RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

A Mutation in the Kozak Sequence of *GATA4* Hampers Translation in a Family With Atrial Septal Defects

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Manuscript Received: 7 October 2013; Manuscript Accepted: 2 July 2014

Atrial septal defect (ASD) is the most common congenital heart defect clinically characterized by an opening in the atrial septum. Mutations in *GATA4*, *TBX5*, and *NKX2-5* underlie this phenotype. Here, we report on the identification of a novel -6 G > C mutation in the highly conserved Kozak sequence in the 5'UTR of *GATA4* in a small family presenting with two different forms of ASD. This is the first time a mutation in the Kozak sequence has been linked to heart disease. Functional assays demonstrate reduced GATA4 translation, though the GATA4 transcript levels remain normal. This leads to a reduction of GATA4 protein level, consequently diminishing the ability of GATA4 to transactivate target genes, as demonstrated by using the GATA4-driven *Nppa* (ANF) promoter. In conclusion, we identified a mutation in the *GATA4* Kozak sequence that likely contributes to the pathogenesis of ASD. In general, it points to the importance of accurate protein level regulation during heart development and emphasizes the need to analyze the entire transcribed region when screening for mutations. © 2014 Wiley Periodicals, Inc.

Key words: GATA4 protein; congenital heart defects; Kozak sequence; atrial septal defects

How to Cite this Article:
Mohan RA, van Engelen K, Stefanovic S, Barnett P, Ilgun A, Baars MJ, Bouma BJ, Mulder BJ, Christoffels VM, Postma AV. 2014. A mutation in the Kozak sequence of *GATA4* hampers translation in a family with atrial septal defects.
Am J Med Genet Part A 164A:2732–2738.

INTRODUCTION

Congenital heart defects (CHD) are the most common developmental anomalies and a major cause of prenatal loss. Approximately 1% of all newborns are diagnosed with CHD [Hoffman and Kaplan, 2002; Van der Linde et al., 2011]. Atrial septal defect (ASD) is the most common form of CHD. It is clinically characterized by an opening in the atrial septum. ASDs can be subdivided into various types depending on the position of the opening [Rojas et al., 2010]. Sinus venosus defects are positioned cranial to the fossa ovalis and its developmental origin is uncertain [Van Praagh et al., 1994].Type II ASD usually arises from an enlarged foramen ovale and is thought to be the result of an insufficient growth of the septum secundum [Posch et al., 2010]. So far, the genetic factors involved in ASD are poorly understood. Nonetheless, several genes have been implicated in causing ASD, including transcription factor encoding genes such as *NKX2-5*, *TBX5*, and *GATA4* [Garg et al., 2003b; Posch et al., 2010].

GATA4 encodes a member of the GATA family of zinc-finger transcription factors. The GATA family is characterized by its DNA binding domain that recognizes the (A/T)GATA(A/G) motive [Molkentin, 2000]. During development and in adulthood, GATA4 is an important regulator of gene expression in a wide spectrum of tissues, the most important being the liver, gonads, small intestines, lung, and the heart [Molkentin, 2000; Peterkin et al., 2005]. In the heart, GATA4 regulates the transcription of downstream genes such as the atrial natriuretic factor (*Nppa*) and gap junction protein alpha 5 (*Gja5*), as well as genes involved in differentiation, proliferation, and apoptosis [Lee et al., 1998; Singh

Conflict of interest: none

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Article first published online in Wiley Online Library (wileyonlinelibrary.com): 5 August 2014
DOI 10.1002/ajmg.a.36703

et al., 2010]. Regulation of these genes occurs in combination with other transcription factors such as NKX2-5 and TBX5 that act as cofactors [Durocher et al., 1997; Lee et al., 1998; Garg et al., 2003b].

