Research Article
Novel Mutation of *LRP6* Identified in Chinese Han Population Links Canonical WNT Signaling to Neural Tube Defects

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Background: Neural tube defects (NTDs), the second most frequent cause of human congenital abnormalities, are debilitating birth defects due to failure of neural tube closure. It has been shown that noncanonical WNT/planar cell polarity (PCP) signaling is required for convergent extension (CE), the initiation step of neural tube closure (NTC). But the effect of canonical WNT//β-catenin signaling during NTC is still elusive. LRP6 (low density lipoprotein receptor related proteins 6) was identified as a co-receptor for WNT/β-catenin signaling, but recent studies showed that it also can mediate WNT/PCP signaling. **Methods:** In this study, we screened mutations in the *LRP6* gene in 343 NTDs and 215 ethnically matched normal controls of Chinese Han population. **Results:** Three rare missense mutations (c.1514A>G, p.Y505C); c.2984A>G, p.D995G; and c.4280C>A, p.P1427Q) of the *LRP6* gene were identified in Chinese NTD patients. The Y505C mutation is a loss-of-function mutation on both WNT/β-catenin and PCP signaling. The D995G mutation only partially lost inhibition on PCP signaling without affecting WNT/β-catenin signaling. The P1427Q mutation dramatically increased WNT/β-catenin signaling but only mildly loss of inhibition on PCP signaling. All three mutations failed to rescue CE defects caused by *lrp6* morpholino oligos knockdown in zebrafish. Of interest, when overexpressed, D995G did not induce any defects, but Y505C and P1427Q caused more severe CE defects in zebrafish. **Conclusion:** Our results suggested that over-active canonical WNT signaling induced by gain-of-function mutation in *LRP6* could also contribute to human NTDs, and a balanced WNT/β-catenin and PCP signaling is probably required for proper neural tube development.

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Key words: LRP6; mutation; neural tube defects; WNT/β-catenin signaling; PCP signaling

Introduction

Neural tube defects (NTDs) are the second most common birth defect, affecting 0.5 to 2 per 1000 established pregnancies all around the world (Mitchell, 2005), and it has been reported that Shanxi province of China had a much higher prevalence rate in NTDs in the past few decades (Li et al., 2006). NTDs result from a failure of neural tube closure (NTC), which occurs during the 3rd and 4th weeks of gestation. Depending on which region of the neural tube is unclosed, a variety of malformations are included under the term NTD, such as exencephaly, anencephaly, craniorachischisis, myelomeningocele (spina bifida), and encephalocele (Copp and Greene, 2013; Wallingford et al., 2013). Infants with exencephaly, anencephaly, and craniorachischisis are often stillborn or die shortly after birth, whereas many infants with spina bifida can survive but suffer severe and lifelong physical disabilities and are at risk for psychosocial maladjustment (Botto et al., 1999). Genetic factors contribute significantly to the etiology of NTDs (Leck, 1974). However, the precise nature of this genetic contribution remains unclear.

The initiation step of NTC is called convergent extension (CE), which is the process of lengthening and narrowing of the initially disc-shaped neural plate (Ybot-Gonzalez et al., 2007). The noncanonical WNT signaling planar cell polarity (PCP) pathway is critical for the CE process (Copp and Greene, 2013; Nikolopoulou et al., 2017). Mouse models with mutations in the core genes of the PCP pathway showed very severe NTDs (summarized in Copp et al., 2013) and putative mutations in PCP genes, such as *VANGL1*

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(Kibar et al., 2007) and *VANGL2* (Lei et al., 2010), are also reported to be associated with NTDs in human. On the other hand, the genetic association of the canonical WNT/β-catenin pathway genes to human NTDs is still elusive.

LRP6 (low density lipoprotein receptor related proteins 6) was identified as an essential co-receptor with Frizzled (Fzd) for the WNT/β-catenin signaling pathway (Schweizer and Varmus, 2003; Tamai et al., 2004). Several studies based on mouse models clearly showed that Lrp6 is involved in neurulation and neural tube formation. Both *Lrp6*$^{-/-}$ mutant mice (Pinson et al., 2000) and *ringelschwanz* (*rs*) mice with a hypomorphic mutation on *Lrp6* (R886W) (Kokubu et al., 2004) exhibit spina bifida phenotype. A mouse line *Crooked tail (Cd)* with a gain-of-function point mutations of *Lrp6* (G494D) has a cranial NTD and crooked tail (Carter et al., 2005). *Skax26*^{m1Jus} mice (Lrp6 I681R) have kinked/looped tails (Allache et al., 2014). Those data strongly support the hypothesis that mutations in *LRP6* are associated with human NTDs. Two recent studies reported eight rare missense mutations in *LRP6* (Y306H, Y373K, V1386L, Y1541C, A3V, Y544C, P1482L, R1574L) from an Italian NTD cohort (Allache et al., 2014) and an American NTD cohort (Lei et al., 2015). However, there is no report of *LRP6* mutations in Chinese NTDs patients yet.

