Genetic analysis of Wnt/PCP genes in neural tube defects

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Abstract

**Background:** Mouse homozygous mutants in Wnt/planar cell polarity (PCP) pathway genes have been shown to cause neural tube defects (NTDs) through the disruption of normal morphogenetic processes critical to neural tube closure (NTC). Knockout mice that are heterozygotes of single PCP genes likely fail to produce NTD phenotypes, yet damaging variants detected in human NTDs are almost always heterozygous, suggesting that other deleterious interacting variants are likely to be present. Nonetheless, the Wnt/PCP pathway remains a genetic hotspot. Addressing these issues is essential for understanding the genetic etiology of human NTDs.

**Methods:** We performed targeted next-generation sequencing (NGS) on 30 NTD-predisposing Wnt/PCP pathway genes in 184 Chinese NTD cases. We subsequently replicated our findings for the CELSR1 gene in an independent cohort of 292 Caucasian NTD samples from the USA. Functional validations were confirmed using in vitro assays.

**Results:** CELSR1, CELSR2 and CELSR3 genes were significantly clustered with rare driver coding mutations (q-value< 0.05) demonstrated by OncodriveCLUST. During the validation stage, the number of rare loss of function (LoF) variants in CELSR1 was significantly enriched in NTDs compared with the LoF counts in the ExAC database (p < 0.001). Functional studies indicated compound heterozygote variants of CELSR2 p.Thr2026Met and DVL3 p.Asp403Asn result in down regulation of PCP signals.

**Conclusions:** These data indicate rare damaging variants of the CELSR genes, identified in ~14% of NTD cases, are expected to be driver genes in the Wnt/PCP pathway. Compound damaging variants of CELSR genes and other Wnt/PCP genes, which were observed in 3.3% of the studied NTD cohort, are also expected to amplify these effects at the pathway level.

**Keywords:** Neural tube defects, PCP (planar cell polarity), Wnt, Variant, CELSR

Background

Neural tube defects (NTDs) resulting from the failure of neural tube closure (NTC) are among the most common and severe forms of developmental defects in humans. The complete closure of the neural tube requires the coordination of neural-plate apical constriction and convergent extension, both of which are directly regulated by the non-canonical Wnt/planar cell polarity (PCP) pathway [1]. Homozygous mouse mutants in the PCP pathway are known to cause NTDs through the disruption of NTC. Loss of function (LoF) alleles of the core components of the PCP pathway, including Celsr1 [2] and Vangl2 [3] produces craniorachischisis in mice. In addition, double but not single knockouts at Fzd3 and Fzd6 [4], as well as Dvl1 [5, 6] result in the NTD known as craniorachischisis in mice. In recent years, functional variants in PCP pathway genes have been identified in human NTDs, including: CELSR1 [7, 8], DVL2 [9], VANGL1 [10], VANGL2 [11], SCRIB [12], LRP6 [13] and FZD6 [14]. PCP genes appear to play an important role in the etiology of these human congenital malformations [15]. Besides these PCP components, alterations in Wnt signaling has previously been associated with NTDs [16].
and Wnt signaling proteins also direct PCP signals in the vertebrate ectoderm [17]. The polarity of Prickle3/Vangl2 complex was disrupted by several Wnt antagonists in the ectoderm of early Xenopus embryos; while Wnt5a, Wnt11, and Wnt11b disrupted Prickle3/Vangl2 complexes in mid-gastrula embryos leading to the development of NTDs [17]. Despite recent advances in genomics and bioinformatics, the genetic contribution of Wnt/PCP pathway genes to human NTDs requires further interrogation. Unlike mutations in many PCP genes that cause NTDs in mice, the variants detected in human NTDs are overwhelmingly heterozygous. Although knock out heterozygotes of single inactivated mouse genes does not typically produce NTD phenotypes, disruption of multiple genes in an individual could produce devastating consequences on embryonic development, as observed in mice [18]. Rare damaging variants were used in identifying interactions with birth defects, such as craniosynostosis [19] and congenital heart disease [20]. These data suggest that the interactions between rare compound damaging variants may contribute to the etiology of NTDs in humans; however, there is a paucity of compound heterozygosity data on human NTDs.

