

A new point mutation in SIX1 causes Branchio-oto syndrome, thymoma and pure red cell aplasia via mitochondrial apoptosis

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Research Article

Keywords: mutation, SIX1, mitochondrial, Branchio-oto syndrome, thymoma, pure red cell aplasia

Posted Date: May 3rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-364292/v1>

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Abstract

A patient was admitted to the hospital with thymoma, branchio-oto syndrome and pure red aplastic anemia. He had both *Six1* and mitochondrial mutations. The *Six1* gene has been linked to branchio-oto-renal spectrum disorder and malignant tumors. Additionally, mitochondrial mutations are known to cause anemia as well. To this end, we designed experiments to explore the relationship between *Six1* mutations, mitochondrial function and these diseases.

Whole-exome sequencing (WES) was used for gene detection, and transfected 293T cells were used for *in vitro* experiments. CCK-8 and flow cytometry were used to detect cell proliferation, cell cycle stage and apoptotic populations. Caspase-3 activity was used as an apoptosis indicator. The effects on mitochondria were examined by ROS and JC-1. Western blotting was used to detect the expression of Bcl-2, Bax and cleaved caspase-3.

WES revealed a Six1c.374A > G point mutation in the proband and deaf members of his family, as well as, a mitochondrial mutation (ND3) in the proband. 293T cells transfected with *Six1* were used as the WT group, the Six1c.374A > G transfection group was used as the MUT group, and the untransfected 293T cells were used as the NC group. A CCK-8 test found that the cell proliferation rates were reduced both in the WT group and the MUT group, and MUT group cells were found to be blocked in S-phase. Due to these findings, apoptosis was investigated. Following investigation, increased levels of ROS, caspase-3 activity and cleaved caspase-3 were detected in WT and MUT groups. Following these results, mitochondrial function was measured. The level of mitochondrial membrane potential in WT and MUT groups was decreased significantly. Western blot showed that and the Bcl-2/Bax ratio decreased.

Six1c.374A > G point mutation can affect cell proliferation through increased apoptosis controlled by the mitochondrial apoptotic pathway. This could be the cause of the patient's pure red cell aplasia.

Background

The members of the Six family are the homologs of the *Drosophila sine oculis (So)*, *optix* and *Dsix4* genes. In *Drosophila*, loss of *So* leads to the elimination of the compound eyes and *optix* induces eye formation when expressed in nonretinal tissues indicating both relate to eye formation. However, *Dsix4* plays an important role in several mesoderm derivatives, including somatic muscles, somatic cells of the gonad, and fat tissue^[1]. The murine Six gene family is composed of six members (Six1-6). Among these, Six homeobox 1 (*Six1*) has been most extensively investigated with important roles in the development of many tissues and organs, such as kidney^[2-3], the auditory system^[4-5], craniofacial structures^[6-7], and thymus^[6,8]. Some research has confirmed that an abnormal *Six1* gene can result in some inherited diseases like Branchio-oto-syndrome (BOS)^[9-10], as well as multiple cancers including: breast^[11], ovarian^[12], hepatocellular carcinoma^[13-14] and colorectal cancer^[15].

Branchio-oto-renal spectrum disorders (BORSD) include Branchio-oto-renal (BOR) syndrome and Branchio-oto (BO) syndrome. BO is a rare genetic disorder characterized by a distinct phenotype which includes branchial arch anomalies and hearing impairment. BORSD is estimated to occur in 1 out of 40,000 births and accounts for 2% of childhood deafness^[16]. Pure red cell aplasia (PRCA) is diagnosed as isolated anemia secondary to the failure of erythropoiesis. T-cell-mediated autoimmune destruction has been proposed as one of the main drivers of PRCA^[17-18]. Mitochondrial DNA (mtDNA) has a very high mutation rate due to the lack of protective histones and an effective DNA repair system. Mutations in mtDNA are associated with hematological diseases, such as myelodysplastic syndromes and acquired aplastic anemia (AA)^[19-20]. Our previous study^[21] suggests involvement of mutation-induced functional impairments of the mitochondrial respiratory chain in the hematopoietic failure of AA patients.

A 70-year-old man presenting with weakness was admitted to our hospital. He was diagnosed with a thymic tumor and anemia. After removal of the thymoma, he was diagnosed with PRCA complicated by BO syndrome. Multiple mutations, including the Six1c.374A > G mutation and multiple mutations in mitochondrial DNA, were found via sequencing. There have been many reports of mutations or deletions of the *Six1* gene affecting organ and tissue development^[2-8,22-24], but few have reported on the hematopoietic function effects. Further testing for genetic mutations in this patient revealed that he had multiple mitochondrial mutations including ND3 missense point mutations and nonsense mutations in other mitochondrial genes. In our previous research, we found that patients with aplastic anemia are prone to mitochondrial mutations^[21]. Therefore, we designed *in vitro* experiments to explore whether the Six1c.374A > G mutation can cause PRCA by affecting mitochondrial function.

