Published in final edited form as:
J Surg Res. 2014 May 15; 188(2): 466–472. doi:10.1016/j.jss.2014.01.015.

Characterization of transcription factor AP-2 beta mutations involved in familial isolated patent ductus arteriosus suggests haploinsufficiency

Wei Ji, MD[#a], **Matthew A. Benson, PhD**[#b], **Shoumo Bhattacharya, MD**[b], **Yiwei Chen, MS**[a], **Jingjing Hu, MD**[a], and **Fen Li, MD**[a,*]

[a]Department of Cardiology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China

[b]Department of Cardiovascular Medicine, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

[#] These authors contributed equally to this work.

Abstract

Background—Patent ductus arteriosus (PDA) is one of the most common congenital heart defects. Transcription factor AP-2 beta (TFAP2B) mutations are associated with the Char syndrome, a disorder associated with PDA, and with facial and fingers abnormalities. Recently, we identified two TFAP2B mutations in two families without Char syndrome phenotype, c. 601+5G>A and c.435_438delCCGG, and these TFAP2B mutations were associated with familial isolated PDA. The aim of this study was to identify the effects of these mutations on TFAP2B function.

Methods—Plasmids containing the wild-type or mutated TFAP2B were constructed and transfected in cells. Plasmids containing the TFAP2B coactivator, Cpb/p300-interacting transactivator 2 (CITED2), was also transfected. TFAP2B expression was detected by luciferase expression and by Western blot analysis.

Results—These mutations resulted in loss of transactivation function, which could not be improved by Cpb/p300-interacting transactivator 2. The c.601+5G>A mutated gene did not express any protein, whereas the c.435_438delCCGG mutation did not impact the transactivation function activated by the wild-type TFAP2B.

Conclusions—These results suggest that a haploinsufficiency effect of TFAP2B could be involved in familial isolated PDA.

Keywords

Patent ductus arteriosus; TFAP2B; Haploinsufficiency; Mutation

[*]*Corresponding author*. Department of Cardiology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai, P. R. China. Tel.: +86 150 2165 5580; fax +86 216 408 5875. jiweimedsci@163.com (F. Li) .

The authors declare that they have no conflict of interest.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

1. Introduction

The ductus arteriosus (DA) is a specific vascular structure between the aorta and the pulmonary artery diverting the blood from the pulmonary artery into the aorta during fetal development. After birth, DA undergoes functional and anatomic closure. In some people, the DA does not close after birth, if present after the age of 3 mo, this condition is known as patent DA (PDA). Untreated PDA usually results in life-threatening conditions, such as congestive heart failure, pulmonary artery hypertension, and neonatal necrotizing enterocolitis [1]. On the other hand, DA patency is essential in some special pathologic conditions. Some DA-dependent congenital heart diseases, such as severe tetralogy of Fallot and single ventricle with severe pulmonary artery stenosis, require palliative systemic pulmonary shunt or stent implantation into the DA to ensure an adequate pulmonary blood flow.

The DA originates from the left dorsum part of the sixth pharyngeal arch and is histologically derived from cardiac neural crest cells (NCCs) [2,3]. Transcription factor AP-2 beta (TFAP2B), is a transcription factor that is enriched in the neural crest and could play an important role in regulating DA closure [4]. Cpb/p300-interacting transactivator 2 (CITED2) acts as a TFAP2B coactivator and enhances its transcriptional efficiency [5]. To date, most TFAP2B mutations [6–9] have been identified in Char syndrome patients, a birth defect characterized by PDA and by deformities in the face and fingers [10]. However, no mutation causing isolated PDA has been identified for a long time. Recently we reported two TFAP2B heterozygous mutations, c.601+5G>A and c.435_438delCCGG, in two unrelated families with PDA, but without features of the Char syndrome [11] (Figs. 1 and 2). In both families, the mutated allele was only observed in affected individuals. This previous study demonstrated that TFAP2B variations are not exclusively associated with the Char syndrome, but may also be associated with isolated PDA. However, although we identified the c.601+5G>A mutation in isolated PDA patients, it was also reported in Char syndrome patients [7], stressing the need for a better understanding of this pathology.

To examine the pathogenic mechanism underlying isolated PDA, we conducted a functional study of the TFAP2B c.601+5G>A and c.435_438delCCGG mutations using a transactivation assay. We hypothesized that the disruption of TFAP2B transcription by these mutations plays a major role in the etiology of isolated PDA.