This study analyzes a panel of Wnt/PCP genes in a population based cohort of human NTD samples. We found that all three CELSR genes (CELSR1, CELSR2 and CELSR3) play driver roles in the etiology of human NTDs. In addition, this study is the first to identify compound PCP rare damaging variants combination in human NTDs, and these genetic combinations are expected to amplify effects at the pathway level and increase the risk of NTDs.

Methods

Human neural tube defects

Our study was conducted utilizing a two-stage design, and detailed diagnostic information on the patients is provided in an Additional file 1: Table S1. Subjects in the capture sequencing stage, consisting of 184 NTD subjects, were all ethnically Han Chinese, as previously described [11, 21, 22]. The Chinese NTD samples were collected from either aborted fetuses (23.4 ± 6.7 weeks) or children with spina bifida (6.4 ± 4.6 years). Subjects for the CELSR1 validation study were primarily collected from Texas (292 cases, including 192 previously reported samples [7] and 100 additional samples) and the NTDs were diagnosed by trained neurosurgeons. The US NTD samples were collected from either new born or children with spina bifida with mean age 7.5 ± 5.4 years. Protocols were reviewed and approved by the Ethics Committee of the School of Life Sciences, Fudan University (EC#216). American NTD study protocols were reviewed and approved by the Institutional Review Board (IRB) of University of Texas at Austin (UT Austin IRB approval number: 2014120041). All of the samples were obtained with the appropriate parental informed consent.

DNA sequencing, genotyping and data analysis

Capture baits were designed to cover all coding regions of 30 Wnt/PCP pathway genes based on KEGG databases [23], and the variants were detected using targeted capture and next generation DNA sequencing (NGS). Variant calling and annotation were conducted as presented in our previously published methods [8]. Based on results from NGS, rare variants were selected to characterize the patterns of Wnt/PCP pathway genes in human NTD cases using the following criteria: (i) minor allele frequency (MAF) in NTDs < 0.01; (ii) coding region and splice sites were included. All variants were further annotated with the Exome Aggregation Consortium (ExAC) [24]. The functional impact of missense mutations were predicted using Sorting Intolerant From Tolerant (SIFT) [25] and Polymorphism Phenotyping version 2 (Polyphen2) [26] via VEP [27].

Plasmids

XE251 pCDNA3.1(zeo)-hDsh3-Flag (DVL3-Flag) was a gift from Randall Moon (Addgene plasmid # 16758) [28]. Plasmid pCDNA-3.0-CELSR2-GFP(CELSR2-GFP) (Clone ID: OHu24729) was purchased from Genescript. VANGL2-HA plasmid was house made by amplification of VANGL2 cDNA and cloning the VANGL2 cDNA into a pCMV-HA plasmid (CloneTech) as previously described [11]. DVL3 c.1207G > A(p.Asp403Asn) and CELSR2 c.6077C > T(p.Thr2026Met) were introduced into DVL3-Flag and CELSR2-GFP plasmids separately by GeneArt® Site-Directed Mutagenesis System (Life Technologies, Carlsbad, CA). M50 Super 8x TOPFlash was a gift from Randall Moon (Addgene plasmid # 12456) [29].

Cell lines, culture conditions and transfection

HEK-293 T (human embryonic kidney), HeLa (human cervical cancer) were grown in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Before transfection, cells were grown to 50-70% confluency. Cell transfection was carried out using Lipofectamine 2000 (ThermoFisher) according to the manufacturer’s protocol.

Luciferase reporter assays

Topflash, a TCF/LEF reporter plasmid, was used for canonical Wnt pathway signaling detection [29]. API reporter plasmid was used to measure activation by Jun-kinase, a downstream effector of the non-canonical Wnt/PCP pathway signaling [13, 30]. For luciferase reporter assays in HEK-293 T cells, cells were transfected
with pcDNA-3.0-Dvl3-Flag (Wildtype or Mutant), pcDNA-3.0-Celsr2-GFP (Wildtype or Mutant) and TOP-Flash (Addgene plasmid ID:12456) [29] or pAP1-Luc (Agilent, Santa Clara, CA) [13] and Renilla-TK plasmid with indicated concentrations. Pools of cells were lysed with passive lysis buffer (Promega), and assayed for luciferase activity using the luciferase system (Promega). All luciferase reporter assays represent the mean ± standard error of the mean (SEM) from three independent measurements of cell pools. Three independent replicates were performed and all statistical tests were using two tailed t-test with \( P < 0.05 \) as the significance level.

