is more hydrophobic and smaller in size than the wild-type residue. According to the analysis of the HOPE online program, the mutation p.Y416C is located within a stretch of residues annotated in a special region which can interact with MYPT1(myosin phosphatase-targeting subunit 1). The differences in amino acid properties may disturb its region function.

With the ROCK2 protein sequence obtained from the Swiss-Model website, the software Swiss-Pdb Viewer was applied to construct the spatial conformation for the mutants and wild type （Fig 2）.115Gln connects to 111His and 112Gly with a hydrogen bond, while the mutation 115His abolish the connection with 112Gly. Similarly, 416Tyr is linked with 86Gln in normal condition, and the mutant 416Cys unusually links with 417Arg and disconnects with 86Gln. Changes of hydrogen bonds and connection objects might influence the functions of ROCK2.

Discussion

Laterality defects are categorized as congenital disorders characterized by a displacement of one or more organs relative to the L-R axis, which causes significant morbidity and mortality. The pathogenesis of the majority of laterality defects cases remains unknown. To elucidate the molecular mechanism of the pathogenesis, we screened *ROCK2* mutations among 65 patients with laterality disorders and identified two novel mutations and a third rare mutation. Based on biological and in silico analyses, the two novel mutations, Q115H and Y416C, may alter protein properties and impact protein functions. These mutations are also more likely to be pathogenic for patients, suggesting that the mutations of *ROCK2* might play a role in the pathogenesis of laterality defects.

ROCK2 contains a kinase domain at the N-terminal, a coiled-coiled region followed by a Rho-binding domain (RBD), and a split-PH domain containing a cysteine-rich C1 domain at the C-terminus[13, 14]. As a serine/threonine kinase, ROCK2 is an important downstream effector of the small GTP-binding protein and participates in actin cytoskeleton organization, smooth muscle contraction, stress fiber and focal adhesion formation, and plays an important role in the initiation of centrosome duplication. Relevant to the current study, ROCK2 acts as a negative regulator of the TGFβ signaling pathway and is part of the ciliary proteome[15, 16] .The knockdown of *Rock2* led to abnormalities in L-R body asymmetry in *Xenopus* and zebrafish by interfering with the cilia's function and disrupting key gene expression in the LPM[16, 17, 20]. In 2011, Wang G. found that knockdown of *rock2b* led to abnormal fluid flow in KV with a random distribution of LR ciliated cells[16]. ROCK2 can also physically bind SHROOM3 to play a part in LR development acting downstream of Pitx2[21]. Furthermore, Khalid A. Fakhro have identified rare genetic CNVs of *ROCK2* in an Htx patient with malpositioned great arteries and coarctation of the aorta (CoA) . They have also demonstrated the effect of *ROCK2* in these CNVs on L-R development using MO knockdown in *Xenopus[17]*.The mutations of *ROCK2* are responsible for laterality defects.

In our research, we found two novel mutations Q115H and Y416C in patients with laterality defects. The two heterozygous mutations are predicted to be pathogenic by biological and in silico analysis. The online program HOPE suggested that the mutant Q115H amino acid residue is more hydrophobic and bigger in size than the wild-type . Rebuilding the 3D conformations of protein chains with swiss-pdb Viewer, we found that 115Gln connects to 111His and 112Gly with a hydrogen bond in the wild type. The mutant 115His abolishes the connection with 112Gly which disrupts the structure of ROCK2.According to the protein secondary structure illustrated in UniPro, the mutation p.Q115H is located at kinase domain which is important to phosphorylation activity[13]. With this in mind, we established the hypothesis the mutant Q115H might change the structure of ROCK2 and interfere with the function of ROCK2 by disrupting normal phosphorylation activity.

The other novel mutation, p.Y416C in *ROCK2,* was also predicted to be "dangerous". HOPE suggested that the mutant Y416C amino acid residue is smaller in size with a different hydrophobicity than that of the wild type. The 3D conformations of protein chains by swiss-pdb Viewer suggest that 416Tyr is linked with 86Gln, and the mutant 416Cys unusually links with 417Arg and disconnects with 86Gln. Changes of connections and hydrogen bonds could, in part, influence the biological activities of ROCK2. Moreover, according to the analysis results of the HOPE , the mutant site is located at the end of an important structural domain that is important for the binding of MYPT1.ROCK2 can form a complex with MYPT1 to inhibit myosin light chain phosphatase (MLCP), facilitating interaction between myosin and actin cytoskeleton and increasing cell contractility[22]. In conclusion, Y146C might change the connections and hydrogen bonds to influence the biological activities of ROCK2 and interfere with the ROCK2 interaction with MYPT1, which can influence cytoskeletal dynamics. It is possible that the novel mutation p.Y416C might be associated with the abnormal cilia development in KV.