Methods

2.1 Patients and families

We used the clinical criteria suggested by Chang et al^[25] in which an individual must fulfil one of the following criteria to be classified as affected with BOR: (A) fulfil at least three major criteria (branchial anomalies, deafness, pre-auricular pits, renal anomalies), or (B) fulfil two major criteria and at least two minor criteria (external, middle or inner ear anomalies, pre-auricular tags, and possibly also have other features such as facial asymmetry or palate abnormalities) or (C) fulfil one major criteria and have an affected first-degree relative meeting the criteria for BOR. All clinical data and specimens were collected with the consent of the patient or family member. The study was approved by the Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (No.2019-020).

2.2 Reagents and instruments

DMEM medium, penicillin and streptomycin were purchased from Gibco. FBS was purchased from Zebio Biotechnology Co., Ltd. (Beijing, China). T25 cell culture flasks and cell culture plates were from JET BIOFIL. Invitrogen (Carlsbad, CA) lipofectamin2000 was used. The apoptosis detection, cell cycle detection, and ROS detection kits were purchased from Solibao Technology Co., Ltd. (Beijing, China). JC-1 came from Yeasen Biological Technology Co., Ltd.

(Shanghai, China). The caspase-3 activity detection kit was from Beyotime Biotechnology (Shanghai, China). The blood DNA kit was from Kangwei Century Biotechnology Co., Ltd. (Beijing, China). Flow cytometry was conducted on the ACEA Biosciences (San Diego, CA) Novocyte flow cytometer.

2.3 Methods

2.3.1 DNA and mtDNA isolation and Exome Sequencing

After the male proband's (II-5) family map was built, DNA was extracted from peripheral blood samples of 3 affected family members (II-5, II-19, II-3) and 2 unaffected family members (II-16, II-20) using standard methods. However, mtDNA was obtained only from the bone marrow of II-5. Oral epithelial cells were collected for normal tissue comparison in mtDNA sequencing test.

DNA was isolated from peripheral blood using a DNA Isolation Kit, and DNA libraries were prepared with KAPA Library Preparation Kit following the manufacturer's instructions. Array capture hybridization of pooled libraries using capture probes and removal of non-hybridized library molecules were completed using the Agilent SureSelectXT2 Target Enrichment System. Sample dilution, flowcell loading and sequencing were performed according to the Illumina specifications. DNA libraries were sequenced on the Illumina Novaseq platform as paired-end 200-bp reads.

2.3.2 293 T cells transfection and culture for experiments

The cDNA of *Six1* was inserted into pRK7-N-Flag between XbaI and EcoRI to construct the vector: pRK7-con1. The c.374A was mutated to c.374G in the pRK7-con1 vector to obtain pRK7-case using the Takara MutanBEST kit. 293 T cells, which were serially transfected with pRK7-con1 and pRK7-case, were cultured in an incubator containing 5% CO₂ at 37°C.

2.3.3 Cell proliferation experiment

Cells in the logarithmic growth phase were collected and trypsinized conventionally, washed with PBS, pelleted by centrifugation, resuspended and counted. The cells were seeded on 96-well plates at 2×10^3 cells/well at a volume of 100 μ l per well. After 12, 24 or 48 h of incubation, 10 μ l of CCK-8 solution was added to each well. After 2h of incubation, the absorbance of each well was measured with a microplate reader at a wavelength of 450 nm and the mean was calculated for each group. These results were then analyzed and recorded.

2.3.4 Apoptosis detection

At corresponding time points after transfection, cells were trypsinized, collected, and apoptosis was detected using flow cytometry according to the following steps. The cells were grouped according to the conditions of each group, harvested, and centrifuged at 1000 rpm at 4°C for 5 minutes to collect the cells. Cells were then, washed twice with pre-chilled PBS, centrifuged at 1000 rpm at 4°C for 5 min, and 5×10^5 cells were collected. The PBS was aspirated and cells were resuspended in 100uL of 1X Binding Buffer. Next, cells were stained with 5uL of Annexin V-FITC and 10uL of propidium iodide (PI) Staining Solution and mixed gently. These cells were stained at room temperature for 10-15min in the dark. Then, 400uL 1X Binding Buffer was added, mixed well, and samples were placed on ice. Apoptosis detected by flow cytometry within 1 hour after samples were put on ice.