2. Materials and methods

2.1. Plasmids

The pcDNA3-TFAP2B expression plasmid (containing the wild-type TFAP2B [NM_003221]), the p3*AP2-Bluc plasmid (containing the luciferase reporter gene with three copies of the TFAP2B recognition sequence), the pcDNA3-lacZ plasmid, the pcDNA3-CITED2 plasmid (containing the TFAP2B coactivator, CITED2), and the empty vector pcDNA3 were all provided by S.B. (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA). The c.435_438delCCGG expression plasmid, CMV-CCGGdel, was developed by site-directed mutagenesis polymerase chain reaction using the primers presented in Table 1. The CMV-Δexon3 vector expressing TFAP2B without exon 3

was made using the primers presented in Table 1; these primers matched the 3′ terminal part of exon 2 and the 5′ terminal part of exon 4. Both mutated TFAP2B vectors were confirmed by sequencing.

2.2. Cell transfection and transactivation assay

U2-OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Sigma), penicillin (100 U/mL, Sigma), streptomycin (10 μg/mL, Sigma) and l-glutamine (4 mM, Sigma). Cells were seeded into a 96-well plate, 5000 cells/well. For transactivation assays, cells were transfected with 20 ng of CITED2 or pcDNA3 vector, 100 ng of p3*AP2-Bluc plasmid, 20 ng of CMV-lacZ plasmid, and 20 ng of wild-type or mutant plasmids using Fugene HD (Promega, Madison, WI). To evaluate the activation of the luciferase reporter gene (p3*AP2-Bluc) by increased doses of CITED2, we transfected 10, 20, or 40 ng of CITED2 together with 20 ng of wild-type or TFAP2B mutant plasmids, and 20 ng of the CMV-lacZ plasmid for normalization. To detect the effects of the CMV-CCGGdel plasmid (30 ng) on cotransfected wild-type TFAP2B (30 ng), cells cotransfected with pcDNA3 (30 ng) and CMV-CCGGdel (30 ng) were compared, and cells transfected with pcDNA3 (60 ng) were used as control. Twenty-four hours after transfection, luciferase expression was measured using the luciferase assay system (Promega, E1500), according to the manufacturer's instructions. LacZ levels were assessed as previously described [12], and luciferase expression levels were normalized to LacZ levels. Results are presented as relative luciferase value from three independent transfection experiments performed in triplicate.

2.3. Western blotting

U2-OS cells were seeded in a 12-well plate (80,000 cells/well) and transfected with 800 ng of pcDNA3, wild-type TFAP2B, c.435_438delCCGG, or c.601+5G>A plasmids using Fugene HD (Promega). Western blotting was performed after 24 or 48 h. We used the TFAP2B N-terminal specific polyclonal antibody (Sigma, AV38282) as the primary antibody (1 μg/mL) and horseradish peroxidase-labeled goat anti-rabbit as the secondary antibody. Proteins were detected using the enhanced chemiluminescence (ECL) advanced Western blotting detection kit (GE Healthcare, Waukesha, WI).

3. Results

3.1. TFAP2B mutations disrupt transactivation

As expected, wild-type TFAP2B-induced luciferase expression, which was increased by CITED2 cotransfection. However, luciferase expression was lowered by TFAP2B mutations. Furthermore, CITED2 could not enhance the transactivation of AP2-luc by mutant TFAP2B (Fig. 3A). Because this decreased luciferase signal may have resulted from an inability of the mutant TFAP2B to activate transcription or from a partial inhibition, we cotransfected mutant or wild-type plasmids with different concentrations of CITED2 to rule out one of these two possibilities. For wild-type plasmids, we observed that the luciferase coactivation was enhanced in a CITED2 dose-dependent manner. However, for both mutants, the luciferase signal was very low, and increasing the CITED2 dose had no effect (Fig. 3B).

3.2. The two TFAP2B mutations have different effects on the protein

The c.601+5G>A mutation causes the deletion of exon 3 of the TFAP2B messenger RNA [11], and we developed a vector lacking exon 3. The c.435_438delCCGG mutation deletes 4 bp in exon 2, inducing a frameshift in the messenger RNA. This frameshift mutation was predicted to encode a truncated 188-amino acid protein because of the presence of a premature stop codon. To confirm the presence of TFAP2B protein expression from the mutant vectors, we transfected the mutant or wild-type vectors in U2-OS cells and analyzed TFAP2B expression by Western blotting (Fig. 4). Because the TFAP2B mutants had sequence variations, we selected a N-terminal specific primary antibody. After transfection (24 or 48 h), we observed that the wild-type TFAP2B was expressed (51 kD). However, the c.435_438delCCGG mutation produced a 21-kD, and no protein was produced by the TFAP2B c.601+5G>A mutant plasmid.