Co-immunoprecipitation assay
Dvl3-Flag and Vangl2-HA were co-transfected into HEK-293 T cells and cell lysates were immunoprecipitated with Anti-Flag Dynabeads (ThermoFisher) overnight at 4 °C, and then washed three times with Lysis buffers and boiled with SDS loading buffer and subjected to Western blotting. Dvl3-Flag and Vangl2-HA were detected, as indicated.

Sub-cellular localization detection
Cells were seeded onto 20 mm Glass Bottom Cell Culture Dish and transfected with Celsr2-GFP, Dvl3-Flag. Cells were washed in PBS prior to fixation in 4% formaldehyde. For Celsr2-GFP transfected cells, GFP was directly detected by a laser scanning confocal microscope (Leica,SP5). For Dvl3-Flag transfected cells, the cells were lysed and blocked by 1% BSA (Sigma Aldrich) for 1 h, after which time they were incubated overnight with 1% BSA and washed three times with cold PBS followed by an hour exposure to a FITC conjugated secondary antibody. Immunofluorescence was visualized by the EVOS cell imaging system (ThermoFisher Scientific).

Statistical analysis
OncodriveCLUST [31], which identifies genes whose alterations tend to cluster in specific protein regions with respect to synonymous alterations [32], was used to identify driver gene mutations in the Wnt/PCP pathway in NTD cases. The Fisher’s exact test was employed for CELSR1 LoF variants association analysis. Statistical analyses were performed using R software.

Results
Genes frequently affected by rare mutations in Wnt/PCP pathway using targeted next-generation sequencing
To better understand the genetic mechanisms underlying the etiology of human NTDs, we investigated the genetic contribution of 30 genes in the Wnt/PCP pathway. Among these 30 Wnt/PCP pathway genes, mouse mutants in 15 genes resulted in NTDs with variable phenotypes and penetrance [33]. There were 264 rare variants (MAF < 0.01) identified in the coding region and in splice sites of 30 Wnt/PCP pathway genes in the 184 human NTD samples. Among these rare variants, 255 were single nucleotide variants (SNVs) and 9 were small insertions or deletions (indels). 62 (33.7%) human NTDs carried 2 or more rare variants, whereas 122 (66.3%) human NTDs had only a single rare variant in the Wnt/PCP pathway. T > C and C > T were the two most common transitions identified in the Wnt/PCP pathway in human NTDs. The top four genes with the highest percentage of mutations in our human NTD samples are CELSR3, EP300, CELSR1 and CELSR2 (Fig. 1). This included 17% cases with CELSR3, 17% cases with EP300, 16% cases with CELSR1 and 16% cases with CELSR2 variants. With the exception of CELSR1, none of the other CELSR genes had previously been observed as NTD mouse candidate genes producing an abnormal phenotype when inactivated.

Rare variants detected in CELSR1-3 tend to be driver genes in Wnt/PCP pathway in human NTDs using OncodriveCLUST
In order to uncover the driver genes in the Wnt/PCP pathway responsible for NTDs, OncodriveCLUST [31] was used to examine those genes with significant positional clustering of mutations. It is a useful method to identify driver genes, whose biased mutations are clustered within the protein sequence for adaptive advances during evolution. Results showed that nine genes were significantly (q-value<0.05) mutated in NTD cases, including: FZD4, WNT11, CELSR1, CELSR3, NFATC4, LRP6, VANG12, CELSR2 and PRICKLE1 (Fig. 1b). Among the top four frequently mutated genes, all CELSR genes (CELSR1, CELSR2 and CELSR3) were significantly (q-value<0.05) mutated in NTDs, while EP300 was not significantly mutated in NTD cases. Mutations in these 30 Wnt/PCP genes were frequently clustered at cadherin repeats, Wnt and strabismus domains (Additional file 2: Figure S1). Our previous study identified the missense mutation CELSR1 p.Phe870Leu in the cadherin repeat domain that induced NTDs via disruption of both the PCP pathway and canonical WNT signaling [8]. This suggested that rare mutations in CELSR genes function as mutational hotspots in the etiology of NTDs. In order to validate our findings, we re-sequenced CELSR1 in 292 NTDs (including 192 previously reported samples [7] and 100 additional samples) collected in the US. Three LoF variants (c. 5050_5051insGT [7], c.5719_5720delTG [7] and c. 4417A > T (p.Gln1473Ter)) were identified in the CELSR1 gene. Compared with the LoF frequency from the ExAC database (13/60693), CELSR1 LoF variants (3/292) in NTDs were significant enriched in our NTD cohort (Fisher test, \( P < 0.001 \)).
Detection of double damaging variants among CELSR genes and other Wnt/PCP genes in NTD cases