In this study, we sequenced all exons of *LRP6* among a Chinese NTD cohort with 343 cases (mainly from Shanxi province) and 215 matched controls, and identified three rare missense mutations, Y505C, D995G, and P1427Q, in our cases. Luciferase reporter assay showed that those three mutations have very different effects on WNT signaling. The Y505C is a loss-of-function mutation in both WNT/β-catenin and PCP signaling. The D995G is a loss-of-function mutation in PCP signaling without affecting WNT/β-catenin signaling. The P1427Q is a gain-of-function mutation in WNT/β-catenin signaling with only a mild effect on PCP signaling. To our surprise, the gain-of-function mutation P1427Q could cause more severe NTD than the p.D995G mutations in zebrafish models when overexpressed, suggesting over-active canonical WNT signaling could also contribute to human NTDs.

Materials and Methods

STUDY SUBJECTS

NTD samples were collected either from aborted fetuses or children with spina bifida who were less than 10 years old from the 1990s to the 2010s. A total of 343 NTD samples (38.8% male, 51.3% female, 9.9% unknown) were collected from Shanxi province (267), Liaoning (25), Heilongjiang (15), Suzhou (12), and Tianjin (24) (Table 1). We enrolled individuals with NTDs who were assessed by clinical geneticists and were placed into at least one of the following diagnostic groups: anencephaly, spina bifida (aperta or cystica), holorachischisis, craniorachischisis, spinal dysraphism, or encephalocele. A total of 309 of the 343 cases were tissue samples from aborted fetuses with severe rostral NTDs, such as craniorachischisis or anencephaly. The remaining 34 cases represented children under the age of 10 with spina bifida from whom blood samples were collected. The 215 controls (40.5% male, 59.5% female) were ethnically and gender-matched unrelated healthy volunteers recruited from Shanxi province (192 tissue samples from aborted fetuses from unrelated healthy volunteers) and Shanghai (32 blood samples from healthy college freshman students) China (Table 1). All samples were collected with the approval of the local ethics committee and the institutional ethics committee of Fudan University. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in

TABLE 1. *Demographic Characteristics in NTD Cohort*

Variable	Case (%)	Controls (%)
Sequencing group	343	215
Region		
Tianjin	24	
Suzhou	12	
Liaoning	25	
Heilongjiang	15	
Shanxi	267	184
Shanghai		31
Age: weeks/years* (mean ± SD)		
Tianjin	3.1 ± 3.4*	
Jiangsu	N.D.	
Liaoning	2.4 ± 4.0*	
Heilongjiang	N.D.	
Shanxi	23.1 ± 6.3	20.0 ± 3.3
Shanghai		18.0*
Gender		
Male	133 (38.8%)	87(40.5%)
Female	176 (51.3%)	128 (59.5%)
Unknown	34 (9.9%)	0 (0)
CRS	19 (5.5%)	
EC	79 (23.0%)	
AE	70 (20.4%)	
EX	2 (0.58%)	
SB	231 (67.3%)	
NTD	1 (0.3%)	

CRS, craniorachischisis; EC, encephalocele; AE, anencephaly; EX, exencephaly; SB, spina bifida; N.D., not determined.

2000 (5). Informed consent was obtained from all patients for being included in the study.

DNA SEQUENCING AND DATA ANALYSIS

Approximately 2 ml of peripheral blood was collected from each test subject. Genomic DNA was prepared and targeted exome sequencing was conducted as previously described (Qiao et al., 2016). To confirm the genotyping results of *LRP6* from next generation sequencing, three case-specific coding mutations of *LRP6* within NTD samples were amplified by polymerase chain reaction and confirmed by Sanger DNA sequencing. *LRP6* variants were checked in absence in two public databases: the 1000 genome project (http://www.1000genomes.org), and the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/)(Lek, Karczewski et al., 2016). Mutations were also evaluated by using the PolyPhen-2 (Adzhubei et al., 2010) (Polymorphism Phenotyping version 2.1.0; http://genetics.bwh.harvard.edu/pph/) and SIFT (Kumar et al., 2009; Sim et al., 2012) (Sorting Intolerant from Tolerant; http://sift.jcvi.org/) programs.

PLASMIDS AND SITE-DIRECTED MUTAGENESIS

LRP6-vsvg plasmid was kindly provided by Dr. Xi He (Harvard Medical School, Boston, MA). Mutations to *LRP6* plasmids were made using the KOD Site-Directed Mutagenesis strategy (TOYOBO, Japan). All plasmids were verified by DNA sequencing. Topflash-luciferase reporter plasmid was a gift from Dr. Tao Zhong at Fudan University. pFR-luciferase reporter and Pfa2-c-Jun plasmids were obtained from Stratagene. DNA concentrations were examined by using NanoDrop 2000 (Thermo Scientific, NC).