3.3. Truncated TFAP2B protein does not inhibit wild-type TFAP2B transactivation

To determine if the truncated protein produced by the c.435_438delCCGG mutation inhibits wild-type TFAP2B transactivation, we cotransfected the c.435_438delCCGG and wild-type TFAP2B plasmids into U2-OS cells. Compared with pcDNA3 and wild-type TFAP2B cotransfection, the protein encoded by the gene bearing the c.435_438delCCGG mutation had no impact on the transactivation of the wild-type TFAP2B (Fig. 3C). Therefore, the c. 435_438delCCGG mutation did not competitively inhibit the function of the wild-type TFAP2B allele.

4. Discussion

The aim of this study was to assess the effects of two TFAP2B mutations associated with familial PDA in cultured cells. Our results showed that the c.601+5G>A and c. 435_438delCCGG mutations produced no functional protein and that the TFAP2B coactivator, CITED2, was unable to increase their function. In addition, the c. 435_438delCCGG mutation produced a truncated protein, whereas the c.601+5G>A mutation did not produce any detectable protein. Finally, the truncated protein encoded by the c.435_438delCCGG mutation did not inhibit the function of the wild-type protein.

PDA is the main phenotype of the Char syndrome, and TFAP2B plays a key role in the regulation of cardiac NCCs. Recently, a family of six members suffering from PDA without any of the characteristic hand or craniofacial abnormalities of the Char syndrome was identified, and their TFAP2B gene was sequenced. All six members were found to carry a single-base substitution in exon 3, IVS2-2A>T8 [9]. This study suggested that mutations in TFAP2B are not only associated with the Char syndrome but also associated with isolated PDA, which was confirmed by our previous study [11]. Although the c.601+5G>A was previously reported to be associated with Char syndrome [7], we identified this mutation in isolated PDA patients. Presently, we do not understand the exact mechanisms by which the same mutation may result in two different phenotypes. We may only hypothesize that these different phenotypes result from a different phenotype penetrance or from complex interactions between environmental and other genetic factors.

TFAP2B encodes a neural crest–derived transcription factor [4], and it affects NCCs biology mainly through the regulation of several target genes. Our study demonstrated that both c.601+5G>A and c.435_438delCCGG mutants could not stimulate transcription and that cotransfected CITED2 was unable to enhance the activation of mutant TFAP2B. Furthermore, increasing the dose of coactivator could not improve this poor activation, indicating that these mutations had a completely deficient function. We observed that the c.601+5G>A mutant could not produce any protein, which indicated a haploinsufficient effect involved in the development of isolated PDA. The c.435_438delCCGG mutant produced a truncated protein with the loss of its DNA-binding domain. A recent study suggests that coactivators, such as p300, bind with the N-terminal end of TFAP2B to coactivate target genes [13]. In addition, TFAP2B binds to its DNA recognition sequence and dimerizes with another TFAP2 molecule on the DNA *via* its C-terminal domain, activating the target gene by the N-terminal transactivation domain [14,15]. Thus, the protein encoded by the c.435_438delCCGG allele contained the N-terminal part and might affect the function of the wild-type protein through competition for coactivators. In this case, the c.435_438delCCGG mutant would exhibit a dominant negative effect. However, cells cotransfected with c.435_438delCCGG and wild-type plasmids had no change in the luciferase signal, suggesting that this mutation also affects the N-terminal coactivator-binding domain. These results are consistent with a haploinsufficient effect involved in the pathogenesis of familial isolated PDA.

