An Axin2 mutation and perinatal risk factors contribute to sagittal craniosynostosis: evidence from a Chinese female monochorionic diamniotic twin family

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Abstract
Background: Craniosynostosis, defined as premature fusion of one or more cranial sutures, affects approximately 1 in every 2000–2500 live births. Sagittal craniosynostosis (CS), the most prevalent form of isolated craniosynostosis, is caused by interplay between genetic and perinatal environmental insults. However, the underlying details remain largely unknown.

Methods: The proband (a female monochorionic twin diagnosed with CS), her healthy co-twin sister and parents were enrolled. Obstetric history was extracted from medical records. Genetic screening was performed by whole exome sequencing (WES) and confirmed by Sanger sequencing. Functional annotation, conservation and structural analysis were predicted in public database. Phenotype data of Axin2 knockout mice was downloaded from The International Mouse Phenotyping Consortium (IMPC, http://www.mousephenotype.org).

Results: Obstetric medical records showed that, except for the shared perinatal risk factors by the twins, the proband suffered additional persistent breech presentation and intrauterine growth restriction. We identified a heterozygous mutation of Axin2 (c.1181G > A, p.R394H, rs200899695) in monochorionic twins and their father, but not in the mother. This mutation is not reported in Asian population and results in replacement of Arg at residue 394 by His (p.R394H). Arg 394 is located at the GSK3β binding domain of Axin2 protein, which is highly conserved across species. The mutation was predicted to be potentially deleterious by in silico analysis. Incomplete penetrance of Axin2 haploinsufficiency was found in female mice.

Conclusions: Axin2 (c.1181G > A, p.R394H, rs200899695) mutation confers susceptibility and perinatal risk factors trigger the occurrence of sagittal craniosynostosis. Our findings provide a new evidence for the gene-environment interplay in understanding pathogenesis of craniosynostosis in Chinese population.

Keywords: Sagittal craniosynostosis, Axin2, Perinatal risk factors, Mutation, Monochorionic diamniotic twin
Introduction
Craniosynostosis (CS), defined as premature fusion of one or more cranial sutures, affects approximately 1 in every 2000–2500 live births [1]. CS contains an isolated condition (non-syndromic craniosynostosis, NCS) and complex syndromes (with other malformations, syndromic craniosynostosis) [2]. Sagittal craniosynostosis is the most prevalent form of NCS, accounting for 40–58% of all NCS cases [3]. Sagittal suture premature closure restricts the widen of the skull and then causes the scaphocephaly deformity and other adverse neurologic outcomes [4].

Pathoetiology of NCS involves interplay between genetic and environmental factors [5–8]. FGF (fibroblast growth factor), BMP (bone morphogenic protein), Wnt (wingless-type integration sites) pathways are major regulators in suture biology [9, 10]. Pivotal component mutations in these pathways, including \textit{FGFR2} (fibroblast growth factor receptor 2), \textit{TWIST1} (twist, drosophila, homolog of 1) and \textit{Axin2} (axis inhibitor 2), have been regarded as the origin of craniosynostosis [1, 9]. Non-genetic risk factors, like intrauterine constraint, twin gestation, breech delivery, low birth weight, malnutrition, premature delivery, maternal thyroid disorders, gestational diabetes, virus infectious, can either cause or exacerbate craniosynostosis [7, 11–14]. Although several findings demonstrate the interactions between genetic and environmental risk factors contribute to premature fusion of cranial sutures [5, 15], more evidence are still need.

Monochorionic (MC) twins, sharing almost the same genome, offer a unique opportunity to study the gene-environment interactions, for the healthy twin as an ideal control. Discordant phenotypes between MC twins emphasize the interplay between genetic and environmental influences in etiologies of the disease [16].

