Identification of a novel de novo RUNX2 frameshift mutation associated with cleidocranial dysplasia

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Abstract

Background Cleidocranial dysplasia (CCD) is a rare genetic disorder affecting bone and cartilage development. Clinical features of CCD comprise short stature, delayed ossification of craniofacial structures with numerous Wormian bones, underdeveloped or aplastic clavicles and multiple dental anomalies. Several studies have revealed that CCD development is strongly linked with different mutations in runt-related transcription factor 2 (RUNX2) gene.

Objective Identification and functional characterization of RUNX2 mutation associated with CCD.

Methods We performed genetic testing of a patient with CCD using whole exome sequencing and found a novel RUNX2 frameshift mutation: c.1550delT in a sporadic case. We also compared the functional activity of the mutant and wild-type RUNX2 through immunofluorescence microscopy and osteocalcin promoter luciferase assay.

Results We found a novel RUNX2 frameshift mutation, c.1550delT (p.Trp518Glyfs*60). Both mutant RUNX2 and wild-type RUNX2 protein were similarly confined in the nuclei. The novel mutation caused abrogative transactivation activity of RUNX2 on osteocalcin promoter.

Conclusions We explored a novel RUNX2 deletion/frameshift mutation in a sporadic CCD patient. This finding suggests that the VWRPY domain may play a key role in RUNX2 transactivation ability.

Keywords RUNX2 · Novel mutation · Cleidocranial dysplasia · Genetic disorder

Introduction

Cleidocranial dysplasia (CCD), also referred as a Scheuthauer syndrome, is a rare autosomal dominantly inherited disorder which is represented by various skeletal abnormalities (Lotlikar et al. 2018). The global CCD occurrence rate is usually one in every millions of newborns without gender preponderance (Offiah et al. 2019). Clinical features of CCD is diverse and involves short stature, delayed ossification of craniofacial structures with numerous Wormian bones, underdeveloped or aplastic clavicles, multiple dental defects such as teeth hypoplasia, delayed primary teeth exfoliation, prolonged eruption of permanent teeth and malocclusion (Konishi et al. 2019). Other skeletal abnormalities, such as hypoplastic iliac wings, distal phalanx dysplasia, knock-knees and malformations of spine can be also observed (Farrow et al. 2018). The CCD can be diagnosed prenatally, from early childhood till late adolescence and diagnostic tools ranges from ultrasound investigations (in prenatal period) to panoramic radiography, although early
diagnosis is considered as a key problem in CCD management (Zeng et al. 2018).

Several studies have revealed that CCD development is strongly linked with different mutations affecting runt-related transcription factor 2 (RUNX2) gene (Jaruga et al. 2016; Xuan et al. 2008), which is a transcription factor involved in osteoblastic differentiation and skeletal morphogenesis. Human RUNX2 gene consisting 8 coding exons, is located in chromosome 6p21 (Levanon et al. 1994). RUNX2 gene comprises Q/A domain on N-terminal part, RUNT domain which is crucial for DNA-binding to a specialized motif and heterodimerization with core-binding factor subunit beta (CBFβ) (Zhang et al. 2017), as well as proline-serine/threonine-rich (PST) domain on its C-terminus (Yoshida et al. 2002). The core-binding factor subunit alpha-1 (CBFα1) protein, that RUNX2 gene encodes is expressed densely in skeletal structures and considered as a key transcription factor for numerous stages of osteogenesis (Sun et al. 2016). Noticeably, mice with targeted RUNX2 disruption have bone formation failure owing to osteoblast deficiency (Zhong et al. 2016). Moreover, it also has been suggested that RUNX2 represents crucial role in tooth development, and its transcriptional failure could promote dental lamina excess activation leading to supernumerary teeth with consequent effect on permanent teeth (Wen et al. 2020). Till recent years, almost 194 causative mutations in RUNX2 have been identified, and this number is still rising (Otto et al. 2002). However, RUNX2 mutations can be found only in two-thirds of patients with CCD, and 30%–40% of cases are triggered by novel mutations (Hordyjewska-Kowalczyk et al. 2019).

Although we have known more about the clinical and functional characteristics of RUNX2 because of recent studies, there are still some potential unknown factors that urge further exploration. RUNX2, as the key contributory gene in CCD, is conceivable to be a therapeutic target for CDD. In current study, we explored a novel RUNX2 deletion mutation: c.1550delT (p.Trp518Glyfs*60) in a sporadic case and also showed its clinical features, possible pathogenesis and functional characteristics.

Materials and methods

Subjects

The subjects were examined in the Department of Endocrinology, Qilu Hospital of Shandong University, China. The peripheral blood samples were obtained for genetic testing from all participants, including 15 year-old boy with suspected CCD and his unaffected parents. We also carried out clinical and radiological examinations on patient, and collected his medical history. Current study was accepted by the ethics committee of Qilu hospital of Shandong University (ethical approval number KYLL-2019-2-111). All subjects in our study signed consent form voluntarily with the review of the ethical committee. The study methods were performed according to the ethical committee accepted guidelines.