There were 27 rare mutations of CELSR genes identified in 26 (14.1%) NTD cases that were predicted to be damaging variants by both PolyPhen-2 and SIFT (Fig. 2). These included eight (4.3%) cases with damaging variants of CELSR1, nine (4.9%) cases with damaging variants of CELSR2, and 10 (5.4%) cases with damaging variants of CELSR3. In addition, six (3.3%) cases were observed to have double Wnt/PCP heterozygous damaging mutants, and all of these cases have both damaging CELSR mutants concurrent with other Wnt/PCP mutations. These six cases were associated with diverse NTD phenotypes (Table 1). These double mutation cases included: case NTD_27 with CELSR2 p.Thr2026Met and DVL3 p.Asp403Asn, case NTD_39 with CELSR2 p.Arg2480Cys and FZD7 p.Leu383Gln, case NTD_56 with CELSR2 p.Arg2626Cys and FZD9 p.Arg714His, case NTD_19 with CELSR1 p.Thr1086Met and VANGL1 p.Arg207His presented with spina bifida. Case NTD_122 with CELSR2 p.Arg1990His and CELSR3 p.Argy1194His had encephalocele. Case NTD_15 with LRP6 p.Arg386Cys and CELSR1 p.Arg714His had both anencephaly and spina bifida. Amongst the 12 damaging variants in these six cases, eight of them existed in the ExAC database with MAF < 0.001, while four variants were novel and did not exist in the ExAC database.

Functional validation of CELSR2 p.Thr2026Met and DVL3 p.Asp403Asn

To determine the combined effect of compound heterozygosity damaging variants on PCP signaling, the functional impact of CELSR2 p.Thr2026Met and DVL3 p.Asp403Asn on canonical Wnt signaling and PCP signaling was examined in case NTD_27. These two heterozygous variants were validated by Sanger sequencing (Fig. 3a). Neither of these two variants affected the protein subcellular localization (Additional file 3: Figure S2). CELSR2 p.Thr2026Met downregulated the PCP pathway signaling in our pAP1-Luc luciferase assay (Fig. 3b). DVL3 p.Asp403Asn reduced interactions between DVL3 and VANGL2 (Fig. 3c). The DVL3 p.Asp403Asn affected both canonical and non-canonical Wnt signaling in our luciferase assay (Fig. 3d). It upregulated canonical Wnt signaling in our TopFlash assay and down regulated non-canonical Wnt/PCP signaling in our pAP1-Luc luciferase assay when 100 ng plasmid was transfected. The two variants demonstrated a combined effect on down regulating PCP pathway signals in our pAP1-Luc luciferase assay (Fig. 3e).

Discussion

Mutations in over 300 mouse genes are associated with diverse NTD phenotypes [33], and a large number of these genes cluster in the PCP pathway [18, 34]. Recent mouse genetic studies have identified that double-
heterozygote combinations of Vangl2LP, ScribCRC and Celsr1CRC are associated with a variety of NTDs, including anencephaly and spina bifida [18]. While homozygous PCP knockout mice demonstrated an NTD phenotype, single PCP heterozygote mutant mice likely fail to produce NTD phenotypes. Instead, double or multiple PCP genes in heterozygous combinations appears to be required in order to express the NTD phenotype. We propose that compound heterozygosity of genes in the PCP pathway in human populations,