In our study, we found that a heterozygous mutation of \textit{Axin2} (c.1181G > A, p.R394H, rs200899695) exist in Chinese female monochorionic sisters and their father. However, only the proband, suffering a persistent breech presentation and intrauterine growth restriction, was diagnosed with sagittal craniosynostosis. Public database indicated that \textit{Axin2} (c.1181G > A, p.R394H, rs200899695) mutation was not detected in Asian population. According to data from The International Mouse Phenotyping Consortium (IMPC, \url{http://www.mousephenotype.org}), about 22% female \textit{Axin2} heterozygous knockout mice developed abnormal head shape before delivery. Thus, we speculate that this particular \textit{Axin2} mutation leads to haploinsufficiency in female with incomplete penetrance, and additional environmental insults eventually trigger the occurrence of sagittal craniosynostosis.

Materials and methods

Clinic Examination and Information
All participants signed the informed consent and received physical examination by two experienced surgeons in Children's Hospital of Nanjing Medical University. All samples used in our study were in compliance with the informed consent and agreement of patients. This study was approved by the ethics committee of Nanjing Medical University.

Clinical information of pregnancy history and infant clinical data were obtained from hospital medical records. Head CT scan of the healthy child was performed upon the request of the parents.

Whole Exome Sequencing and Data Analysis
Genomic DNA, extracted from peripheral blood samples (proband, proband's co-twin sister, the parents) and proband's skull periosteum tissue, was subjected to whole-exome sequencing (WES) on the platform of Genery Biotechnology, Shanghai, China. Raw reads were aligned to the human genome reference assembly (GRCh37/hg19) using the Burrows-Wheeler Aligner [17]. The Picard software was employed to remove PCR duplicates and evaluate the quality of variants. DNA variants was called and analyzed using the Genome Analysis Toolkit [18]. The variants with read depths less than $4 \times$ were filtered out. All variants were further annotated [19–25]. The workflow of genetic analysis was shown in Fig. 1.

Sanger Sequencing
\textit{Axin2} mutation was validated by Sanger sequencing in Tsingke (China) using the following primers: Forward: 5'-CGCACACCCCTAAGCGACCCCAT-3' and Reverse: 5'-ACCGCCCCACCTAAGCCCTGCTGAA-3'. Results were visualized using FinchTV (Geospiza) software.

Conservation Analysis
Multiple-species amino acid sequences were obtained from National Center for Biotechnology Information (NCBI), and were analyzed by WebLogo (Version 2.8.2, \url{http://weblogo.berkeley.edu})[26].

Structural Analysis
Three-dimensional models of the wild-type and mutant \textit{Axin2} protein were constructed by I-TASSER [27] and visualized using the PyMOL software (PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA).
Functional Annotation

Functional annotation of Axin2 (c.1181G > A: p.R394H, rs200899695) was conducted on FAVOR functional annotation online portal (http://www.favor.genohub.org/) [28] or the University of California, Santa Cruz Genome Browser (UCSC, http://www.genome.ucsc.edu).

Phenotype analysis of Axin2 knockout mice

Phenotypes of Axin2 homozygous (Axin2−/−) and heterozygous (Axin2+/−) knockout mice were obtained from The International Mouse Phenotyping Consortium (IMPC, http://www.mousephenotype.org) [29, 30].

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**Fig. 1** Workflow for identifying Axin2 (c.1181G > A, p.R394H, rs200899695) mutation in pedigree diagnosed with sagittal craniosynostosis

**Fig. 2** Clinical features of the pedigree with sagittal craniosynostosis. a Pedigree of the family. Proband is marked with an upward arrow. Open squares/circles denote unaffected individuals; squares denote males and circles denote females. b Schematic diagram of fetal position before delivery. Proband (II-1) was in breech presentation at left side of the uterus, while her healthy sister (II-2) was in cephalic presentation at right side of the uterus. c Computerized tomographic (CT) scan revealed premature closure of sagittal suture (solid circle) and digital impressions (arrowheads) in proband's head (left panel). Cranial index of the proband is 70.9%. The co-twin sister was also received CT examination under the request of the parents. Her sagittal suture remains patent (dotted circle) and cranial index is 83.2% (right panel).
Results
Clinical information
The pedigree came from Wuhan, Hubei Province, China. Female proband (II-1), the elder monochorionic diamniotic (MCDA) twin (Fig. 2a-c and Supplementary Fig. S1-3), was diagnosed sagittal craniosynostosis.