2.3.5 Cell cycle stage analysis

1×10^5 transfected cells were added to each tube, 1mL of pre-chilled 1xPBS (pH 7.2–7.3) was added, mixed well, and centrifuged at 1000rpm for 5min to wash the cells. Next, the 1X PBS was aspirated and 500 uL pre-cooled 70% ethanol was slowly added, and mixed by pipetting gently. Cells were fixed at 4°C for 16h and washed again. Next, 200uL of pre-chilled 1X PBS was added with 2uL of RNase and mixed by gently pipetting. These cells were incubated at 37°C for 30min to remove RNA. 5uL of PI staining solution was added and the cells were incubated at room temperature for 30min in the dark. Finally, 100uL of this solution was taken (around $1 \times 10^4 \sim 1 \times 10^5$ cells) and the cell cycle stage was examined by flow cytometry.

2.3.6 Caspase-3 activity test

Cells were collected at the corresponding time point after transfection, trypsinized, collected, and washed once in PBS. After aspirating the supernatant, lysing solution was added at a ratio of 100uL/2 million cells, the pellet was resuspended, and lysed for 15 minutes in an ice bath. The lysate was then centrifuged at 3500 rpm at 4°C for 10–15 min. The supernatant was collected and Ac-DEVD-pNA (2mM) was added, mixed, and solution was incubated at 37°C for 60–120 min. Samples were measured with an ELISA reader at an absorbance of 405nm.

2.3.7 ROS staining

Cells were collected at the proper time point, the medium was discarded, and the cells were washed three times with 1 ml of PBS buffer. 1ml of 1:1000 diluted fluorescent probe DCFH-DA was added and the cells were incubated at 37 degrees for 30 min. Then cells were washed three times with 1ml of PBS buffer, counterstained with DAPI, and washed another 3 times. A confocal laser scanning microscope was used to take pictures to record the distribution and expression of green fluorescence (ROS).

2.3.8 JC-1 detects mitochondrial membrane potential

Cells were collected at the corresponding time point after transfection, trypsinized, harvested, added to JC-1 the staining working solution, mixed and incubated. 1X JC-1 buffer was created according to the kit instructions. After incubation, the supernatant was aspirated, washed three times with 1X JC-1 buffer, and the pellet was collected by centrifugation. The proportion of the JC-1 polymer or monomer was determined using flow cytometry.

2.4 Western blotting test

Cells were harvested and total protein was collected after lysing the cells. The protein electrophoresis was run on 10% SDS-PAGE gels. 20µg of each protein sample was loaded, concentrated at 80 V for 20 min and separated at 120 V for 1 h. Electrophoresis was stopped as soon as the bromophenol blue color marker had run off. The samples were then transferred to PVDF at 110V for 100 min at 4°C by using the wet blotting method.

Each immunoblot was blocked in 5% nonfat milk in TBST for 2h prior to being incubated overnight at 4°C with primary antibodies against Six1, Bcl-2, Bax, cleaved-caspase 3 or β-tubulin. Then the membrane was washed 3 times with TBST for 10 min, and then incubated with diluted secondary antibodies in blocking solution for 2h at room temperature. After the secondary antibody solution was fully washed away, PTG ECL chemiluminescence detection kit was used for color rendering, then the blot was transferred to the X-ray film for exposure and analysis.

2.5 Statistical analysis

The experimental results were statistically analyzed using SPSS 19.0 and GraphPad Prism 8.0. The results were expressed as mean ± standard deviation and analyzed by ANOVA test. P < 0.05 indicated that the difference was statistically significant.

Results

3.1 Case report

The male proband (Ⅱ-5) presented with weakness and chest congestion and was admitted to our hospital. He was diagnosed with thymoma via enhanced computed tomography (CT) scan (Fig. 1a) and pathological examination (Fig. 1b). After his thymoma was excised, we examined both of his kidneys by ultrasound (Fig. 1c). Additionally, anemia was tested for by routine blood testing, a CD55/CD59 test and a bone marrow examination including bone marrow cytology, biopsy, immunophenotyping, and chromosome analysis (Fig. 1d). Results of the routine blood testing showed: WBC 7.18, HGB 67, PLT 163, Ret% 0.2%, and HCT 23.4. Bone marrow aspiration smears in this patient revealed a normal cellular marrow. An absence of erythroblasts was noted, but more immature erythrocyte progenitors were present indicating maturation arrest. WBCs and platelet maturation were normal. Ultrasound examination showed that the size and shape of both kidneys was normal, the contour line of both kidneys was continuous and the structure was clear. The human parvovirus B19 test was negative. When pure red cell aplasia was diagnosed in this patient we found that he had facial malformation and was deaf since childhood. Then we found out that he had multiple deaf family members, and that some of these deaf relatives died of cancer. Therefore, we constructed his family map (Fig. 1e) and examined genes associated with deafness and cancer using blood samples from him and several family members, as well as, additional mitochondrial genes using his bone marrow sample. The mutation of *Six1* gene (c.374A > G) was found in the patient and family members (Fig. 1f). Additionally, a missense mutation of ND3 (340,ACC = > GCC) was found in his bone marrow cells when compared with his oral epithelial cells. This mutation would cause an amino acid change from Thr into Ala (Table 1). According to our data [21], this mitochondrial related mutation may explain his abnormal level of hematopoietic function.