How the disruption of the transactivation causes PDA? We know that two main processes are involved in DA closure: functional DA closure by constriction and anatomic closure caused by DA occlusion by intima thickening. During anatomic closure, endothelium is detached from internal elastic lamina, and ductus smooth muscle cells migrate from media into intima [16]. Because ductus smooth muscle cells are differentiated from NCCs [2,3], proliferation, differentiation, and migration of NCCs are important for DA closure. TFAP2B is expressed in NCCs during early embryogenesis [17]. Many genes are regulated in NCCs during the embryonic life and are modulated by TFAP2B, such as bone morphogenetic protein 2 and bone morphogenetic protein 4 [18,19], activin receptor-like kinases 2 (ALK2) [20], many pivotal potassium and calcium ion channels (such as CACNA1G/alpha1G, CACNB2/CaL-beta2, and KCNA2/Kv1.2) [21–23], hypoxia-induced transcription factor, and endothelin 1 [17]. All these factors constitute a complex network that modulates functional and anatomic DA closure. Based on these previous studies and the results from the present study, we speculated that TFAP2B could be a key modulator of this network. Consequently, TFAP2B mutations disrupting transactivation should result in the downregulation of these target genes, leading to the disruption of this network and to a disrupted DA closure. However, many studies are still required to correctly understand the complex relations between these factors and to explain why the same mutation may cause Char syndrome or isolated PDA.

5. Conclusions

The alleles bearing the c.601+5G > A and c.435_438delCCGG mutations were unable to activate the transcription of their target sequences. These results suggest that a haploinsufficiency effect could be involved in familial isolated PDA.

Acknowledgment

The authors thank all the patients who participated in our study. We are deeply grateful to the people at the Shanghai Children's Medical Center and to the members of Dr Bhattacharya's group for their kind assistance. This study was supported by grants from the Wellcome Trust and the National Natural Science Foundation of China (NSFC) (81170152). The authors would like to thank Dr Bardfield from the Project HOPE for the revision of the manuscript.

REFERENCES

[1]. Giliberti P, De Leonibus C, Giordano L. The physiopathology of the patent ductus arteriosus. J Matern Fetal Neonatal Med. 2009; 22(Suppl 3):6. [PubMed: 19718587]

[2]. Waldo KL, Lo CW, Kirby ML. Connexin 43 expression reflects neural crest patterns during cardiovascular development. Dev Biol. 1999; 208:307. [PubMed: 10191047]

[3]. Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. Development. 2000; 127:1607. [PubMed: 10725237]

[4]. Moser M, Imhof A, Pscherer A, et al. Cloning and characterization of a second AP-2 transcription factor: AP-2 beta. Development. 1995; 121:2779. [PubMed: 7555706]

[5]. Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, Livingston DM. Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. Genes Dev. 1999; 13:64. [PubMed: 9887100]

[6]. Satoda M, Zhao F, Diaz GA, et al. Mutations in TFAP2B cause Char syndrome, a familial form of patent ductus arteriosus. Nat Genet. 2000; 25:42. [PubMed: 10802654]

[7]. Mani A, Radhakrishnan J, Farhi A, et al. Syndromic patent ductus arteriosus: evidence for haploinsufficient TFAP2B mutations and identification of a linked sleep disorder. Proc Natl Acad Sci U S A. 2005; 102:2975. [PubMed: 15684060]

[8]. Zhao F, Weismann CG, Satoda M, et al. Novel TFAP2B mutations that cause Char syndrome provide a genotype-phenotype correlation. Am J Hum Genet. 2001; 69:695. [PubMed: 11505339]

[9]. Khetyar M, Syrris P, Tinworth L, Abushaban L, Carter N. Novel TFAP2B mutation in nonsyndromic patent ductus arteriosus. Genet Test. 2008; 12:457. [PubMed: 18752453]

[10]. Char F. Peculiar facies with short philtrum, duck-bill lips, ptosis and low-set ears–a new syndrome? Birth Defects Orig Artic Ser. 1978; 14:303. [PubMed: 728571]

[11]. Chen YW, Zhao W, Zhang ZF, et al. Familial nonsyndromic patent ductus arteriosus caused by mutations in TFAP2B. Pediatr Cardiol. 2011; 32:958. [PubMed: 21643846]

[12]. Bamforth SD, Braganca J, Eloranta JJ, et al. Cardiac malformations, adrenal agenesis, neural crest defects and exencephaly in mice lacking Cited2, a new Tfap2 co-activator. Nat Genet. 2001; 29:469. [PubMed: 11694877]

[13]. Braganca J, Eloranta JJ, Bamforth SD, Ibbitt JC, Hurst HC, Bhattacharya S. Physical and functional interactions among AP-2 transcription factors, p300/CREB-binding protein, and CITED2. J Biol Chem. 2003; 278:16021. [PubMed: 12586840]