Translation of any gene is executed by the ribosomes which bind mRNA and translate it into the primary sequence of the corresponding protein. Within the mRNA, the Kozak sequence is involved in the initiation of translation and determines the translation efficiency [Kozak, 1987a, 1987b]. The Kozak consensus sequence differs between species, but is conserved for most genes within the species. In humans, the consensus is GCCGCC(A/G)(C/A)CAUGGCG, with the start codon being essential and other positions within the Kozak sequence acting to enhance the affinity for the ribosome, such as G +4, a purine on -3, and a G on -6 [Kozak, 1987b; Nakagawa et al., 2008]. Not surprisingly, it has been shown that mutations of the Kozak sequence can cause disease, an example being the G-base to C-base conversion of position -6 of the gene *β-globin* that leads to the blood disorder β-thalassaemia [Kozak, 2002; De Angioletti et al., 2004; Wolf et al., 2011].

In this study, we identified a mutation, a -6 G-base to C-base conversion within the Kozak sequence of *GATA4*, in a small family with four carriers, two of them affected with ASD. All family members were negative for mutations in *NKX2-5* and *TBX5*. The *GATA4* mutation was functionally analyzed, and *in vitro* assays show that the mutation severely hampers translation of GATA4 and results in a diminished transactivation of downstream target genes.

MATERIALS AND METHODS

Clinical Evaluation

The proband and available family members were clinically evaluated by analysis of medical records, physical examination with attention to syndromic features, 12-lead electrocardiogram (ECG), and two-dimensional echocardiography. The cardiologic examinations were assessed by a cardiologist who was blinded for the mutational status. This study was approved by the Medical Ethical Committee at the Academic Medical Center in Amsterdam. Written informed consent was obtained from all participants.

Mutational Screening

Genomic DNA has been isolated from blood of the patients according to standard procedures. Exons of *NKX2-5*, *TBX5*, and *GATA4* were PCR amplified according to standard methods using Hot Fire Polymerase (Solis Biodyne). Sequences of intronic primers and PCR conditions are available upon request. Sequencing was performed using the BigdyeTerminator v3.1 Kit.

Plasmid Constructs, Cell Lines and Transfections

Human *GATA4* cDNA (ENST00000532059) was PCR amplified and inserted into pcDNA3.1(+). The reporter plasmid pGL3-Nppa-luciferase has been described previously [Postma et al., 2008]. The reporter plasmid pGL3SV40 is a modified form of pGL3-basic (Promega). Mutations were introduced using site-directed mutagenesis (Quickchange, Strategene). PCR generated constructs were fully verified by sequencing. The human cell line HeLa and H10 were cultured according to standard methods. Transfections were performed using polyethylenimine (PEI) in a plasmid to PEI ratio of 1:4.

RNA Isolation and Quantitative PCR

H10 cells ($3.0^{*}10^{6}$ cells/6-wells plate) were transfected with 2.5 μg of either pcDNA3.1(+)-GATA4-wt, pcDNA3.1(+)-GATA4-mutant, or pcDNA3.1(+)-empty as a negative control. RNA was isolated using Nucleospin RNA II kit (Clontech). Isolated RNA (1 μg) was reverse transcribed using oligo(dT) primer and Superscript II RT–PCR kit (Invitrogen). Expression from the vectors and Hprt from the cells were quantified using quantitative PCR (qPCR) on a LightCycler480 (Roche). Samples were measured in triplicate. Quantification was performed using LinReg software [Ruijter et al., 2009].