Table 1
Mutation of the proband

Base mutation	Mutation cite	Sense mutation	Gene
-->CC	chrM-302	-	D-loop
-->C	chrM-310	-	D-loop
ACC = > GCC	chrM-10398	Thr(T) = > Ala(A)	ND3
GGG = > RGA	chrM-15043	Gly(G)=>	CYTB

3.2 Linkage analysis.

Linkage analysis and mutation detection were performed in the proband’s family. Figure 1. f shows the mutation that affects this family’s deaf living members. All three patients (Ⅱ-5, Ⅱ-19,Ⅱ-3,) had the same *Six1* gene point mutation, whereas the two unaffected family members (Ⅱ-16, Ⅱ-2) did not, suggesting this rare point mutation was a new dominant mutation linked to deafness. Additionally, the linkage analysis suggested that all five brothers(Ⅱ-1,2,3,4,5) and their mother had the same mitochondrial mutations. This could explain there were many cancer patients(Ⅱ-1, Ⅱ-4, Ⅱ-5) in this generation of this family. Ⅱ-1 died of esophageal cancer, Ⅱ-4 died of liver cancer, and now Ⅱ-5 has presented with thymoma.

3.3 293T cell proliferation and apoptosis experiments

3.3.1 Transfection of 293T cells

The Six1c.374A > G plasmid map is shown in Fig. 2. a. Microscopy and western Blot test confirmed successful transfection (Fig. 2. b-d).

3.3.2 CCK-8 detection of cell proliferation

CCK-8 results showed that compared with the NC group, the cell proliferation levels of the WT and MUT groups decreased at 24h and 48h (P < 0.05), and that there was no significant difference between them (Fig. 2. e).

3.3.3 Apoptosis detection

Apoptosis detection is shown in the following figure (Fig. 2. f-g). Compared with the NC group, the percentage of apoptotic cells in the WT group and the MUT group was significantly increased (Q2-2% + Q2-4%), and it had a significant time effect. This indicates that the percentage of apoptotic cells increased after transfection of WT and MUT plasmids, and that WT *Six1* induced less apoptosis than mutated *Six1*.

3.3.4 Cell cycle stage

Compared with 24h, the S-phase of the MUT group was still higher at 48h ($p < 0.01$), indicating that the cells of the MUT group had problems progressing to the G2 phase, and that cells were arrested in S-phase (Fig. 2h-i, Table 2).

Table 2
Percentage of cells in each group for 48h at each phase

Group	G1	S	G2
NC	24.81	43.43	29.09
WT	14.57	44	41.44
Mut	14.06	61.52	24.42

3.3.5 Caspase-3 activity test

As shown in the figure (Fig2. j), compared with the NC group, the activity of Caspase-3 in the WT group and the MUT group was significantly increased($p<0.05$), and the effect of the WT group was lower than that of the MUT group.

3.4 293T cell mitochondrial function test

3.4.1 ROS level in 293T cells

As shown in the figure (Fig. 3. a), compared with the NC group, the fluoescence intensity of DCF increased in the WT group and the MUT group. This showed that after transfection of the WT plasmid and the MUT plasmid that the intracellular reactive oxygen level increased. Additionally, the MUT group had slightly higher levels of ROS compared to the WT group.

3.4.2 Flow cytometry detection of mitochondrial membrane potential

Compared with the NC group, the percentage of Q4-4% cells (monomer JC-1 group) was significantly increased in the WT and Mut groups, and increase in the population demonstrated a time effect. Increase of this group indicates that the mitochondrial membrane potential of the cells decreased after transfection of the WT and MUT plasmid, and the effect was slightly lower for the WT group (Fig. 3. b-c). Decreased mitochondrial membrane potential, which indicates a reduced level of mitochondrial productivity, suggests that 293T cells transfected with WT or MUT *Six1* may have mitochondrial damage.

3.4.3 Determination of Bcl-2, bax and cleaved caspase-3 levels

Compared with the NC group after transfection, the proportions of Bcl-2 and Bax in the WT and MUT groups decreased($p < 0.01$), the overall levels of Bcl-2 decreased, and the level of cleaved caspase-3 increased ($p < 0.01$)(Fig. 3. d-e).