[14]. Williams T, Tjian R. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev. 1991; 5:670. [PubMed: 2010091]

[15]. Williams T, Tjian R. Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins. Science. 1991; 251:1067. [PubMed: 1998122]

[16]. Bokenkamp R, DeRuiter MC, van Munsteren C, Gittenberger-de Groot AC. Insights into the pathogenesis and genetic background of patency of the ductus arteriosus. Neonatology. 2010; 98:6. [PubMed: 19955832]

[17]. Ivey KN, Sutcliffe D, Richardson J, Clyman RI, Garcia JA, Srivastava D. Transcriptional regulation during development of the ductus arteriosus. Circ Res. 2008; 103:388. [PubMed: 18635823]

[18]. Stottmann RW, Choi M, Mishina Y, Meyers EN, Klingensmith J. BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. Development. 2004; 131:2205. [PubMed: 15073157]

[19]. Zhao W, Wang J, Shen J, et al. Mutations in VEGFA are associated with congenital left ventricular outflow tract obstruction. Biochem Biophys Res Commun. 2010; 396:483. [PubMed: 20420808]

[20]. Dudas M, Sridurongrit S, Nagy A, Okazaki K, Kaartinen V. Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. Mech Dev. 2004; 121:173. [PubMed: 15037318]

[21]. Reese J, Waleh N, Poole SD, Brown N, Roman C, Clyman RI. Chronic in utero cyclooxygenase inhibition alters PGE2-regulated ductus arteriosus contractile pathways and prevents postnatal closure. Pediatr Res. 2009; 66:155. [PubMed: 19390487]

[22]. Waleh N, Reese J, Kajino H, et al. Oxygen-induced tension in the sheep ductus arteriosus: effects of gestation on potassium and calcium channel regulation. Pediatr Res. 2009; 65:285. [PubMed: 19092721]

[23]. Akaike T, Jin MH, Yokoyama U, et al. T-type Ca2+ channels promote oxygenation-induced closure of the rat ductus arteriosus not only by vasoconstriction but also by neointima formation. J Biol Chem. 2009; 284:24025. [PubMed: 19567868]

Europe PMC Funders Author Manuscripts

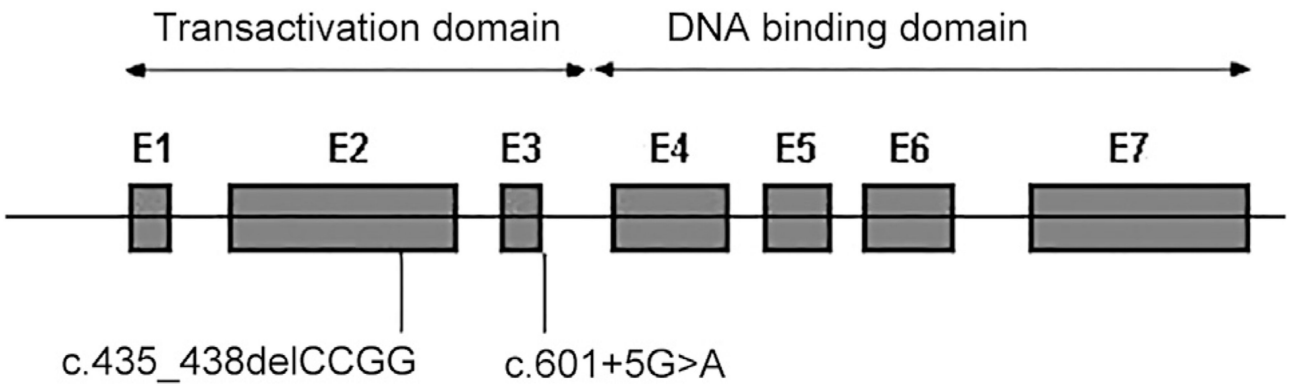

Fig. 1.

Schematic representation of the TFAP2B gene and position of the two mutations. The c. 601+5G>A mutation is located in the junction area of exon 3 and intron 3. The c. 435_438delCCGG is located in the C-terminal part of exon 2. Boxes represent the exons.