Western Blot

HeLa cells ($2.6^{*}10^{6}$ cells/6-wells plate) were transfected with either pcDNA3.1(+)-GATA4-wt, pcDNA3.1(+)-GATA4-mutant, or pcDNA3.1(+)-empty as a negative control. Cells were harvested 20 hr post-transfection and lysed in lysis buffer (10 mM Tris, pH8.0; 150 mM NaCl; 1% Nonidet p-40; 10% Glycerol; 5 mM EDTA, pH8.0; ¼ Protease tablet (Roche)) for 30 min. Cell lysates were centrifuged to clear it from cell debris and protein concentration was measured using the BCA kit from Pierce. From each sample 1 mg of total protein was run on a 4% stacking and 10% running polyacrylamide gel (SDS-PAGE) after diluting with Laemmli buffer. Afterwards, the gel was blotted onto 0.45 μm polyvinylidene fluoride membrane (PVDF; immobilon P, Millipore). Pre-incubation (blocking) was performed in 2.5% milk (Campina). Immunodetection was performed using polyclonal goat-α-GATA4 (Santa Cruz), monoclonal mouse-α-GAPDH (Santa Cruz), and alkaline phosphatase (AP) conjugated secondary antibody. Incubation with primary antibody was done overnight at 4°C and secondary antibody for 2 hr at room temperature. Analysis of the images was performed by image analysis software (AIDA v4.26.038, Raytest). Results of three independent experiments were subjected to statistical analysis. $P < 0.05$ was considered significant using a two tailed Student's *t* test.

Luciferase Assay

HeLa cells in standard 12-wells plates were transfected in triplicate. 800 ng pGL3-Nppa-luciferase reporter vector was co-transfected with 1 ng pRL-CMV, as normalization control (Promega), and 125 ng of the wildtype or mutant expression vector. The wildtype and mutant modified pGL3SV40 vectors were transfected in an amount of 1 μg with 3 ng phRG-TK as normalization control (Promega). Forty eight hours after transfection, HeLa cells were harvested and Luciferase activity measurements were performed on a Glomax E9031 luminometer. Triplo transfection experiments were repeated at least three times for each condition and data were corrected for intersession variation and statistical analysis was performed [Ruijter et al., 2006]. $P < 0.05$ was considered significant using a two tailed Student's *t* test.

RESULTS

Clinical Details

At 33 years of age, the proband (II-2) was diagnosed with a sinus venosus ASD and aberrant pulmonary vein draining into the right atrium (Fig. 1a and Table I). The co-existence of these conditions is well documented [Davia et al., 1973]. The ASD was surgically corrected due to the presence of a large left-right shunt. Her medical history reported six spontaneous abortions prior to the 12th week of pregnancy, for which no cause had been found. Chromosomal analysis displayed a normal female karyotype with a chromosome 22q11 microdeletion being excluded by FISH analysis. The probands' mother (I-2) had been diagnosed with ASD type II at the age of 59 years, which was surgically closed percutaneously with a device at age 80 years because of dyspnea. She also suffered from paroxysmal atrial fibrillation (AF). The probands' father (I-1) had died of myocardial infarction at 55 years of age. Cardiologic examination of the daughters as well as the available siblings of the proband showed no evidence of CHD. Moreover, the children of III-1 also showed no evidence of CHD under cardiologic examination. The family was of Dutch descent.

Mutation Screen

Sequencing of *GATA4* in the proband revealed a mutation within the *GATA4* Kozak sequence (Fig. 1b). As described, the Kozak sequence is involved in the translation initiation process of protein synthesis [Kozak, 1987a]. The mutation is located at -6 converting a G-base residue into a C-base (-6G > C). This position just upstream of *GATA4* is highly conserved between species (Fig. 1c). The mutation is not present in either dbSNP, Genome of the Netherlands (GoNL), the 1000genome project, or the exome variant server [Sherry et al., 2001; Abecasis et al., 2012; Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) (April 2013 accessed)]. The probands' mother and two of her daughters also carried the