Fig. 3 Sequence analysis of Axin2 (c.1181G>A, p.R394H, rs200899695). a-e Integrative Genomics Viewer (IGV) of the sequences around Axin2 (c.1181G>A, p.R394H, rs200899695) and the results of Sanger sequencing were shown. The gDNA samples were extracted from peripheral leucocytes of the parents and twin sisters (a-d) and from skull periosteum tissue of the proband (e). Please note that the DNA sequences are shown in + strand, but Axin2 is located on-strand. The father and two twins have this missense mutation (a, c, d, e), but the mother does not carry this mutation (b).
at the age of 9 months in the Department of Neurosurgery, Children's Hospital of Nanjing Medical University. She was born to non-consanguineous parents without family history of craniosynostosis. Her mother, a 30-year-old Chinese female, conceived MCDA twins by frozen embryo transfer (FET) (Supplementary Fig. S1-3). Her father was 36-year-old at that time. Twenty-six days after FET, two embryos inside a gestational sac (approximately 22 mm × 13 mm) was confirmed by four-dimensional ultrasound scan (Supplementary Fig. S1). Embryo length were 2.8 mm and 3.3 mm, respectively. Fetal heart rate were 107 per minute and 118 per minute, respectively (Supplementary Fig. S1 ).

At 13 weeks of gestation, IgG of cytomegalovirus and herpes simplex virus were detected in the mother's serum (Supplementary Table S1 ). At 14 weeks of gestations, dietary assessment indicated that the mother had inadequate intakes of energy, protein, fat, several vitamins and minerals, while excessive consumption of carbohydrate (Supplementary Table S2 ). At 17 weeks of gestations, decreased thyroid-stimulating hormone (TSH) level and increased level of urinary iodine were detected (Supplementary Table S3 ). At 27 weeks of gestations, the mother was diagnosed with gestational diabetes mellitus (Supplementary Table S3 ).

The ultrasound scan detection indicated that the proband (II-1) had been in persistent breech position on the left side of uterus, while the younger sister (II-2) had been in cephalic position (Fig. 2a, b). At 28 weeks of gestations, the proband (II-1) and twin younger sister (II-2) were born via spontaneous vaginal delivery (Fig. 2a, b). Birth weight of proband (II-1) and co-twin (II-2) was 880 g (ranking 50th - 90th centiles for postnatal weight of infants [31]) and 990 g (ranking 90th - 97th centiles for postnatal weight of infants [31]), respectively. The proband (II-1) were diagnosed with sagittal craniosynostosis and intrauterine growth restriction, while no signs of craniofacial deformity were detected in parents (I-1, I-2) and co-twin sister (II-2) (Fig. 2c). Cranial index, which represents the ratio of maximum cranial width to maximum cranial length, is decreased in patients with sagittal craniosynostosis [32]. In our case, the cranial index of the proband and co-twin sister was 70.9%, 83.2%, respectively. In addition, tooth agenesis, oral clefts or colorectal cancer were not detected in this family through physical examination by clinical doctor as well as medical history inquiry. And the other family members have no history of tooth agenesis, oral clefts or colorectal cancer. Taken together, clinical records indicate that the proband suffered sagittal craniosynostosis, persistent breech presentation and intrauterine growth restriction, except for other shared perinatal risk factors of the twins.