IN VITRO TRANSCRIPTION OF AMPLIFIED CDNA

The *LRP6* vectors were linearized with *Not*I restriction enzyme (NEB, USA) and transcribed with the Sp6 mMESSAGE mMACHINE kit (Ambion, TX). The reaction was carried out at 37°C for 2 hr, followed by the addition of DNase I and incubation for 15 min. Ammonium acetate was added, and RNA was isolated by phenol/chloroform extraction and isopropanol precipitation. After centrifugation, the RNA pellet was resuspended in RNase-free water. The pelleted RNA was resuspended in RNase-free water, and the quantity and purity were determined by ultraviolet spectrophotometry and electrophoresis.

ZEBRAFISH EMBRYO MICROINJECTIONS

The wild-type (WT) AB zebrafish strain was maintained and bred under standard conditions. All institutional and national guidelines for the care and use of laboratory animals were followed. All of the *LRP6* mRNA was diluted in RNA-free water. Morpholino oligos (MOs) acted as a means to inhibit gene function in embryos (Heasman, 2002). To examine their effect of rescuing, we injected the 300 pg of mRNA of WT or *LRP6* mutants mixed with 3 ng of *Lrp6* MOs, respectively, into zebrafish embryos during the one- to two-cell stage. After injection, the embryos were allowed to develop at 28.5°C for 48 hr and were observed by microscopy. Phenotypes were scored, and images were taken using a Leica MZ95 microscope system. Statistical significance was calculated using chi-squared analysis in Microsoft Excel. The sequences of zebrafish *lrp6* MOs and control MOs are: Control MO 5′ CCTCTTACCTCAGTTACAATTTATA 3′

zlrp6 MO 5′ AGAGAGTCTGAAGCACGGCACCCAT 3′.

CELL CULTURE AND LUCIFERASE ASSAY

HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. In Topflash luciferase reporter experiment, HEK293T cells were transfected (24 hr after 1 × 10e5 seeding in 24-well plate, 80% confluence) with 250 ng of WT/*LRP6* mutants/empty vector plasmids, 250 ng of Topflash luciferase reporter, and 25 ng of the Renilla luciferase expression plasmid (Promega, China) as an internal control per well using Lipofectamine2000 (Invitrogen, China). After 36 hr in transfection media, cells were overlaid with WNT3a conditioned media. Conditioned medium was harvested from WNT-3a secreting L cells per manufacturer's instructions (ATCC). Cells were harvested after 10-hr incubation, and the Promega Dual Luciferase Assay kit was used per manufacturer's instructions. pFR-luciferase reporter plasmid was used to evaluate PCP signaling. Cells were transfected with 100 ng of pFR-luciferase reporter, 100 ng of Pfa2-c-Jun construct, and 200 ng of various *LRP6* constructs with 50 ng of Renilla-luciferase plasmid. The firefly and Renilla luciferase activities in cell lysates were measured 36 hr after transfection. Three independent transfection experiments were performed, and each luciferase assay was performed in triplicate. Statistical significance was calculated using a two-tailed student's *t* test in Microsoft Excel.

WESTERN BLOT

To test the effect of each mutation on LRP6 phosphorylation, Western blot analysis was carried out. HEK293T cells were transfected with WT *LRP6* or *LRP6* mutants in six-well plates using Lipofectamine 2000 reagent (Invitrogen, USA) per manufacturer's instructions with a 0.5:1 ratio of reagent to DNA. After 36 hr, HEK293T cells were treated with WNT-3a CM for 1 hr. Then cells were lysed in RIPA buffer (Beyotime, China) containing a cocktail of protease inhibitors and heated for 5 min at 100°C. Cell lysates were analyzed by sodium dodecyl sulfate gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Germany). Blocking of the membrane was done with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20. Then we incubated the membrane with anti-vsvg (V4888, Sigma St. Louis, MO), anti-LRP6 (phospho S1490) (ab76417, Abcam), anti-Beta-actin

TABLE 2. *Genotype and Clinical Phenotypes of Patients Carrying the Three Missense Mutations of LRP6 That Are Absent in Controls*

Mutation	Amino acid	SIFT	PolyPhen2	Sex	Age (weeks)	NTD type
c.1514A>G	Y505C	Damaging	Probably damaging	F	40	Open spina bifida
c.2984A>G	D995G	Tolerated	Possibly damaging	F	104	Cervical spina bifida occulta
c.4280C>A	P1427Q	Tolerated	Possibly damaging	M	39	Open spina bifida

For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence NM_002336.2.

antibody (Sigma), secondary antibodies, and visualized with an Tanon 5200 (Tanon, China).