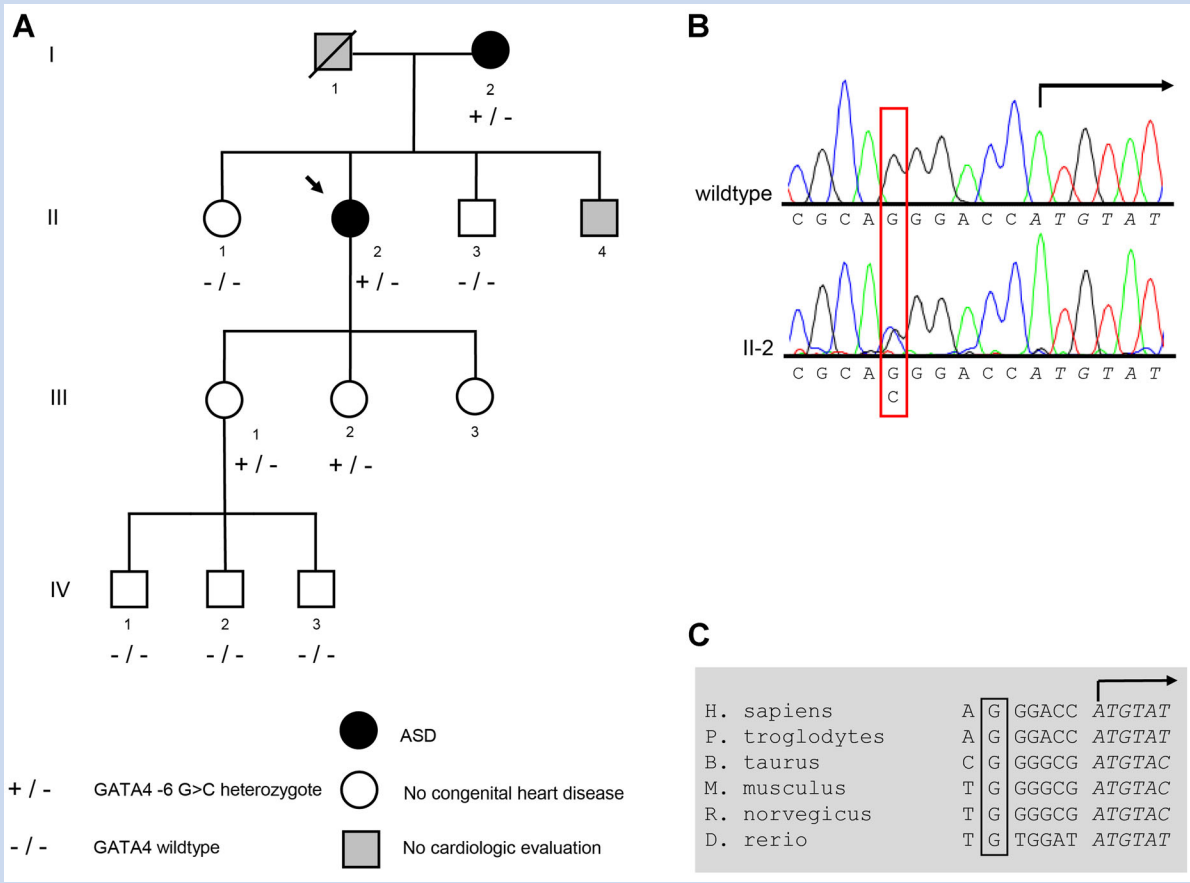

FIG. 1. ***GATA4*** **mutation segregates with familial atrial septal defect. a: Kindred with four generations indicated in Roman numerals. Carriers of the -6 G > C conversion are noted with +; individuals with wildtype *GATA4* are noted -. ASD affected in black, not affected in white and no cardiologic evaluation in gray. The arrow points to the proband b: Sequence chromatogram displaying the -6 G > C conversion in the affected proband (II-2). c: Alignment of GATA4 sequence surrounding the *ATG* start codon with various species showing the highly conserved -6 G. [Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833.]**