Table 1 Combined rare damaging variants among Wnt/PCP genes in human NTD samples

| Sample  | Gene   | Variant     | Chr. | Positiona | Minor/major allele | Sex | Phenotypeb | SIFTc | PP2d | MAF in ExACe |
|---------|--------|-------------|------|-----------|--------------------|-----|------------|-------|------|--------------|
| NTD_27  | CELSR2 | p.Thr2026Met| 1    | 109,810,233 | T/C                | M   | SB         | D     | D    | 0.000009415  |
| DVL3    | Asp403Asn| 3       | 183,885,376 | A/G    |                    |     |            |       |      |              |
| NTD_39  | CELSR2 | p.Arg2480Cys| 1    | 109,813,177 | T/C                | M   | SB         | D     | D    | 0.0003766   |
| FZD7    | p.Leu383Gln| 2   | 202,900,518 | A/T    |                    |     |            |       |      |              |
| NTD_122 | CELSR2 | p.Arg1990His| 1    | 109,808,784 | A/G                | M   | EC, SB     | D     | D    | 0.0001318   |
| CELSR3  | p.Arg1194His| 3 | 48,696,487 | T/C    |                    |     |            |       |      | 0.00002825  |
| NTD_15  | LR6P6  | p.Arg386Cys | 12   | 12,334,194  | A/G                | F   | AE, SB     | D     | D    | 0.000273    |
| CELSR1  | p.Arg714His| 22 | 46,930,927 | T/C    |                    |     |            |       |      |              |
| NTD_56  | CELSR2 | p.Arg2626Cys| 1    | 109,814,294 | T/C                | NA  | SB         | D     | P    | 0.0004425   |
| FZD5    | p.Trp242Leu| 2       | 208,632,739 | A/C    |                    |     |            |       |      |              |
| NTD_19  | VANGL1 | p.Arg207His | 1    | 116,206,697 | A/G                | F   | SB         | D     | D    | 0.0001883   |
| CELSR1  | p.Thr1086Met| 22 | 46,929,811 | A/G    |                    |     |            |       |      | 0.0001318   |

*aPositions are given in bp from GRCh37

*bSB, spina bifida; EC, encephalocele; AE, anencephaly

*cSIFT predictions: D, deleterious

*dPolyPhen2 (PP2) predictions: D, probably damaging; P, possibly damaging

*eMAF from Exome Aggregation Consortium (ExAC) database

*fNot available

Fig. 2 Mapping of rare damaging mutations in the CELSR1, CELSR2, CELSR3 genes in human NTDs
where multiple PCP gene functional variants work together in the heterozygous state, can result in NTDs. Previously reported human NTDs with missense PCP mutations were all single heterozygotes, suggesting that the ‘second’ or ‘the other’ deleterious interacting variant might be present [15]. Understanding the genetic mechanisms of Wnt/PCP pathway is essential for appreciating the genetic architecture of human NTDs.

In this study, we focused on the genetic hotspots of rare coding mutants in the Wnt/PCP pathway genes, in regard to determining their genetic impact on susceptibility to NTDs. Overall, rare coding mutations were observed to be frequently mutated in CELSR1-3 genes. Moreover, we identified 84 (46%) cases with rare missense mutations, and 26 (14.1%) cases with rare damaging missense mutations in CELSR genes, respectively. Importantly, we determined that six (3.3%) of these cases had both damaging CELSR mutations and additional deleterious mutations of other Wnt/PCP genes, which were associated with diverse NTD phenotypes in humans.

These findings suggest that rare mutations of CELSR genes are identified as driver genes in human NTDs. CELSR, or Flamingo (Fmi) genes are a special subgroup of adhesion G protein-coupled receptors (GPCRs) that have three homologs, CELSR1, CELSR2 and CELSR3. CELSR genes were first identified in Drosophila for regulating planar cell polarity in the wing [35]. Robinson and co-workers detected missense variants in CELSR1 increased risk of craniorachischisis in humans [36]. Allache and co-workers identified pathogenic CELSR1 mutations in 2.9% of Italian and Canadian NTD cohort [37]. Lei and colleagues detected deleterious CELSR1 variants in about 3% of spina bifida cases collected in the U.S. cohort [7]. In contrast to CELSR1, the genetic evidence of CELSR2 and CELSR3 contributing to NTDs...
risk was previously lacking. Our previous study identified the missense mutation CESLR1 p.Phe870Leu in the cadherin repeat domain induced NTDs via disruption of both the PCP pathway and canonical WNT signaling [8]. It is implied that CESLR2 and CESLR3, clustered at cadherin repeat domain, may also contribute to the human NTD risk. In the present study, we found that CESLR2 p.Thr2026Met, which is located in the HornR domain, affected PCP signaling in the HEK293T cell line (Fig. 3). Taken together, we found that in addition to CESLR1, both CESLR2 and CESLR3 also contribute to genetic etiology of human NTDs.