Mutation analysis

The whole-exome sequencing (WES) was carried out on DNA from venous blood sample. Fragmentation of the genomic DNA, paired-end adaptor ligation, amplification and purification were implemented, and the all human exons together with 50 bp bases in their adjacent introns were captured by xGen® Exome Research Panel. The DNA library was performed post-capture amplification and purifying, and then arrayed by the Illumina HiSeq sequencing platform. All test and sequence analysis were supplied by the Beijing Fujun Gene Biotechnology Co., Ltd (Beijing, China).

In silico assays for a Runx2 frameshift

Using phyer2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), the WT-RUNX2 and W518Gfs-RUNX2 structural conformation were comprehensively analyzed and predicted using the threading method and the heavy-head prediction method, respectively, and the WT-RUNX2 and W518Gfs-RUNX2 structural models were established as the reference instruction (Kelley et al. 2015). Additionally, SAVE5.0 3D Structure Viewer (https://saves.mbi.ucla.edu/) to visualize 3D structure has been used.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium basic (DMEM basic, Gbico, Grand Island, NY, USA, cat: C1199500BT) supplemented with 10% fetal bovine serum (FBS, Gbico, Grand Island, NY, USA, cat: 10,099,141), penicillin (100 IU/mL), and streptomycin (100 μg/mL) as formerly detailed (Hu et al. 2014; Wang et al. 2014). Cells transfection with the plasmids carrying needed genes by using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was performed to investigate protein expression and other related studies.

Western blotting analysis

We used the human embryonic kidney (HEK) 293 cells to study the WT- RUNX2 and W518Gfs-RUNX2 expression as previously described. For western blotting analysis, the cells were seeded in 10 cm² plates. One day later, the HEK293 cells were transfected with pcDNA3.1-GFP, pcDNA3.1-WT-RUNX2-GFP and pcDNA3.1 W518Gfs-RUNX2-GFP. After 48 h transfection, the cells were cultured and subjected to
SDS–PAGE. Western blotting was performed with a rabbit polyclonal anti-GFP antibody (1:1000, proteintech, Wuhan, Hubei, China, cat: 50,430-2-AP) and a horse radish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (1:10,000, Zhongshan Golden Bridge, Beijing, China, Cat: ZB-2301) as a second antibody. Signals were detected using a chemiluminescence kit (Millipore, California, USA, Cat: WBKLS0050). Each experiment was repeated three times.

Luciferase reporter assays

Previously, seeded the HEK-293 cells into 96-well culture plate 24 h before this assay, 10,000/well, 200 ul cell suspension, culture at 37 °C, 5% CO₂ (Sun et al. 2020). On the second day, pRL-TK and pGL3-basic-osteocalcin promoter plasmid has been added to each group, and each group were also transfected separately with different concentrations of maxilla and mandible. d Chest X-ray showed bilateral hypoplastic clavicles hypoplasia of iliac bones, wide symphysis pubis with a bell-shaped thoracic cavity and scoliosis. e, f No abnormalities were seen in the bones of the bilateral elbow joints, and the epiphyseal line showed closure.

Fig. 1 Typical and radiological findings in the CCD patient. a Frontal facial view of patient representing midline depression of forehead and bilateral hypoplastic clavicles. b Hypoplasia of the clavicles abnormal facility in the opposing shoulders. c Panoramic radiography revealed primary teeth retention, numerous impacted permanent teeth in both maxilla and mandible. d Chest X-ray showed bilateral hypoplastic clavicles hypoplasia of iliac bones, wide symphysis pubis with a bell-shaped thoracic cavity and scoliosis. e, f No abnormalities were seen in the bones of the bilateral elbow joints, and the epiphyseal line showed closure.
Sequencing results and biochemical characterization of \textit{RUNX2} (c.1550delT) gene mutation

Sequencing analysis was implemented in the coding region of \textit{RUNX2} gene. \textit{RUNX2} (c.1550delT) mutant was detected in the proband, but not in his parents (Fig. 2A). The c.1550delT mutation was clustered in the terminal VWRPY of highly conserved PST domain (Fig. 2B–C). It is predicted that mutations at this site may change the protein activity (Fig. 2D).