Results

IDENTIFICATION OF THREE NOVEL *LRP6* MUTATIONS IN CHINESE NTD CASES

Three case-specific heterozygous missense mutation c.1514A>G (p.Y505C), c.2984A>G (p.D995G), and c.4280C>A (p.P1427Q) in *LRP6* were identified by targeted exome sequencing in three patients from our cohort (Table 2). Each of these variants was detected in a heterozygous state in NTD case as confirmed by Sanger sequencing (Fig. 1A). All three mutations were not identified in our 215 ethnically matched normal controls. According to the ExAC database (Exome Aggregation Consortium databases) (http://exac.broadinstitute.org) (Lek et al., 2016), which currently contains whole exome sequencing data of 60,706 unrelated individuals, the D995G and P1427Q are novel mutations (not reported in ExAC), while Y505C is a very rare mutation with only two allele count (allele frequency = 1.648e-05). All three mutations occurred in the highly conserved amino acids. Y505 and D995 are located in the second and fourth YWTD-EGF-like repeat of LRP6 extracellular domain, respectively; while P1427 locates near the phosphorylation motif of LRP6 intracellular domain (Fig. 1C). Those mutations are predicted to be damaging by PolyPhen2 (Table 2).

LRP6 MUTATIONS AFFECT BOTH WNT/β-CATENIN AND PCP SIGNALING

To investigate the effect of human *LRP6* mutations on protein function, we first examined the effect of these mutants in both WNT/β-catenin and PCP signaling with luciferase reporters. The Topflash reporter, which is a β-catenin-responsive reporter, was used to examine the activity in WNT/β-catenin signaling; while the pFR and Pfa-c-Jun reporter system was used as indicator for PCP signaling. Each reporter system was co-transfected with either WT or LRP6 mutants into HEK293T cells. Similar to previous reports (Carter et al., 2005; Lei et al., 2015), our results also showed that WT LRP6 could activate the WNT/β-catenin signaling and inhibiting PCP signaling in HEK293T cells (Fig. 2A,B). The Y505C mutant resulted in a significantly lower activation of WNT/β-catenin and less inhibition of PCP signaling. Surprisingly, the D995G only lost inhibition on PCP signaling without affecting WNT/β-catenin signaling and the P1427Q mutant significantly up-regulated WNT/β-catenin signaling (Fig. 2A) with only mild effect on PCP inhibition (Fig. 2B).

Y505C AND P1427Q MUTANTS SERIOUSLY AFFECTED LRP6 PHOSPHORYLATION

It has previously been shown that WNT3a treatment quickly induces plasma membrane-associated LRP6 aggregates, which induce LRP6 phosphorylation (Pan et al., 2008). The phosphorylation of LRP6 is essential for WNT/β-catenin signaling pathway (Veeman et al., 2003; Tamai et al., 2004). As the P1427Q is very near the phosphorylation motif of LRP6 intracellular domain, we examined whether the LRP6 mutants were affected in this process. Our results showed that WNT3a-conditioned medium induced robust and rapid phosphorylation of the WT and D995G LRP6. The P1427Q mutant showed a significantly up-regulation in LRP6 phosphorylation, while the Y505C showed significantly down-regulation in LRP6 phosphorylation (Fig. 3). The phosphorylation levels of those LRP6 mutants correlated well with their ability in canonical WNT signaling (Fig. 2A).

THESE HUMAN *LRP6* MUTATIONS CAUSED CE DEFECTS IN ZEBRAFISH

Next we investigated the in vivo effect of these human *LRP6* mutations on neural tube development in zebrafish models. Antisense MOs for zebrafish *lrp6* were injected into one- to two-cell stage zebrafish embryos. Forty-eight hours postinjection, the MO-injected embryos exhibited high ratio (83.7%) of abnormal curving due to defects in CE process (Fig. 4A,B), which is similar to previous report (Jiang et al., 2012).

A rescue experiment was conducted with either WT or mutant human LRP6. We co-injected embryos with a mixture of *lrp6* MOs and with either WT or mutant human LRP6 plasmid. The frequency of NTDs caused by MOs knockdown was significantly reduced by LRP6-WT and all three mutations showed significantly less rescuing ability than WT (Fig. 4B). Of interest, the P1427Q displayed the least rescuing ability than the LRP6-WT under the same concentrations (Fig. 4B).