TABLE I. Clinical Details of the Family Members

Family member	Sex/Age (years)	Age at evaluation (years)	Mutation	CHD	Rhytm- and conduction abnormalities
I-2	F/86	80	Heterozygous	ASDII	Paroxysmal AF, RBBB
II-1	F/60	49	Absent	None	None
II-2	F/56	55	Heterozygous	Sinus venosus ASD	None
II-3	M/52	47	Absent	None	1st degree AV block
III-1	F/30	22	Heterozygous	None	None
III-2	F/27	19	Heterozygous	None	None
III-3	F/24	17	ND	None	None
IV-1	M/6	6	Absent	None	None
IV-2	M/5	5	Absent	None	None
IV-3	M/2	2	Absent	None	None

ND, not determined; CHD, congenital heart defect; ASD, atrial septal defect; AF, atrial fibrillation; RBBB, right bundle branch block.

same mutation (Fig. 1a). In addition, we screened the *TBX5* and *NKX2-5* genes in the proband; no mutations were identified.

The GATA4 -6G > C Mutation Significantly Lowers GATA4 Protein Level

Since the location of the mutation is within a sequence known to be of great importance for efficient protein translation, we first assessed GATA4 protein expression levels in both wildtype and mutant GATA4 transfected cells. GATA4-specific bands could be observed after transfection with either the GATA4-wt or –mut expression plasmids in HeLa cells (Fig. 2a), GATA4 being absent in the control. Importantly, substantially less protein was synthesized from the mutant expression plasmid in comparison with the wildtype (Fig. 2a). Quantification, using optical density, of the amount of protein normalized against GAPDH showed a significant reduction of almost 50% ($P = 1.37 \times 10^{-5}$) (Fig. 2b). In addition, it seems that a smaller GATA4 protein product is being synthesized more extensively than the full-length (based on the coding sequence) protein thereby changing the ratio between the two bands. It is known that multiple fragments can be synthesized from a single transcript, a process known as leaky scanning [Kozak, 1994]. During leaky scanning, the first Kozak sequence is skipped and translation starts from a downstream Kozak sequence. The GATA4 transcript contains three putative in frame Kozak sequences. The difference in the ratio between the two bands observed after transfection with either GATA4-wt or –mut expression plasmids would seem to emphasize weakening of the first Kozak sequence.

Since the Kozak sequence is involved in translation, but not in transcription, we hypothesized that there would be no difference in the rate of transcription from either the GATA4-wt or GATA4-mutant expression plasmid. Indeed, no difference in the amount of mRNA of either expression plasmid was observed using quantitative PCR, indicating that the mutation does not affect the level of mRNA expression (Fig. 2c).

Quantifying the Effect of the Mutation on Translation

To more accurately determine the effect of the Kozak mutation on the protein level, we employed a Luciferase assay using a modified reporter plasmid. To this end, we modified the standard pGL3-SV40 reporter by replacing the Kozak sequence of the *Luciferase* gene with the Kozak sequence of *GATA4*. The region that was replaced starts at position -9 to +6. The Kozak consensus sequence is from position -9 to +4 (Fig. 3a; Kozak, 1987b). Figure 3a shows that substituting the Luciferase Kozak sequence for the *GATA4*-wt Kozak sequence resulted in a general reduction in luciferase activity. Importantly, introduction of the *GATA4* -6G > C mutation in this construct resulted in a significant reduction of the luciferase activity (approximately 75%, $P = 2.15 \times 10^{-8}$) in comparison to the *GATA4*-wt Kozak sequence. This is in line with the observation that less GATA4 protein is produced from the mutant Kozak sequence and demonstrates that the -6G > C conversion indeed results in a significant reduction of the protein level.