Europe PMC Funders Author Manuscripts

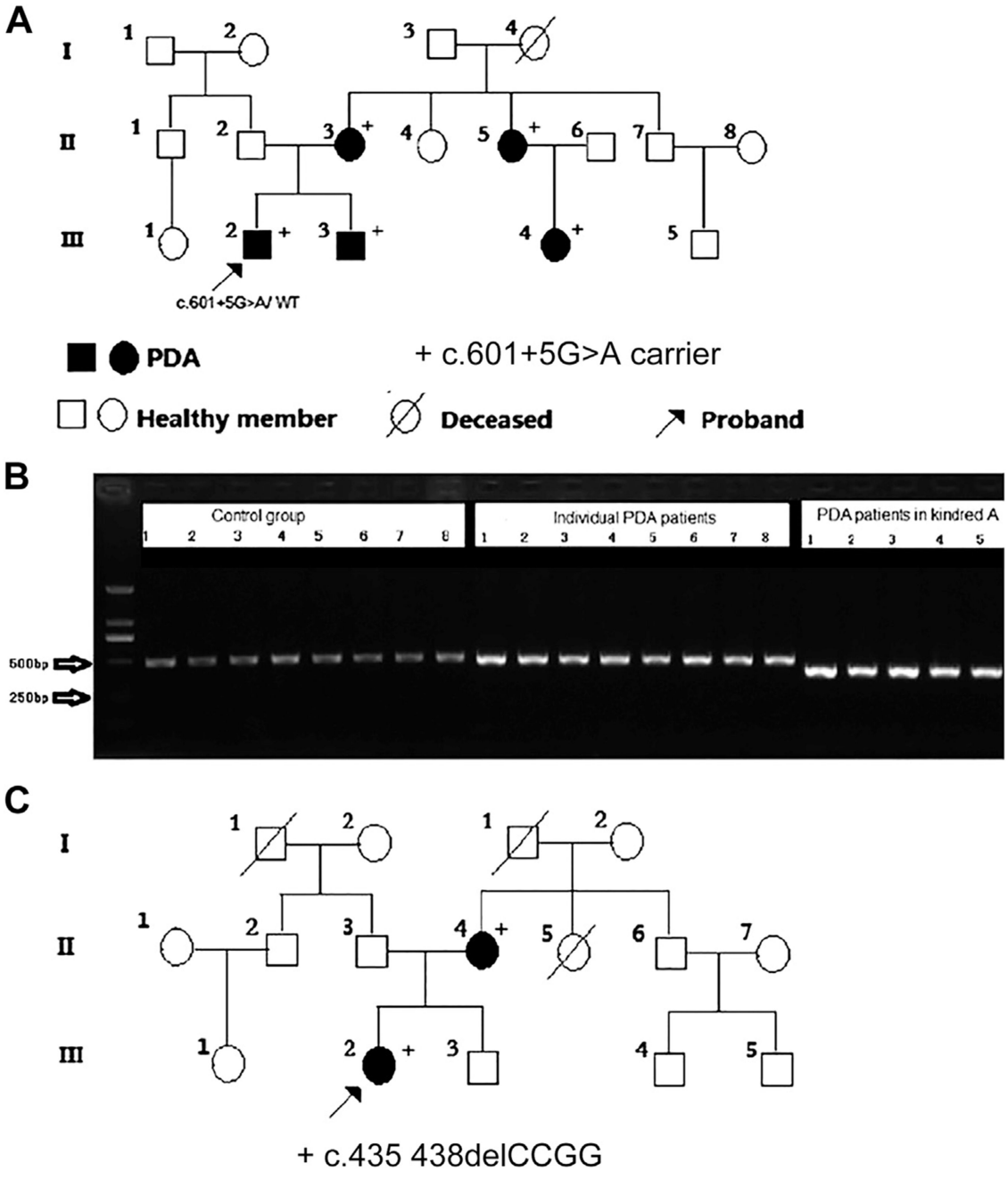

Fig. 2.

(A) Identification of the (C)601+5G>A mutation in a family. (B) The (C)601+5G>A mutation causes a 61-bp deletion in TFABP2B. (C) Identification of the (C)435_438delCCGG mutation in an unrelated family. This figure was from our previous study reporting these two mutations [11].