An over-expression experiment was also performed with either WT or mutated LRP6 injected alone into zebrafish embryos. A significantly higher frequency of NTDs was observed in zebrafish embryos injected with either Y505C or P1427Q compared with those injected with WT LRP6 at the same concentration (Fig. 4B). Again, P1427Q

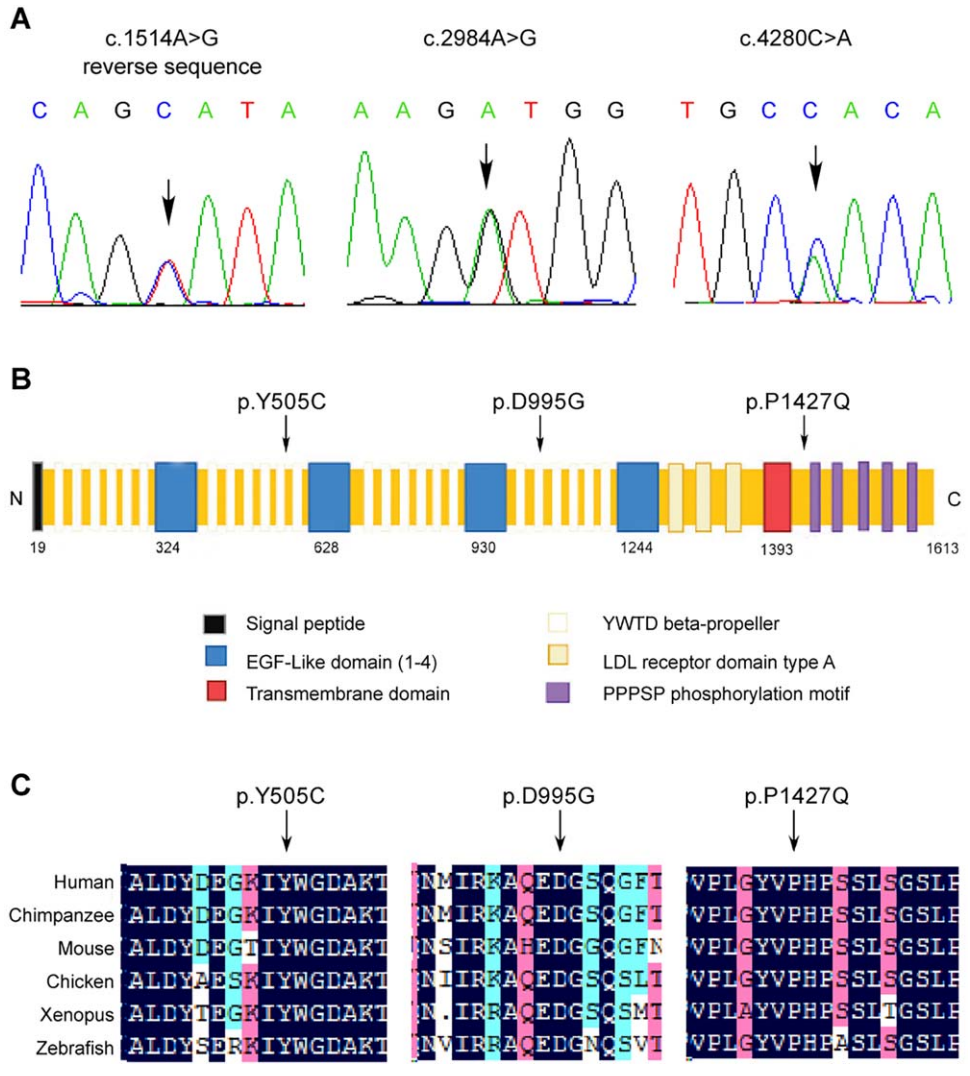

FIGURE 1. *Three rare/novel mutations in LRP6 were identified in our NTDs cohort.* **(A)** *Sequencing results of the LRP6 mutation. The arrows indicate the heterozygous nucleotides of each mutation.* **(B)** *Illustration of LRP6 structure with novel/case-specific SNVs identified in NTD cohorts.* **(C)** *A partial alignment of LRP6 amino acid sequence between human and several other vertebrates. The LRP6 variants found in NTDs affect conserved residues. Residues conserved between human LRP6 and other homologue members are highlighted in dark blue. National Center for Biotechnology Information accession numbers are NP_002327.2 for human LRP6, XP_001152103.1 for Pan troglodytes (chimpanzee) LRP6, NP_032540.2 for Mus musculus (mouse) Lrp6, XP_015146392.1 for Gallus (chicken) LRP6, NP_001079233.1 for Xenopus laevis lrp6, NP_001128156.1 for Danio rerio (zebrafish) lrp6.*