In our study, we found two novel mutations and a rare mutation in patients with laterality defects. The two novel mutations Q115H and Y416C may alter protein properties and impact protein functions, based on our in silico analyses. Our study suggests that the mutations Q115H and Y416C may be pathogenic in patients and be partially responsible for laterality defects. Functional experiments for further confirmation of the pathogenic significance of these mutants should be conducted in future studies.

Abbreviations

AP anteroposterior

ASD Atrial septum defect

ASS Atrial situs inversus

CHD congenital heart defects

CT Computed Tomography

D dextrocardia

DSA digital subtraction angiography

HTX heterotaxy

IRAA isomerism of right atrial appendage

L levocardia

LPM lateral-plate mesoderm

L-R left-right

MRI Magnetic Resonance Imaging

NF not found

PDA patent ductus arteriosus

PA pulmonary atresia

PS pulmonary stenosi

RBD Rho-binding domain

ROCK2 Rho-associated coiled-coil containing protein kinase 2

RSS right-sided stomach

SI situs inversus(SI)

SNV single nucleotide variation

SV single ventricle

TGA translocation of great arteries,

VSD ventricle septum defect

Statements And Declarations

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Ethics approval: This research conformed to the ethical guidelines of the 2013 Declaration of Helsinki with the local ethics committee of Xinhua Hospital's ethical approval.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent to publish: The authors affirm that human research participants provided informed consent for publication of the information in the article.

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Figures

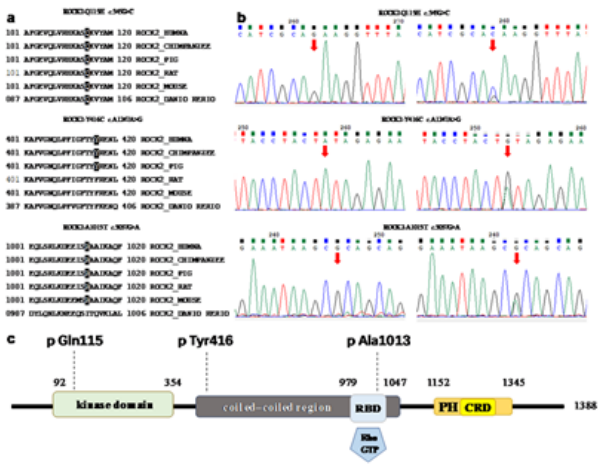

Figure 1

Biological analysis of ROCK2 and mutants a DNA sequence chromatograms of patients and controls. **b** homology analyses of ROCK2 mutations by cross-species sequence alignment. **c** Schematic diagram of ROCK2 structure with approximated localization of the mutations.

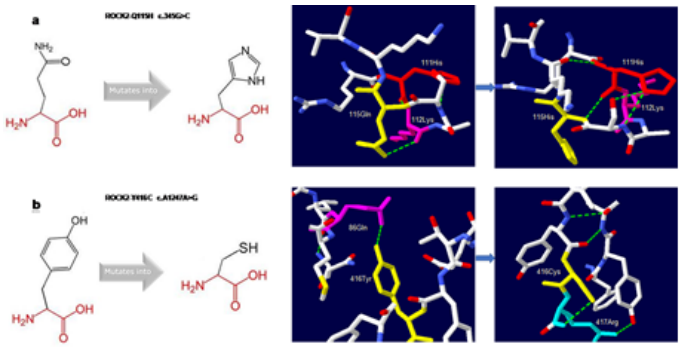

Figure 2

Spatial conformation of ROCK2 **a** 115Gln(yellow) connects to 111His(red) and 112Gly(pink) with a hydrogen bond in normal condition (3D conformation of native protein chain is shown on the middle); the mutation 115His abolish the connection with 112Gly (the 3D conformation of mutant is shown in the on the right). **b** 416Tyr(yellow) is linked with 86Gln(pink) in normal condition (3D conformation of native protein chain is shown on the middle); the mutant 416Cys(yellow) unusually links with 417Arg (blue)and disconnects with 86Gln (the 3D conformation of mutant is shown in the on the right).

Supplementary Files

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- TableS1.xlsx