Functional characterizations of \textit{RUNX2} (c.1550delT) gene mutation

Western blot analysis confirmed \textit{RUNX2} (c.1550delT) mutant gene did not cause reduction in \textit{RUNX2} protein expression level (Fig. 3A, B). Some studies have illustrated that \textit{RUNX2} is predominantly located in the cell nucleus and little perinucleolar region (Javed et al. 2000; Young et al. 2007). In order to verify the \textit{RUNX2} mutation nuclear localization, we transfected both pGFP-\textit{RUNX2} and pGFP-\textit{RUNX2}-Trp518Glyfs plasmids into HEK293T cells. The subcellular localization of the \textit{RUNX2} and the Trp518Glyfs mutation was observed by in situ immunofluorescence microscopy (Young et al. 2007). Both \textit{RUNX2} mutation and wild-type \textit{RUNX2} accumulated in the nuclei of HEK293T cells (Fig. 3C). This denoted that the subcellular compartmentation of the \textit{RUNX2} mutation was not affected. It has been reported in previous studies that \textit{RUNX2} is responsible for the transactivation of the osteoblast-specific osteocalcin gene in osseous cells (Zaidi et al. 2001). Luciferase assays demonstrated the \textit{RUNX2} transactivation activity and the transcriptional regulation of the osteocalcin promoter. The \textit{RUNX2} (c.1550delT) variant induced the osteocalcin promoter activity lower than \textit{RUNX2}-WT (Fig. 3D).

Discussion

Human \textit{RUNX2} gene comprises Q/A domain on N-terminal part, RUNT domain, as well as PST domain on its C-terminus. More noticeably, RUNT domain include NLS on its C-terminus, and this part has been reported to be important for the protein nuclear transportation (Ryoo et al. 2010). It also has been found that PST domain, to be more specific, its nuclear matrix targeting sequence (NMTS)-associated subnuclear foci is engaged in both activation of downstream factors such as osteocalcin gene and subnuclear localization of \textit{RUNX2} (Zaidi et al. 2001). Similarly, Q/A domain also participates in transactivation activity of \textit{RUNX2} target genes (Kauffenstein et al. 2016).
In our study, we found a novel RUNX2 (c.1061G > T) variant, causing frameshift starting from 518 codon, eventually leading to aberrant VWRPY domain in C-terminus. The protein sequence of RUNX2 WT 518WRPY521 is replaced by 518GDHIEIPQQWPSGIWGPHPTRINIYIYRESAYICISISYLQASAYFLEDFSFTHSVMILQP577. The local spatial structure of the protein is changed because the local secondary structure of the protein changes from alpha helix to random coil. Stop codon of RUNX2 WT is TGA at gene site 1566. Stop codon of c.1550delT is TAA at gene site 1734. Considerably, neither RUNX2 protein expression, nor its subcellular distribution was impaired in current study, which might be explained by intact RUNT domain. Several studies have proposed that osteocalcin promoter activation
is VWRPY-dependent (Qin et al. 2017). In the present study, abnormal VWRPY domain led to aberrant downstream activation of osteocalcin promoter that further supported previous findings. Zaidi et al. suggested that VWRPY is not crucial for RUNX2 nuclear retention (Zaidi et al. 2001). Since we have not found defective nuclear retention of RUNX2, our findings approved the point of Zaidi et al.

Currently, more than 48 phenotypic characteristics of CCD have been registered by OMIM (Qin et al. 2017). Although, numerous previous studies aiming to reveal genotype–phenotype association have been performed, controversy related to this point is still exist (Quack et al. 1999; Zhang et al. 2010). Additionally, some studies failed to find association between RUNX2 mutation and the severity of CCD (Lou et al. 2009). Moreover, threshold level of RUNX2 mutation that initiates CCD remains unidentified (Xu et al. 2017). Although CCD is considered as autosomal dominant disease, as stated in recent studies, it can be present in a sporadic pattern almost in 30% of cases (Huang et al. 2013). Notably, the genetic testing for possible mutations revealed that both of patient’s parents had normal RUNX2 in our study, suggesting that mutation occurred de novo. Although VWRPY is not hotspot for mutations and aberrant VWRPY did not lead to impaired RUNX2 protein synthesis, subcellular distribution in present research, it precipitated CCD with classical phenotype. This might be explained by possible influence of other determinants on CCD phenotype.

To conclude, we explored a novel RUNX2 deletion/frameshift mutation in a sporadic CCD patient. This finding emphasizes on crucial role of VWRPY domain in RUNX2 transactivation ability. Further studies are awaited to explore more RUNX2 mutations for revealing potential contributors as well as genotype–phenotype association.

Author contributions JR and XH conceived and designed the study. FH, FL and YS contributed partly to sample collection and genotype data. NZ and XZ contributed reagents and materials. JY and SW collected data and performed the analyses. LG and BO interpreted the data and wrote the paper. All authors have read and approved the manuscript. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interests All authors declare that they have no competing interests.

Ethics approval and consent to participate All case information were collected with informed consent of the patient’s parents. Prior to genotyping and analysis, all samples were stripped of personal identifiers (if any existed).

Consent for publication Not applicable.

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