The GATA4 -6G > C Mutation Significantly Lowers Transactivation of Target Genes

The GATA4 transcription factor works dose dependently [Pu et al., 2004]. Therefore, we expected a diminished transactivation of GATA4 target genes in the presence of this mutation. In order to test this, we performed a luciferase assay using the well characterized -638bp *Nppa* promoter fragment [Grepin et al., 1994; Postma et al., 2008], which is highly sensitive to GATA4. This pGL3-NPPA-luciferase reporter plasmid was co-transfected with identical amounts of either a GATA4-wt or GATA4-mutant expression plasmid. Both, the wildtype and mutant GATA4 were able to transactivate expression of luciferase. However, the GATA4-mutant plasmid showed a significantly decreased transactivation of the *Nppa* promoter ($P = 0.011$, Fig. 3b), effectively showing that a reduction in translated GATA4 directly leads to diminished target gene activation.

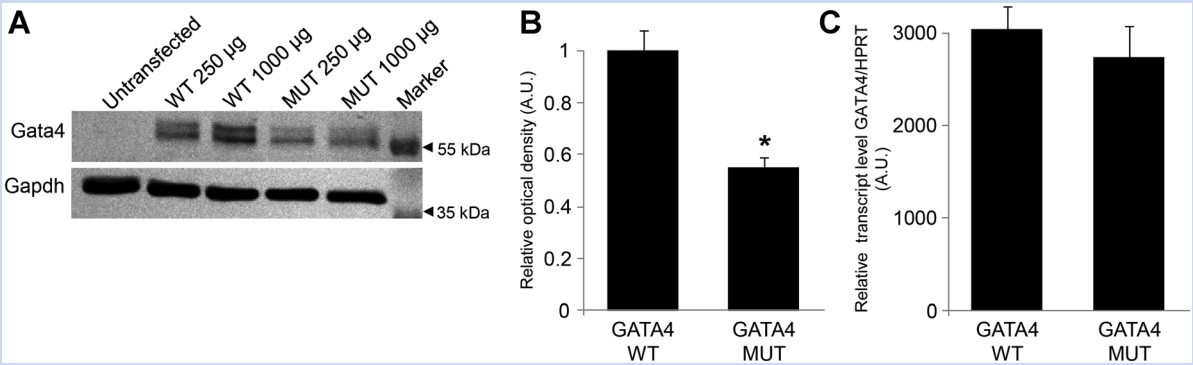

FIG. 2. The GATA4 -6 G > C mutation significantly lowers GATA4 protein level. a: Western blot on cell lysates of HeLa cells transfected with GATA4-wildtype (WT) or GATA4–mutant (MUT) expression plasmid shows less GATA4 protein due to the mutation. b: Densitometric quantification of GATA4 bands on western blot. Normalized for GAPDH bands on the same western blot. Significantly, less GATA4 protein is synthesized from mutant expression plasmid compared to wildtype. Student's *t* test, $P < 0.05$. Values are expressed as average ± SEM, representing independent transfections (n = 12) c: Transcription levels of GATA4-wildtype or GATA4–mutant expression plasmid are not different. Normalized for Hprt. Values are expressed as average ± SEM, representing 2 independent transfections (n = 2).