Europe PMC Funders Author Manuscripts

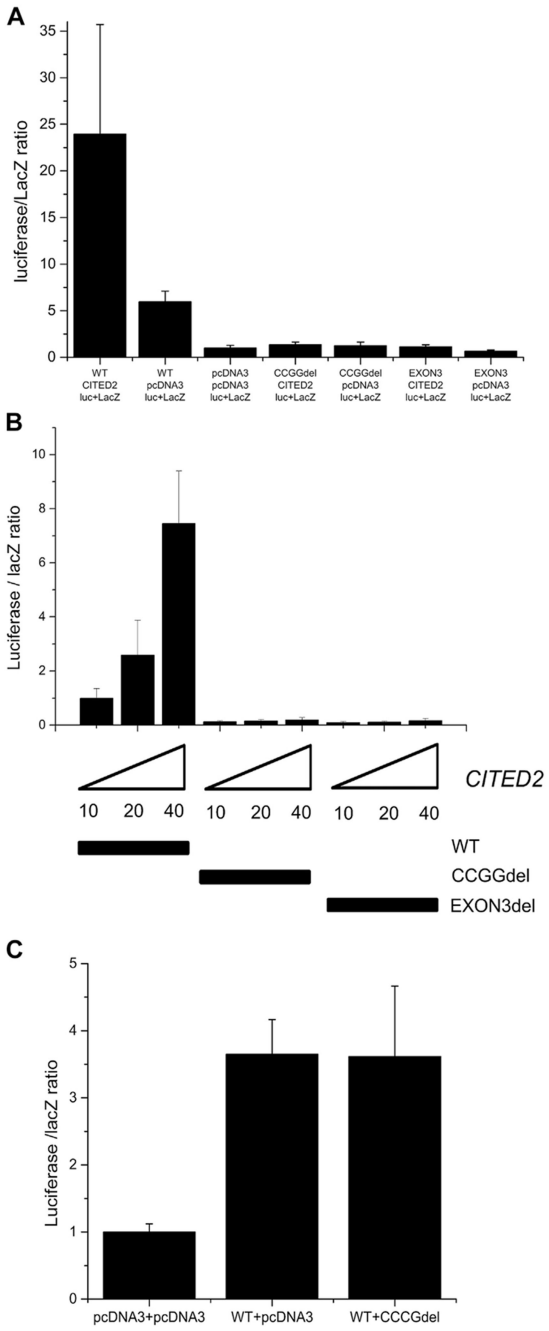

Fig. 3.

Transactivation assay of wild-type and mutant TFAP2B. (A) Transcriptional assay. Cells were transfected with 100 ng of p3*AP2-Bluc and 20 ng of lacZ plasmids. About 20 ng of wild-type or mutant TFAP2B plasmid was cotransfected with 20 ng of CITED2 or pcDNA3 plasmid. The control value (third column from the left) was set to 1.0. (B) Transactivation assay with increasing doses of CITED2 plasmid. Cells were transfected with 100 ng of p3*AP2-Bluc and 20 ng of lacZ plasmids. The wild-type or mutant TFAP2B (20 ng) and different doses of CITED2 plasmid (10, 20, or 40 ng) were cotransfected. The left column

was set at 1.0 as control. (C) To determine if the product of the (C)435_438delCCGG mutation would compete with wild-type TFAP2B proteins, 80 ng of p3*AP2-Bluc and 40 ng of lacZ were transfected into U2-OS cells, as well as 30 ng of pcDNA3, wild-type or CCGGdel plasmid. The left column was set to 1.0 as control. Twenty-four hours after transfection, cells were lysed, and the luciferase expression was determined. Luciferase expression was normalized to lacZ. Results are expressed as mean ± standard error from three independent experiments performed in triplicates. WT: wild-type.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