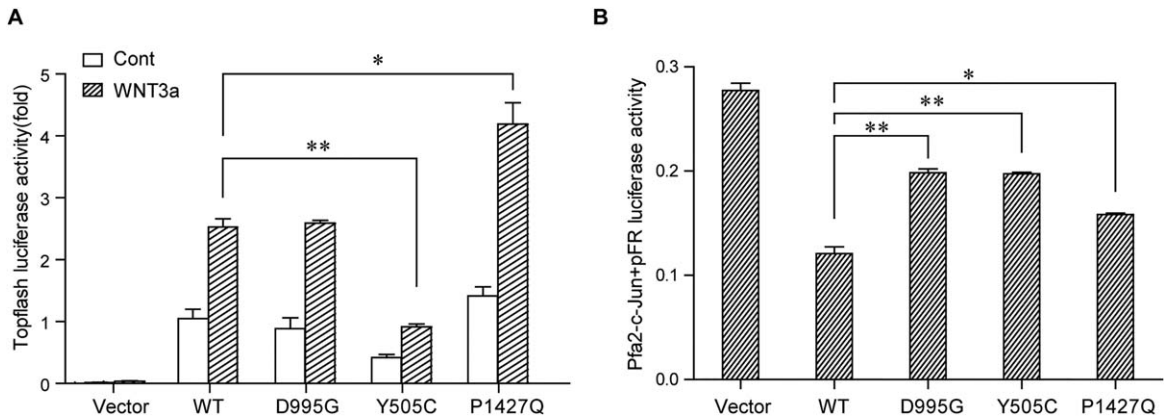

FIGURE 2. *Those three mutations affect WNT/β-catenin and PCP signaling activity in luciferase reporter assay.* **(A)** *TopFlash Reporter was used to indicate WNT/β-catenin signaling. WT or mutant LRP6, and empty vector (as control) were cotransfected into HEK293T cells and 36 hr after transfection the cells were treated with either control medium or Wnt3a conditional medium and incubated for 1 hr before harvesting the cells for a Luciferase assay. The Y505C mutation decreased WNT/β-catenin signaling; while the P1427Q mutation showed a significant up-regulation in WNT/β-catenin signaling compared with WT LRP6.* **(B)** *The Pfa2-c-Jun and JNK-responsive pFR-luciferase reporter was used to indicate PCP signaling. WT LRP6 significantly inhibited PCP signaling while all LRP6 mutants lost some inhibition on PCP signaling. The asterisk indicates a significant difference between WT and mutant LRP6. All experiments repeated for 3 times (N = 3, *p < 0.05, **p < 0.01, two-tailed t test).*

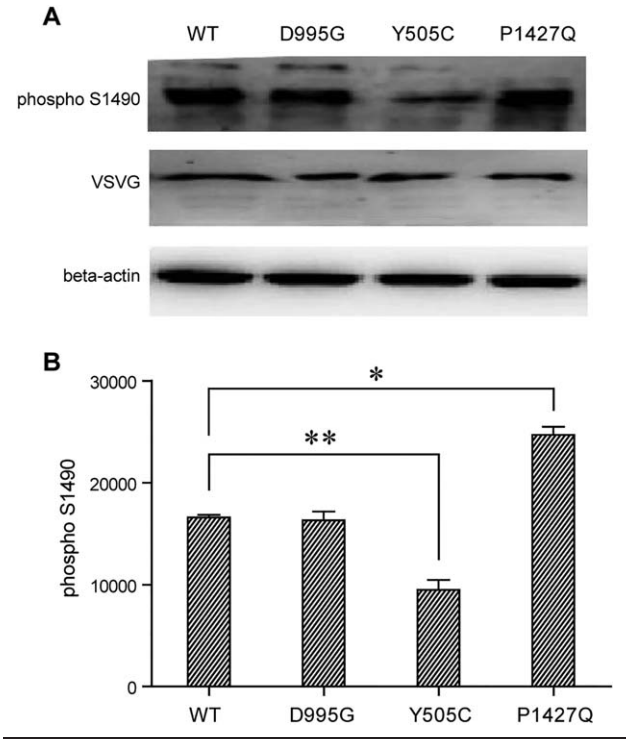

FIGURE 3. *The phosphorylation level of LRP6 is affected by mutations. HEK293T cells transfected with either WT or mutant LRP6 (vsvg-tagged) were treated with WNT-3a CM for 1 hr. Cell lysates were blotted by a VGVG antibody or PPPSP-phosphorylated LRP6 antibody Ab1490. The representative Western blot images are shown in* **(A)**, *and the statistical analysis is shown in* **(B)**. *(N = 4, *p < 0.05, **p < 0.01, two-tailed t test).*

had significantly higher ability to induce NTDs in zebrafish under the same condition. Surprisingly, the D995G did not have an effect on NTDs in zebrafish when overexpressed under the same condition (Fig. 4B).

Discussion

In this study, we identified three case-specific rare mutations (Y505C, D995G, P1427Q) in *LRP6* in Chinese NTD cases. To our knowledge, this is the first report of *LRP6* mutations associated with Chinese NTDs. Furthermore, we showed that those mutations affected both in vitro and in vivo functions of LRP6. In addition to our study, eight different missense mutations (Y306H, Y373K, V1386L, Y1541C, A3V, Y544C, P1482L, R1574L) in the *LRP6* gene have also been identified in NTDs from Italian (Allache et al., 2014) and Americans (Lei et al., 2015). Taken together, we can conclude that mutations in *LRP6* are associated with human NTDs worldwide.