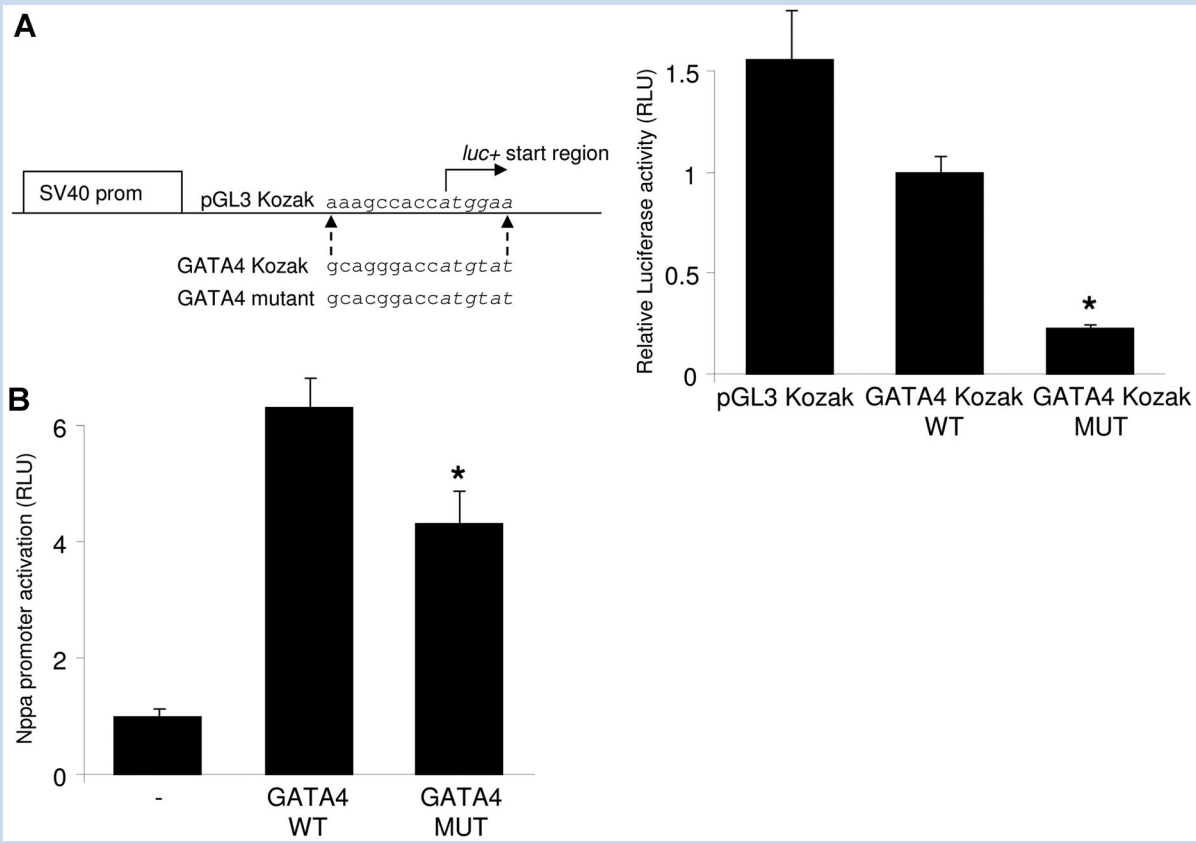

FIG. 3. GATA4 translation is severely hampered resulting in diminished activation of *Nppa* promoter. a: The pGl3-SV40 reporter is modified by replacing the region surrounding the *ATG* (-9 to +6) of the *Luciferase* gene by the same region of the human *GATA4* gene. Relative activity of modified pGl3-SV40-luciferase constructs. Replacing the Kozak sequence of *Luciferase* gene by the Kozak sequence of human *GATA4* lowers the activity. Mutation reduces the activity +/− 4-fold. Student's *t* test, $P < 0.05$. Values are expressed as average ± SEM, representing three independent transfections each in triplo (n = 9). b: Transcriptional activation of *NPPA* promoter luciferase constructs by GATA4 protein from wildtype and mutant expression plasmids. Mutant expression plasmid compared to wildtype has significantly less activation of *NPPA* promoter luciferase. Student's *t* test, $P < 0.05$. Values are expressed as average ± SEM, representing 3 independent transfections (n = 8).

DISCUSSION

We have identified a novel mutation within the Kozak sequence of *GATA4* in a patient with a sinus venosus ASD. This mutation was also present in her family, where three additional members are carriers of the mutation. The mother of the proband, a carrier, was diagnosed with ASD type II and suffered from paroxysmal AF. The two other carriers showed no CHD. Our *in vitro* assays demonstrated that the mutation decreases translation efficiency which lead to lower GATA4 protein levels and diminished transactivation of GATA4 target genes.