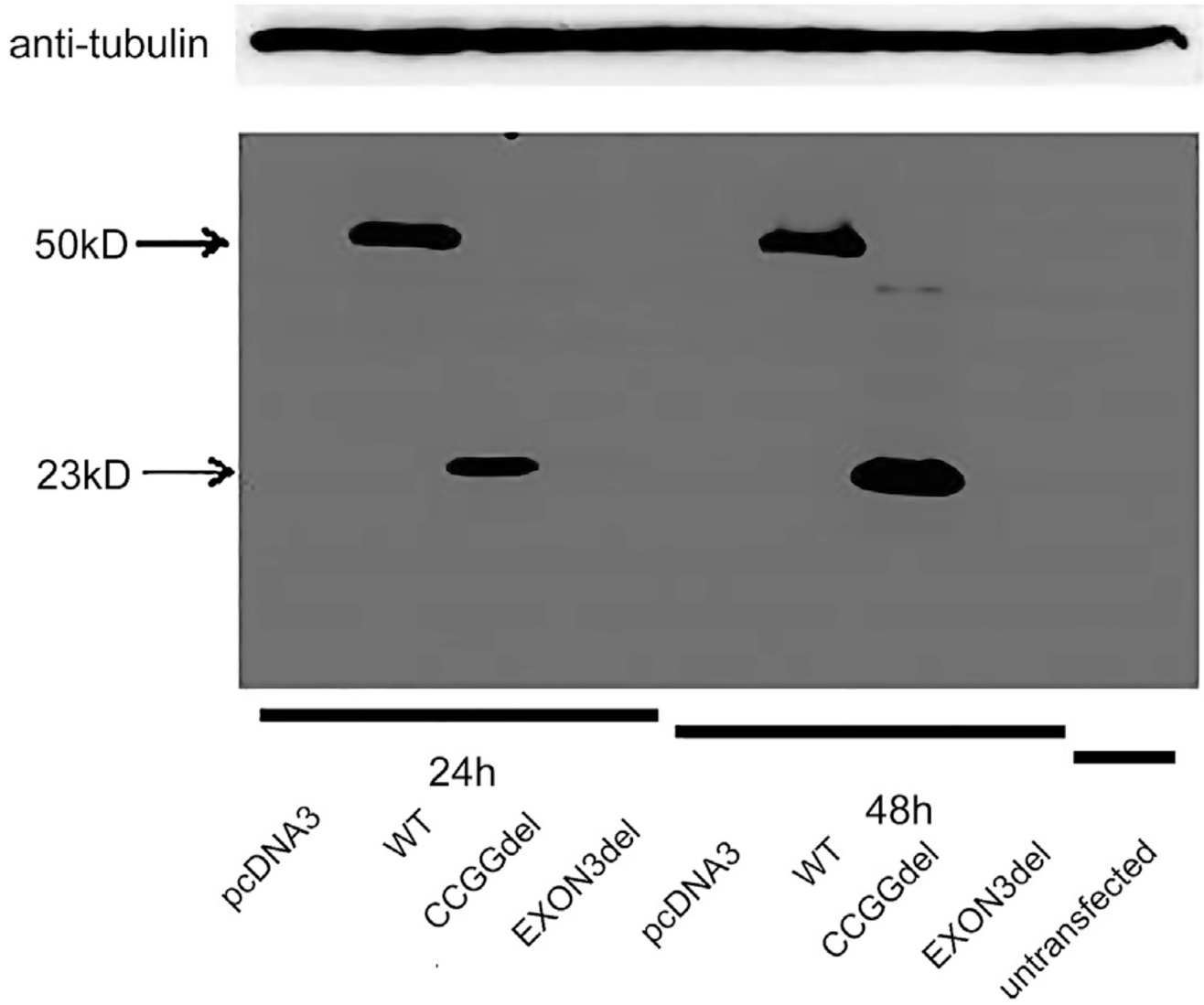

Fig. 4.

Detection of the expression of mutant TFAP2B proteins in transfected U2-OS cells by Western blot analysis. Cells were transfected with 800 ng of pcDNA3, wild-type, CCGGdel or EXON3del TFAP2B plasmids. After 24 or 48 h, cells were collected, and a Western blot was performed using a polyclonal TFAP2B N-terminal antibody.

Europe PMC Funders Author Manuscripts

Table 1

Primers for mutagenesis PCR for the generation of TFAP2B mutant plasmids.

Primer	Sequence
CMV-TFAP2B EcoRV Fwr	5′-GTC AAG TAC GAA GAT ATC TAT GAG G-3′
CMV-TFAP2B SacII Rev	5′-CGC CTG CCC GCG GGT AAA TTC-3′
TFAP2B CCGGdel Fwr	5′-CTA CCA CTC GGT CCG CCG CAC GTG CTG CTG CAT TC-3′
TFAP2B CCGGdel Rev	5′-GAA TGC AGC AGC ACG TGC GGC GGA CCG AGT GGT AG-3′
TFAP2B EXON3del Fwr	5′-AAT GGA AGA CGT CCA GTT CCA GTT CCT CCC AA-3′
TFAP2B EXON3del Rev	5′-TTG GGA GGA ACT GGA ACT GGA CGT CTT CCA TT-3′

Fwr = forward; PCR = polymerase chain reaction; Rev = reverse.