However, how LRP6 is involved in the pathogenesis of NTDs is still not that clear as both mouse models and human mutations showed very complex mechanisms. In mouse models, the study by Gray et al. suggested that Lrp6 mediates RhoA activation and this noncanonical WNT function of LRP6 is critical for the NTC process (Gray et al., 2013). RhoA inhibition exacerbated NTDs in $Lrp6^{-/-}$ mice and partially rescued NTDs in $Lrp6^{Cd/Cd}$ mice (Gray et al., 2013). However, it is difficult to fully exclude the effect of canonical WNT/β-catenin signaling by means of Lrp6 during NTC and neurulation as (1) the RhoA inhibition cannot fully rescue the defects in $Lrp6^{Cd/Cd}$ mice, (2) $Lrp6^{Cd}$ mutant showed decreased β-catenin activation, and (3) there is no RhoA/ROCK activator rescuing experiments performed on $Lrp6^{-/-}$ mice. On the other hand, a cross-talk function of Lrp6 between the canonical Wnt and Notch signaling pathways during somitogenesis was shown, and

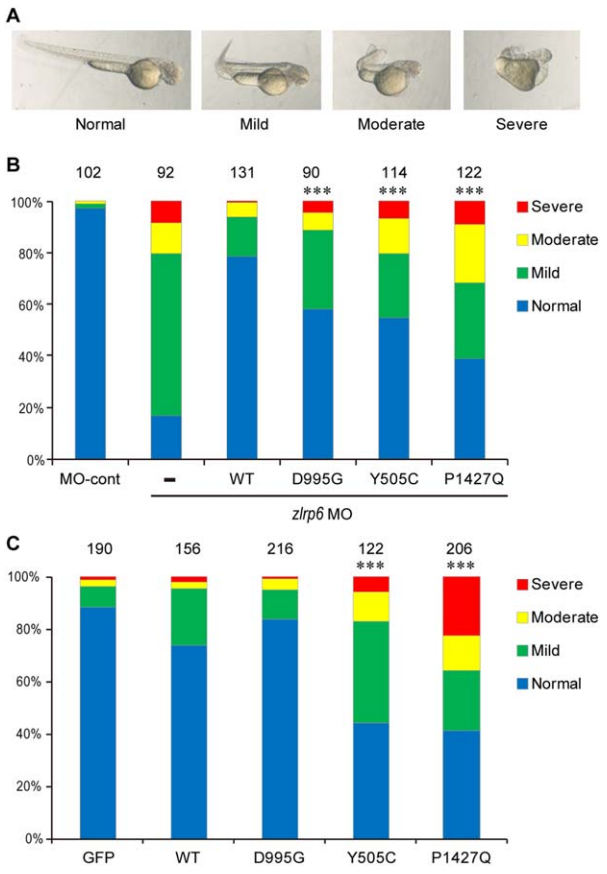

FIGURE 4. *Those LRP6 mutants could induce NTD phenotypes in zebrafish embryos.* **(A)** *The severity of the NTD was determined based on the morphogenesis of the neural tube as either curved NTDs or shorten spine.* **(B)** *Injection of zlrp6 MOs yielded high proportion NTD phenotypes in zebrafish. WT LRP6 exhibited higher efficiency saving ability than three mutations.* **(C)** *Overexpression of WT and mutant LRP6 alone also induced NTDs in zebrafish except D995G mutation. The number above each bar is the total number of embryos examined under each experimental condition. The p-value was calculated by chi-squared analysis. The asterisk indicates a statistically significant difference (***p < 0.001).*

TABLE 3. *Summary of Human LRP6 Mutations Identified in NTDs Cohorts*

			Luciferase reporter assay				
Reference	**cDNA**	**A.A change**	**WNT/β-catenin**	**PCP**	**Group**	**NTDs in zebrafish OE**	**NTDs in zebrafish w/MO[a]**
	LRP6-WT		Activation	Inhibition		+	+
Our cohort	c.1514A>G	Y505C	LoF	partial LoF	II	+++	+++
Our cohort	c.2984A>G	D995G	–	partial LoF	III	++	++
Our cohort	c.4280C>A	P1427Q	GoF	slightly LoF	IV	++++	++++
Allache et al. 2014	c.916T>C	Y306H	LoF	LoF	II	N.A..	N.A..
Allache et al. 2014	c.1118A>G	Y373L	LoF	LoF	II	N.A.	N.A.
Allache et al. 2014	c.4157T>G	V1386L	LoF	LoF	II	N.A.	N.A.
Allache et al. 2014	c.4622G>T	Y1541C	–	–	I	N.A..	N.A..
Lei et al. 2014	c.8C>T	A3V	–	slightly LoF	III	N.A..	N.A..
Lei et al. 2014	c.1631A>G	Y544C	LoF	partial LoF	II	N.A.	N.A.
Lei et al. 2014	c.4445C>T	P1482L	–	–	I	N.A.	N.A.
Lei et al. 2014	c.4721G>T	R1574L	slightly GoF	LoF	IV	N.A.	N.A.