Discussion

Ng et al.^[26] first reported that *Six1* gene is closely associated with tumor recurrence and metastasis of hepatocellular carcinoma with overexpression of *Six1* mRNA observed in about 85% of liver tumor tissue, while *Six1* mRNA was absent in 91.7% of non-tumor liver tissue. Similarly, *Six1* protein was overexpressed in about 60% of all tumor tissues and not detected in non-tumor tissues. Branchio-oto-renal spectrum disorder is an autosomal dominant developmental disorder with hearing impairment and other manifestations. Several previous studies have confirmed that abnormalities in the *Six1* gene can cause Branchio-oto-renal spectrum disorders. *Six1* mutations can eliminate the formation of the SIX1-EYA complex or reduce the ability to bind DNA, leading to a variety of symptoms including hearing impairment^[27–28]. For this family, mutations in the *Six1* gene were present in all tested deaf patients. This implicates the *Six1* mutations as a potential key cause of hearing impairment and malignancy in multiple family members. We further explored the mechanism of action and found that overexpression of *Six1* and mutant *Six1* (*Six1*c.374A > G) can lead to a reduction in 293T cell proliferation levels. Our data suggests that *Six1*c.374A > G point mutation can block cells in S phase—an important step in cell proliferation. When transition from S phase to G2 is blocked, apoptotic mechanisms are triggered. Both overexpression of *Six1* and mutant *Six1* can lead to increase in apoptosis with mutant *Six1* causing a greater level of apoptosis. The role of *Six1* in activating the apoptotic pathway could explain the mechanism behind its role in hearing impairment and malignancy.

Interestingly, the proband presented both BOR and thymoma as well as pure red aplasia. At present, it has also been found that there is a close relationship between *Six1* gene and hematopoietic diseases. Creed et al.^[29] reported that overexpression of human *Six1* gene stimulated erythroid differentiation of human erythroleukemia TF1 cells and primary hematopoietic stem-progenitor cells. And Chu et al.^[30] found an increase *Six1* expression in AML patients and mouse leukemia stem cells. Moreover, it was observed in human patients that the high expression of *Six1* often represents a worse AML prognosis. However, according to our data, the PRCA of proband cannot be defined as the result of *Six1* mutation since this proband has both *Six1* and mitochondrial mutations. Since mitochondria are maternally inherited, members of the 3rd generation have different mitochondria. This is likely why there is no anemia in members of the 3rd generation while severe anemia was found in the 2nd generation (II-5). Multiple studies show that mitochondrial gene mutations cause mitochondrial function damage which is a key factor of marrow failure of aplastic anemia^[21]. Therefore, II-5's mitochondrial mutations may explain the patient's anemia. To test this, we designed *in vitro* experiments to explore the relationship between pure red aplastic anemia, *Six1*c.374A > G mutation and mitochondria. Studies have found that the reduction of membrane potential is an important factor for the initiation of mitochondrial apoptosis^[31]. ROS, which mitochondria are the main endogenous source of, can reduce the membrane potential of mitochondria through oxidative stress, leading to mitochondrial dysfunction and apoptosis^[32]. We examined changes in ROS levels and mitochondrial membrane potentials in 293T cells, and found that overexpression of *Six1* and mutant

Six1 (Six1c.374A > G) can lead to the decrease in mitochondrial membrane potential level and the increase in ROS level. However, the effect of mutant *Six1* on mitochondrial membrane potential and ROS level was more potent. Bax and Bcl-2 act as promoters and inhibitors of apoptosis, respectively, and their levels are regulated through the release of cytochrome C and other substances via the mitochondrial apoptosis pathway^[33]. Caspase-3 is an important mediator of apoptosis, and is activated after being cleaved. In this study, by constructing a *Six1* overexpression model and a Six1c.374A > G model, it was found that the Bcl-2/Bax ratio of the *Six1* overexpression group and the Six1c.374A > G point mutation group decreased, and the level of cleaved caspase-3 increased. Combined with the previous experiments, we believe that the pure red aplastic anemia in the proband may be caused by the Six1c.374A > G mutation's effects on the mitochondrial apoptosis pathway.

Additionally, a recent study by Liu et al suggested another PRCA-promoting effect of a *Six1* mutation. PRCA has been demonstrated to be caused, in some cases, by anti-erythroblast antibodies, indicating B cell involvement^[34], and PRCA is routinely treated using rituximab, an anti-CD20 antibody, which targets and kills mature B cells^[35]. Liu et al recently uncovered a link between *Six1* and non-canonical NF-κB signaling, a crucial pathway in B cell survival and maturation. They found that *Six1*, as well as *Six2*, are essential inhibitors of the non-canonical NF-κB pathway^[36]. This suggests that the patient's *Six1* mutation could have further exacerbated his PRCA by enabling a more robust anti-erythroblast B cell response caused by an increase of pro-survival non-canonical NF-κB signaling. This potential connection warrants further investigation.

Conclusion

Our experiment confirmed that the Six1c.374A > G point mutation can increase the apoptosis level of 293T cells, and that this is likely due to increased activity in the mitochondrial apoptosis pathway. Therefore, we believe that the Six1c.374A > G point mutation's effects on mitochondrial apoptosis may be the cause of abnormal hematopoietic function.