Table 1 Allele frequency of Axin2 (c.1181G > A: p.R394H, rs200899695) in human populations (dbGaP, Release Version: 20,200,227,123,210)

| Study       | Population | Sample size | Ref Allele | Alt Allele | BioProject ID | BioSample ID |
|-------------|------------|-------------|------------|------------|---------------|---------------|
| gnomAD—Exomes | Global     | 250,890     | G = 0.999151 | A = 0.000849 | PRJNA398795   | SAMN07488253  |
| gnomAD—Exomes | European   | 134,850     | G = 0.999993 | A = 0.000007 | SAMN0181265   |               |
| gnomAD—Exomes | Asian      | 49,008      | G = 1.000000 | A = 0.000000 |               |               |
| gnomAD—Exomes | American   | 34,584      | G = 0.99396  | A = 0.00604  | SAMN07488255  |               |
| gnomAD—Exomes | African     | 16,244      | G = 1.000000 | A = 0.000000 | SAMN07488254  |               |
| gnomAD—Exomes | Ashkenazi Jewish | 10,072 | G = 1.000000 | A = 0.000000 | SAMN07488252  |               |
| gnomAD—Exomes | Other       | 6132        | G = 0.9995   | A = 0.0005   | SAMN07488248  |               |
| ExAC        | Global      | 120,894     | G = 0.999363 | A = 0.000637 | PRJEB8661     | SAMN07490465  |
| ExAC        | Europe      | 72,950      | G = 0.99999  | A = 0.00001  |               |               |
| ExAC        | Asian       | 25,152      | G = 1.000000 | A = 0.000000 |               |               |
| ExAC        | American    | 11,542      | G = 0.99342  | A = 0.00658  |               |               |
| ExAC        | African     | 10,358      | G = 1.000000 | A = 0.000000 |               |               |
| ExAC        | Other       | 892         | G = 1.000000 | A = 0.000000 |               |               |
| ALFA        | Total       | 62,874      | G = 0.99992  | A = 0.00008  | PRJNA507278   | SAMN10492705  |
| ALFA        | European    | 59,864      | G = 0.99995  | A = 0.00005  | SAMN10492695  |               |
| ALFA        | Other       | 2654        | G = 0.9992   | A = 0.00008  | SAMN1605645   |               |
| ALFA        | African     | 242         | G = 1.000000 | A = 0.00000  | SAMN10492703  |               |
| ALFA        | Asian       | 78          | G = 1.000000 | A = 0.00000  | SAMN10492704  |               |
| ALFA        | Latin American 2 | 22   | G = 1.000000 | A = 0.00000  | SAMN10492700  |               |
| ALFA        | South Asian | 8           | G = 1.000000 | A = 0.00000  | SAMN10492702  |               |
| ALFA        | Latin American 1 | 6    | G = 1.000000 | A = 0.00000  | SAMN10492699  |               |
Mutation analysis of AXIN2 (c.1181G > A, p.R394H, rs200899695)

Whole-exome sequencing was applied to identify the potential genetic etiology leading to sagittal craniosynostosis in our case. Due to the low incidence rate of sagittal craniosynostosis [3], we focused on private and/or rare (minor allele frequency, MAF < 0.01) variants on exons or splicing sites (Fig. 1). However, none of candidate germline or somatic mutations were proband-specific. As gene-environment interactions have been demonstrated in the pathogenesis of craniosynostosis [5, 15], we wonder whether the intrauterine risk exposures (environment factors) triggered the susceptible individual to develop sagittal craniosynostosis. Based on this hypothesis, we re-analyzed our sequencing data and identified a heterozygous missense mutation of Axin2 (c.1181G > A, p.R394H, rs200899695) in the leukocytes of subjects I-1, II-1 and II-2, and skull periosteum tissue of subject II-1. This finding was further validated by Sanger sequencing (Fig. 3a-e). The frequency of Axin2 (c.1181G > A, p.R394H, rs200899695) mutation of global population is 0.000849, 0.000637, 0.00008 in gnomAD, ExAC, ALFA database, respectively, while it is not detected in Asian population (Table 1).

Conservation analysis of Axin2 (c.1181G > A, p.R394H, rs200899695)

G to A transition of Axin2 (c.1181G > A, p.R394H, rs200899695) resulted in the replacement of Arg by His at 394th AXIN2 protein residue (Fig. 4a). Arg394 residue, located at the GSK3β binding domain (amino acid 327 to 413 according to the UniProt Consortium) of Axin2 protein (Fig. 4a) [33], is conserved across species (Fig. 4b,c and Table 2).