[a]The severity of NTDs in zebrafish injected with MO was set as +++++.

OE, overexpressed; N.A., not analyzed; LoF, loss of function; GoF, gain of function.

disruption of such interaction accounts for the spina bifida phenotype in *Lrp6*$^{rs/rs}$ mice (Kokubu et al., 2004).

In human studies, with the 3 mutations we reported in this study, a total 11 rare missense mutations of *LRP6* have been identified in NTD patients to date (summarized in Table 3). However, the effects of LRP6 mutations to WNT/β-catenin and PCP signals are very diverse, which can be divided into four groups. Group I, Y1541C and P1482L have no effect on either pathway. Group II, five mutations (Y306H, Y373K, V1386L, Y544C, Y505C) are loss-of-function mutations in both WNT/β-catenin and PCP signals. Group III, D995G and A3V have no effect on WNT/β-catenin signaling but have a weak loss of inhibition on PCP pathway. And the last group, P1427Q and R1574L showed very different degree of effects on those two pathways (Table 3). Luckily, the three mutations we identified fall into groups II–IV, and we also performed zebrafish experiments to observe the in vivo effects of those LRP6 mutations during the CE process.

The loss-of-function mutation Y505C caused severe CE defects in zebrafish (Fig. 4; Table 3). This mutation is very similar to the previously identified Y544C mutation (Lei et al., 2015). As both mutations affect conserved Y in YWTD motif in the second β-propeller-EGF-like (PE) domain, it strongly suggests that the second YWTD-β-propeller (PE2) is important for both WNT/β-catenin and PCP signaling.

The D995G mutation is located in the fourth PE domain. Although a previous study showed that Wnt3a binds to PEs 3-4 (Tamai et al., 2004), here, we did not observe any changes on Wnt3a induced canonical WNT/β-catenin signaling (Fig. 2A) by the D995G mutation. Only the PCP signaling was affected by this mutation. When D995G was overexpressed alone in zebrafish, this mutation did not induce NTDs (Fig. 4B), suggesting that it does not function as a dominate negative mutant competing with the lrp6 in zebrafish. The fact that this D995G mutation still showed loss-of-function in rescue experiment in zebrafish (Fig. 4C) indicates that loss of inhibition on PCP signaling affected its rescue ability. This observation correlates with previous reports that showed that the PCP signaling mediated by means of LRP6 is important for the NTC process (Gray et al., 2013; Lei et al., 2015).

The most interesting mutation is the P1427Q mutation. Different from the previously reported mutation R1574L, which increased the canonical WNT/β-catenin signaling and completely lost inhibition on PCP signaling (Lei et al., 2015), our biochemistry assay showed that the P1427Q mutation is a gain-of-function mutation in the canonical WNT pathway and has a very mild effect on PCP signaling (Fig. 2). Unlike R1574L, which is in the middle of the PPPSP motifs, the P1427Q is near a juxtaposed casein kinase 1 (CK1) site S1430, which is a negative regulated phosphorylation site of CK1ϵ (Swiatek et al., 2006). A previous study showed that S1430A mutation caused higher phosphorylation on the PPPSP motif and increased the canonical WNT/β-catenin signaling (Swiatek et al., 2006). We also observed dramatically increased phosphorylation level of LRP6 with P1427Q. The distinctive cyclic structure of the proline's side chain at P1427 causes a special conformation of the protein structure. As the mutation changes this proline to glutamine, it probably affected the CK1ϵ binding to the LRP6 cytoplasmic domain and lost the

inhibition on LRP6 phosphorylation on PPPSP sites. This correlates with our observation of the up-regulated phosphorylation level of this mutant LRP6 (Fig. 3).

The fact that this P1427Q mutation showed the strongest NTD-inducing ability when overexpressed in zebrafish (Fig. 4B) and that the D995G mutation did not induce NTDs under similar condition suggests that the hyperactive canonical WNT/β-catenin signaling (not the mild loss-of inhibition on the PCP signaling observed in this P1427Q mutation) caused abnormal CE when overexpressed.

In conclusion, here we reported that three rare *LRP6* mutations identified in 336 Chinese NTDs are probably associated with NTD in human. We used a zebrafish model to show that the hyperactive *LRP6* mutation in canonical WNT/β-catenin signaling induces NTDs in vivo. Our results also strongly suggested that a balanced canonical and non-canonical WNT signaling is critical for NTC process, as both loss of inhibition on PCP signaling and hyperactive WNT/β-catenin signaling can induce NTDs.

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