List Of Abbreviations

WES, Whole-exome sequencing; SO, sine oculis; Six 1, Six homeobox 1; BOS, Branchio-oto-syndrome; BORSD, Branchio-oto-renal spectrum disorders; BOR, Branchio-oto-renal; BO, Branchio-oto; PRAA, Pure red cell aplasia; AA, aplastic anemia; PI, propidium iodide.

Declarations

Ethics approval and consent to participate

This experiment was approved in writing by all study participants. The study was approved by the Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (No.2019-020).

Consent for publication

All participants (including the proband and his family members) agreed to publish the paper.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing financial interests.

Funding

This work is supported by the National Natural Science Foundation of China (No.81774080) for the purchase of experimental materials, the Taishan Scholar Program (tsqn201812145) supports the testing part of the experiment, the Shandong Province Key Research and Development Program (No.2019GSF108162) provides financial support for the writing of this paper and the labors participating in the experiment.

Authors' contributions

Dr. XC designed and performed the experiments, obtained the bone marrow samples, analyzed the data, and wrote the manuscript; Pr. YG and JS conducted clinical examination and surgical treatment; Master JX, XD and WZ obtained the bone marrow samples and performed experiments; Master RS, YZ and XS provided vital new reagents and performed experiments; Dr. FS and MP analyzed the data and wrote the manuscript. All authors have read and approved the manuscript.

Acknowledgments

Thank you for the support of the Study Abroad Funding by the People's Government of Shandong Province for the experimental fund. Thanks for the experimental site and equipment supported by the Affiliated Hospital of Shandong University of traditional Chinese medicine. Thanks for the sequencing service provided by Beijing Hightrust Diagnostic.

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Declarations

A new point mutation causes Branchio-oto syndrome, thymoma and pure red cell aplasia. This declaration applies to the feature manuscript.

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Figures

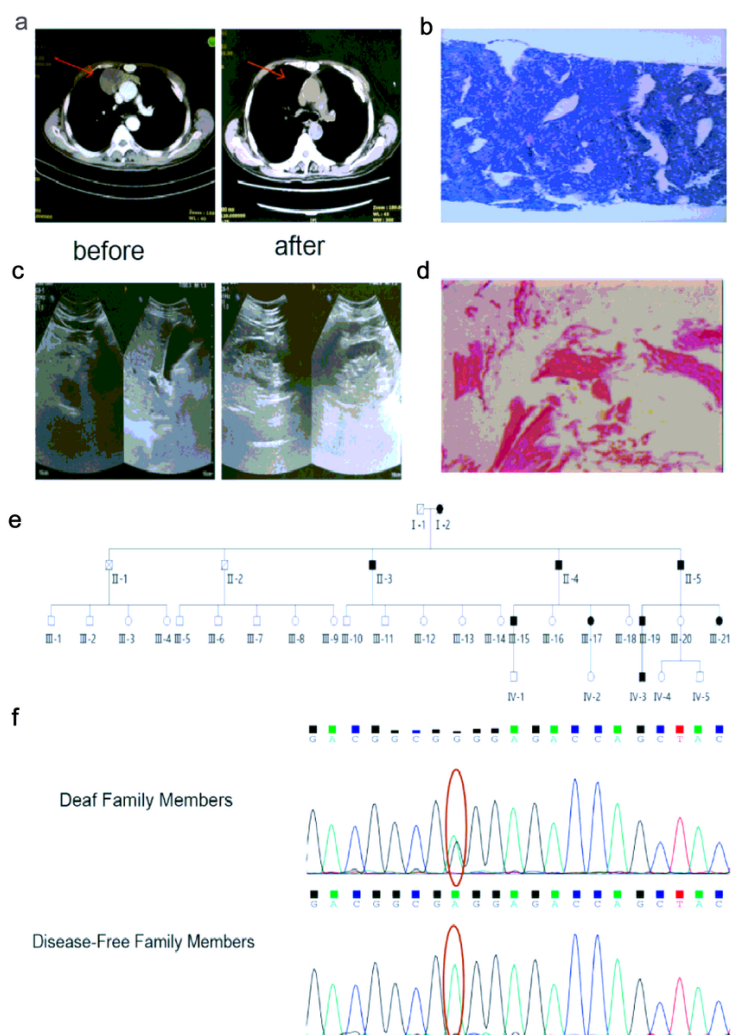


Figure 1

Characters and six1 mutation of patient. (a) The CT of the proband showed that he had a thymic tumor before the operation, and the CT showed that the thymoma was removed after the operation. (b) Pathological microscopy of the proband reveals he had a thymoma (type A). IHC:CK-pan(3+),CK19(3+),CD3 dim(+),CD5 seldom(+), CD34(-),CK20(-),Bcl-2(+/-),EMA(-),P53(-),Desmin(-),S-100(-),Ki67(2%+). (c) No obvious abnormality was found in the kidney of the proband. (d) Bone marrow biopsy showed that bone marrow hyperplasia was extremely low, the percent of hematopoietic area was less than 5%, there was no proliferation of primary cells and proliferation of fibrous tissue. (e) Proband's family pedigree. Deaf family members are indicated in black. (f) Six1 gene test in this family revealed a new mutation (c.374A>G).