GATA4 encodes for the zinc finger transcription factor GATA4 [Molkentin, 2000]. During development, it plays an important role in many tissues. In the heart, GATA4 is necessary for correct development and homeostasis [Molkentin, 2000; Peterkin et al., 2005]. To date, many mutations in the coding region of *GATA4* have been identified that affect the function of the GATA4 protein, resulting in a wide spectrum of CHD, including ASD type II and a sinus venosus ASD, as seen in our family [Garg et al., 2003b; Rajagopal et al., 2007; Posch et al., 2010]. Thus far, no mutations within the *GATA4* Kozak sequence have been described.

The Kozak sequence is involved in the initiation of translation and determines the translation efficiency. A spectrum of diseases, including cancer and autoimmune disease, have been associated with Kozak sequence mutations [Cooper, 1993; Kozak, 2002; Wolf et al., 2011]. To date, over 40 mutations are known, and have been shown to occur in every position (-6/+4) within the Kozak sequence [Wolf et al., 2011]. The mutation reported in our family occurs at position -6 at which two mutations have been previously identified. One of these is a -6 G > C conversion in the *β-globin* gene, that has been linked to β-thalassaemia, a disease known to be caused by defects in post-transcriptional modifications [De Angioletti et al., 2004]. The other is a -6 C > A conversion in the *leukaemia inhibitory factor* gene, this mutation being associated with infertility in females [Giess et al., 1999]. Corroboration for the importance of a G on position -6 comes from computational analyzes of the Kozak sequence of 10,012 human mRNAs. The resulting consensus sequence shows the highest prevalence for a G with a very low prevalence for a C [Nakagawa et al., 2008]. Therefore, it is likely that a mutation at the -6 position can impair translation efficiency.

Our experiments indicate that the mutation in the Kozak sequence of *GATA4* affects the amount of GATA4 protein. Mouse studies have shown that a reduction in the amount of GATA4 protein can lead to CHD, including ASD as well as ventricular septal defects (VSD), atrioventricular septal defects (AVSD), right ventricle hypoplasia and cardiomyopathy [Pu et al., 2004; Rajagopal et al., 2007]. However, the severity- and the spectrum of defects resulting from a reduction in Gata4 protein in mice is strongly dependent on the genetic background, as 24% of C57BL6/J mice and 70% of FVB mice carrying a Gata4 mutation show no phenotype [Rajagopal et al., 2007]. Likewise, the initial study in which human *GATA4* mutations were linked to CHD demonstrated that the same mutation in one family can lead to a large spectrum of defects including ASD and VSD, but, importantly, also included one carrier without CHD [Garg et al., 2003a]. Taken together, it is likely that genetic modifiers ultimately determine the phenotype in the presence of a *GATA4* mutation, including non-penetrance. A similar mechanism seems to occur in the family described here, as four members are carriers of the -6G > C conversion, but only two of them have a CHD. We therefore hypothesize that this is caused by an incomplete penetrance of the mutation and likely depends on the genetic background, such as variant risk allele combinations of the individual, as demonstrated in mice.

Taken together, we believe it is plausible that the -6 G > C conversion in the *GATA4* sequence has a pathogenic role in the formation of ASD for the following reasons: (1) the mutation seems to associate with ASD; (2) the mutation is absent in all published exomes of healthy individuals (>6000); (3) the mutation occurs at a highly conserved position within the Kozak sequence; (4) the mutation severely hampers translation of GATA4; (5) the mutation results in a diminished transactivation of downstream target genes; and (6) several mutations within the Kozak sequence and more precisely the -6 position have previously been associated with disease.

In conclusion, in this study we identified a mutation within the Kozak sequence of *GATA4* in a small family presenting with ASD. It demonstrates that incorrect regulation of GATA4 protein levels may contribute to ASD. Additionally, it reinforces the notion that the entire transcribed sequence of a gene needs to be analyzed when screening for mutations, as mutations outside the coding regions can also have deleterious and pathogenic consequences.

ACKNOWLEDGMENT

We would like to thank the family for their participation in our study.

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