Functional annotation and structural analysis and of Axin2 (c.1181G > A: p.R394H, rs200899695)

Axin2 (c.1181G > A: p.R394H, rs200899695) mutation is predicted to be potentially deleterious by in silico analysis (Table 3) and the wild type Axin2 (c.1181G) loci is located within a region modified by H3K4Me3 and H3K27Ac (Fig. 5 a). In addition, p. R394H substitution is predicted to affect spatial structure of AXIN2 GSK3β binding domain (Fig. 5 b, c).

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**Fig. 4** Conservation analysis of Axin2 (c.1181G > A, p.R394H, rs200899695). a Schematic diagram depicts structure of AXIN2 protein. The mutation (R to H at 394th amino acid) resides in AXIN2 GSK3β binding domain. (TNKS_binding: Tankyrase binding N-terminal segment of AXIN; RGS: Regulator of G protein signaling; DIX: Disheveled and AXIN interacting) b) Evolutionary conservation analysis revealed that the Arg394 site is conserved from zebrafish to humans. c) WebLogo analysis showed that the Arg394 site was relatively conserved.
Phenotype analysis of Axin2 knockout mice

Data from The International Mouse Phenotyping Consortium (IMPC) documented that homozygous Axin2 knockout (Axin2−/−) mice developed preweaning lethality, while heterozygous Axin2 knockout (Axin2+/−) was alive (Fig. 6 a). The percentage of abnormal craniofacial morphology at embryonic day 12.5 (E12.5) for female Axin2+/+ and Axin2−/− mice was 0.73% (4/547) and 50% (1/2), respectively (Fig. 6 b). In addition, all female (6/6) and male (3/3) Axin2−/− mice had abnormal head shape, whereas 22.22% (2/9) female Axin2+/− mice were with abnormal head shape at E18.5 (Fig. 6 c). However, none of male (0/5) Axin2−/− mice presented with abnormal head shape (Fig. 6 c). Taken together, these results indicate incomplete penetrance of Axin2 haploinsufficiency in female mice.

Discussion

Craniosynostosis, a highly heterogeneous disease, is caused by genetic mutations, adverse environmental exposures and their interactions. Identifying the pathophysiology of craniosynostosis gives light to uncover susceptibility individuals, discern environmental risk factors and establish effective strategies for prevention and early diagnosis. In our study, we demonstrated that a heterozygous Axin2 (c.1181G > A: p.R394H, rs200899695) mutation was presented in the monochorionic twins and their father, but not in the mother. However, only the female proband, who was received additional environmental insults (persistent breech presentation and intrauterine growth restriction), developed sagittal craniosynostosis. We assume that this Axin2 mutation predisposes to sagittal craniosynostosis but extra environmental insults are needed to initiate the disease.

Prenatal risk factors, including intrauterine constraint, twin gestation, premature delivery, maternal thyroid disorders, gestational diabetes, malnutrition, virus infectious, increase the susceptibility of craniosynostosis in genetically predisposed infants [7, 11–14]. Research of monochorionic (MC) twins provide exceptional opportunity to decipher the interplay between genetic and environment risks on the occurrence of premature suture fusion [34]. In our study, monochorionic twins suffered the majority of risk factors prenatally, however, only the infant with breech presentation and intrauterine growth restriction presented sagittal craniosynostosis. Thus, intrauterine growth restriction and breech position deserves particularly attention in causing sagittal craniosynostosis.