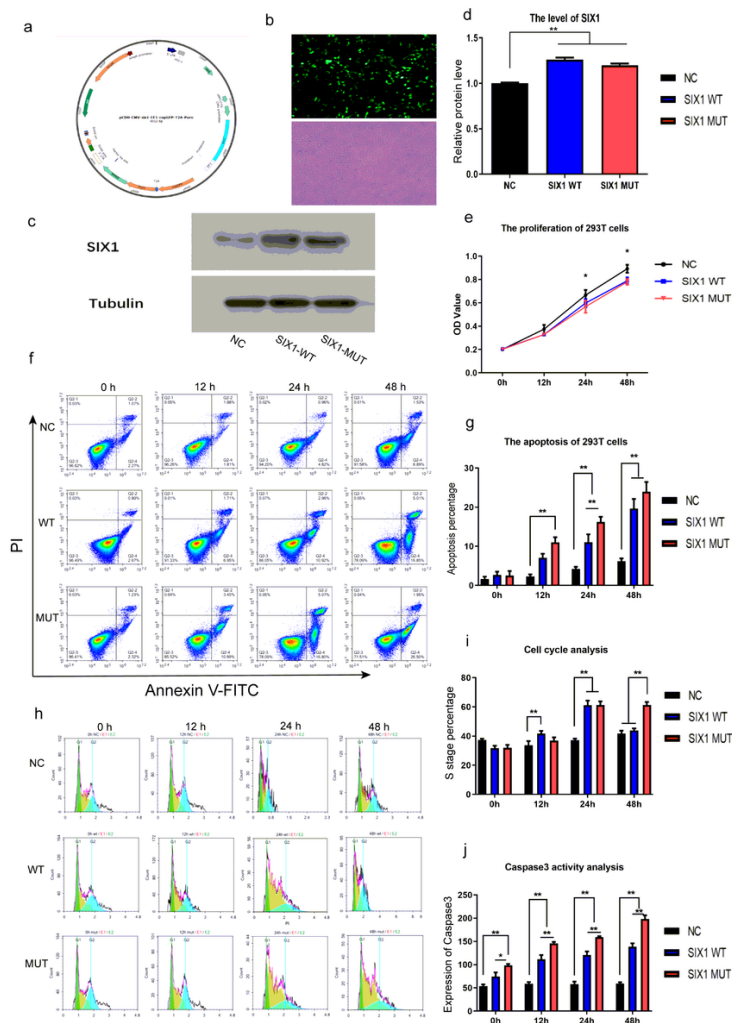


Figure 2

293T cell transfection, proliferation and apoptosis. (a) Plasmid map of Six1c.374A>G. (b) Fluorescence image of Six1c.374A>G transfection under microscope. (c) Western blotting of Six1 in NC group, WT group and MUT group. The picture has been cropped for easy viewing and full-length blots are presented in Supplementary Figure 1. Image J was used to scan gray values and analysis results. (d) Western blotting data comparison of NC group, WT group and MUT group. (e) CCK-8 detected cell proliferation: 0h, 12h, 24, and 48h after seeding 2×10^3 cells/well 293T cell proliferation was detected. Culture medium was changed 6 hours after transfection and 0h was the medium changed time. Compared with NC group, the proliferation level of WT group and MUT group decreased at 24h and 48h, and there was no significant difference between the two groups. (f) Flow cytometric detection of apoptosis: 6 hours after transfection $1-5 \times 10^5$ 293T cells were seeded, and apoptotic rates were detected at 0h, 12h, 24h and 48 h. (g) Flow cytometric analysis of apoptosis confirmed a significant increase of apoptotic 293T cells in WT group and MUT group, and that there was no significant difference between them at 48h. (h) Cell cycle detection: 6 hours after transfection 1×10^5 293T cells were seeded, and the cell cycle stage was detected at 0h, 12h, 24h and 48 h. (i) 293T cell cycle analysis: compared with the other two groups, the S phase of MUT group was still higher at 48h which indicated that the cells were blocked in S phase. (j) Caspase 3 activity of 293T cells: 6 hours after transfection 2×10^6 293T cells were seeded, and caspase 3 levels were detected at 0h, 12h, 24h and 48 h. The caspase 3 activity level of MUT group was significantly higher than the other 2 groups. The date is presented as the mean \pm SD.*P<0.05 and **P<0.01.