Previous studies reported that the damaging mutations of CESLR1 are associated with diverse NTD phenotypes in humans, such as craniorachischisis [36] and spina bifida [7]. Given the different phenotypes, these data suggest that human NTDs could be caused by compound mutant heterozygosity. Celsr2 or Celsr3 regulate forebrain wiring in a Vangl-independent manner and inactivation of Celsr2 or Celsr3 produces abnormal brain morphology but does not produce NTDs in mice [38], also implicating that other unidentified molecules may participate in the genetic interactions leading to human NTDs. The present study further supported this notion by examining the damaging mutations of CESLR genes and other Wnt/PCP genes. Six (3.3%) of NTD cases were identified to have double damaging variants that included CESLR genes and other Wnt/PCP genes, such as CESLR2 p.Thr2026Met and DVL3 p.Asp403Asn. This combination was determined to have the lowest PCP signal in vitro (Fig. 3). In addition, among these eight genes involved in the double damaging heterozygous mutations combinations (Table 1), damaging variants of the CESLR1 [7, 8], VANGL1 [10], LPR6 [13] and Dvl3 [5] are known to be associated with NTDs, indicating that compound heterozygote damaging variants, LRP6 p.Arg386Cys and CESLR1 p.Arg714His (case NTD_15), CESLR1 p.Thr1086Met and VANGL1 p.Asp207His (case NTD_19) may increase human NTD risk. As other combinations of double damaging mutations might also expand the genetic risk factors potentially associated with NTDs, further experiments are warranted to assess their contribution to the risk for human NTDs. Altogether, we added to our understanding of the role of CESLR2 and CESLR3 in human NTDs, and established that the combination of rare damaging mutations in CESLR genes and other Wnt/PCP variants in an individual could increase human NTD risk. Different combinations are associated with diverse NTD phenotypes, including spina bifida, encephaloceles, and the combined phenotype of anencephaly and spina bifida (Table 1). Our findings suggested that combinations of multiple deleterious heterozygous mutations in individuals might better explain the etiology of NTDs in humans. There are two major limitations in this study. First, we found CESLR genes as the driver genes in NTDs using OncoDrive-Clust [31] that is often used for identifying cancer-driving genes. Although all the compound heterozygote mutants found contain the CESLR genes, suggesting that CESLR genes are possibly the genetic hotspots in NTDs, the validity of this method needs further evaluation in human NTD cases. Second, further studies of whole genome or exome sequencing with larger sample sizes are warranted to better appreciate the genetic contributions of CESLR genes and additional PCP gene-gene interaction in the development of NTDs.

Conclusions

In conclusion, this study demonstrates that damaging mutations of CESLR genes are genetic hotspots in the development of NTDs in our human population. We also emphasized that the increased risk of double damaging variants of CESLR genes and other Wnt/PCP genes, which was observed in 3.3% of our human NTD samples. The variable outcomes of mutations in CESLR genes that interact with other Wnt/PCP genes are direct evidence linking these genes to the etiology of human NTDs, indicating that multiple genetic mutations are important for understanding the genetic etiology of human NTDs.

Additional files

Additional file 1: Table S1. Demographic characteristics in NTD cohorts. (DOCX 17 kb)

Additional file 2: Figure S1. Most frequent Pfam domains affected by Wnt/PCP genes in human NTD samples. (TIFF 2976 kb)

Additional file 3: Figure S2. CESLR2 p.Thr2026Met and DVL3 p.Asp403Asn did not affect the protein subcellular localization. (A) CESLR2 p.Thr2026Met did not affect CESLR2 subcellular localization in HEK293T & MDCKII cells transfected with CESLR2-GFP and CESLR2 (p.Thr2026Met)-GFP expression plasmids. (B) DVL3 p.Asp403Asn did not affect DVL3 subcellular localization in HEK293T. (TIFF 4540 kb)

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.
The authors declare that they have no competing interests.

Competing interests
Not applicable.

Consent for publication
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Authors’ contributions
HW, TZ and RF directed and designed the study. ZC performed the statistical and bioinformatics analyses. YL, XC, FW, YB and RP performed the experimental analysis. HW, ZC, YL, YZ and RF prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Chinese NTD study protocols were reviewed and approved by the Ethics Committee of the School of Life Sciences, Fudan University (ECR216). American NTD study protocols were reviewed and approved by the Institutional Review Board (IRB) of University of Texas at Austin (UT Austin IRB approval number: 2014120041). All of these samples were obtained with the approval of parental informed consents.

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