It has been well accepted that AXIN2 is essential for normal calvarial morphogenesis by directly targeting β-catenin, orchestrating the crosstalk of Wnt, BMP, FGF signaling pathways and maintaining suture cell stemness [35–37]. Deletion or mutation of Axin2 attribute to craniosynostosis in humans and mice [6, 36]. Moreover, phenotype data available in The International Mouse Phenotyping Consortium (IMPC, https://www.mousephenotype.org/) show that all female and male homozygous Axin2 knockout (Axin2−/−) mice present abnormal head shape malformation. For heterozygous Axin2 deletion (Axin2+/−) mice, a total of 2/9 females develop abnormal head shape at E18.5;
Fig. 5 Functional annotation and structural analysis of AXIN2 (c.1181G > A; p.R394H, rs200899695). a UCSC database predicted that wild type AXIN2 (c.1181G) locates in the region modified by H3K4Me3 or H3K27Ac in seven cell lines by ChIP-Seq assay. b Location of Arg394 residues within the GSK3β binding domain of AXIN2. c Location of the His 394 residues within the GSK3β binding domain of AXIN2. Arrowheads indicate the hydrogen bond in the domain.

Fig. 6 Phenotype analysis of Axin2 homozygous (Axin2−/−) and heterozygous (Axin2+/−) knockout mice. Data of Axin2−/− and Axin2+/− mice were obtained from IMPC. a Axin2−/− mice could not be alive due to preweaning lethality. Number and percentage of Axin2+/− and matched Axin2+/+ mice at early adult stage. b Number and percentage of female mice (Axin2+/+, Axin2−/−) with abnormal craniofacial morphology at E12.5. c Number and percentage of mice (Axin2+/+, Axin2+/−, Axin2−/−) with abnormal head shape at E18.5.
however, the male Axin2+/− mice are not. These results indicate haploinsufficiency of Axin2 in female mice with incomplete penetrance.

In this study, an Axin2 heterozygous missense mutation (c.1181G > A: p.R394H, rs200899695) was identified in peripheral blood samples of subjects I-1, II-1 and II-2 (Fig. 1a), suggesting that the proband inherits the mutation from her father. The wild type Axin2 (c.1181G) loci is conserved across species and in the region may be modified by H3K4Me3 or H3K27Ac in seven cell lines by ChIP-Seq assay. H3K4Me3 modification is known to mark genes that are essential for the identity and function. H3K4Me3 breadth contains information that ensures transcriptional precision at the identified genes [38]. H3K27Ac, a robust mark of active enhancers and promoters, has been demonstrated to be strongly correlated with gene expression and transcription-factor binding [39]. However, much more functional annotation and experiments are needed to clarify whether Axin2 (c.1181G > A: p.R394H, rs200899695) could affect the H3K4Me3 or H3K27Ac modification. In addition, Axin2 missense mutation (c.1181G > A: p.R394H, rs200899695) is likely to be deleterious by in silico predication; however, only the proband received additional risk factors (persistent breech presentation and intrauterine growth restriction) (intrauterine growth restriction) developed sagittal craniosynostosis in this Chinese female monochorionic diamniotic twin family. These findings provide new evidence for the gene-environment interplay in understanding etiologies of NCS.

**Conclusion**

Based on the results of monochorionic twins, we demonstrated Axin2 (c.1181G > A, p.R394H, rs200899695) mutation led to haploinsufficiency with incomplete penetrance in female, and additional prenatal risk factors (intrauterine growth restriction and breech position) were indispensable to trigger the occurrence of sagittal craniosynostosis in this Chinese female monochorionic diamniotic twin family. These findings support the hypothesis.

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**Authors’ contributions**

AHG, GW designed the study. GW, QY, LZ provided patients’ data and performed clinical assessments. JX, CCS, JIL, ZKW, CX, QL, SQX, LYZ, YS conducted the experiments. JX, QY, CCS, JIL, XZ, ZKW analysed the data. JX, QY, CCS, JIL wrote the manuscript. All authors critically revised the manuscript for important intellectual content. AHG, GW supervised the study. The authors read and approved the final manuscript.

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**Availability of data and materials**

All the data generated in the present research is contained in this manuscript.

**Declarations**

**Ethical approval and consent to participate**

This research was carried out according to the principles of the Declaration of Helsinki and was approved by the Nanjing Medical University Ethics Committee.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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