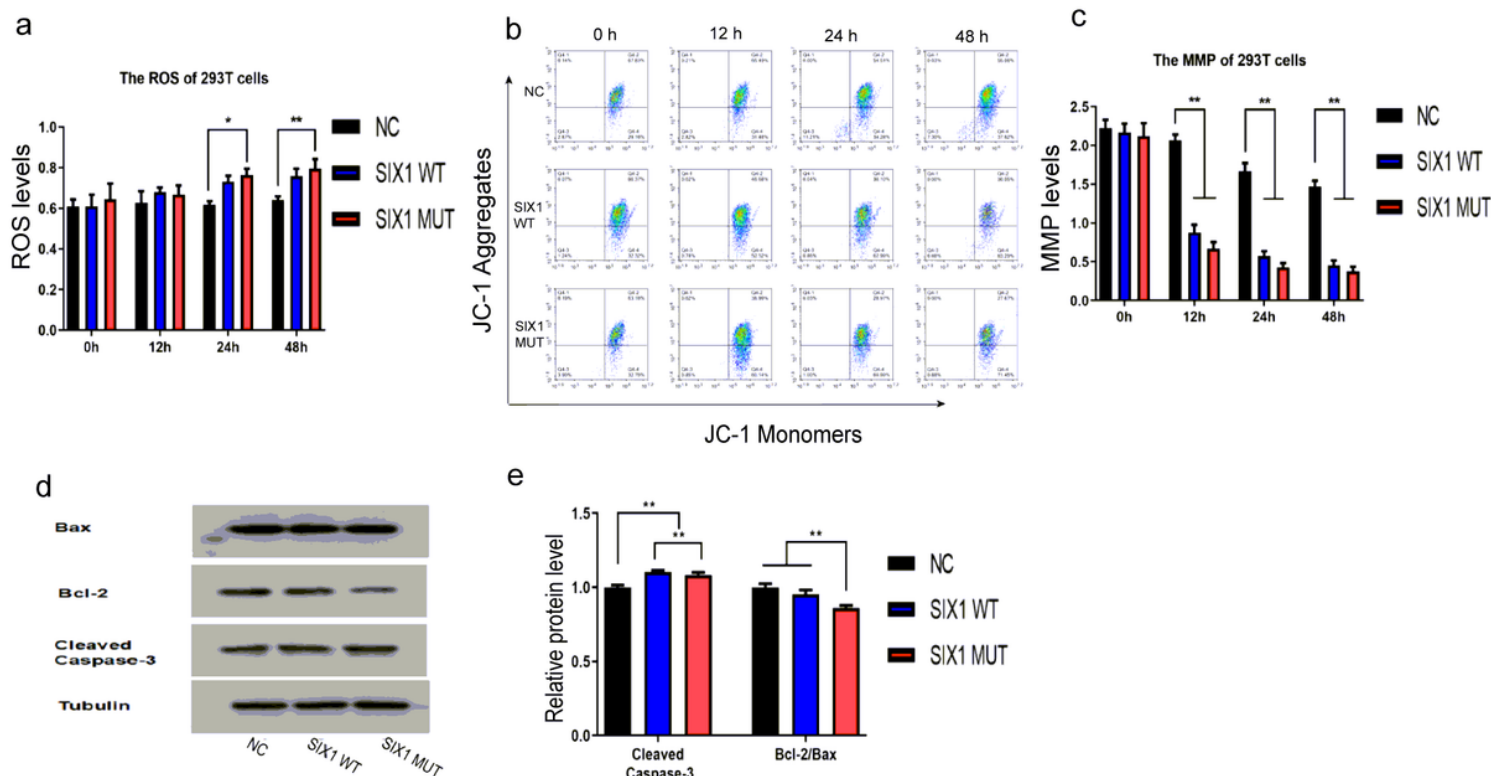


Figure 3

293T cell mitochondrial function (a) ROS fluorescence data (DCF) showed that at 48h, WT group and MUT group ROS levels increased significantly, and MUT group ROS was significantly higher than WT group. (b) JC-1 fluorescence was seen in Q4-2% and Q4-2% of NC. Compared with the NC group, WT and MUT cell populations had a significantly increased proportion of the monomer JC-1 group (Q4-4%). (c) The MMP of WT group and MUT cells decreased, and the effect of WT group was lower than that of MUT group. (d) Western blotting detection of Bcl-2, Bax, and cleaved caspase-3 expression in NC group, WT group and MUT group. The picture has been cropped for easy viewing and full-length blots are presented in Supplementary Figure 2. Image J was used to scan gray values and analysis results. (e) Western blot analysis: Compared with the NC group, the Bcl-2 / Bax ratio in the WT group and the MUT group was reduced, and cleaved caspase-3 increased. The data is presented as the mean \pm SD. *P<0.05 and **P<0.01.

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