European Journal of Human Genetics (2009) 17, 344 – 351

www.nature.com/ejhg

ARTICLE

Mutations in mammalian tolloid-like 1 gene detected in adult patients with ASD

Paweł Stańczak[1], Joanna Witecka[2], Anna Szydło[2], Ewa Gutmajster[2], Małgorzata Lisik[2], Aleksandra Auguściak-Duma[2], Maciej Tarnowski[2], Tomasz Czekaj[2], Hanna Czekaj[2] and Aleksander L Sieroń*[,2]

[1]*Swietokrzyskie Center for Cardiology, Regional Hospital, Kielce, Poland;* [2]*Department of General and Molecular Biology and Genetics, CoE for Research and Teaching of Molecular Biology of Matrix and Nanotechnology, CoE Network, BioMedTech 'Silesia', Medical University of Silesia, Katowice, Poland*

Atrial septal defect (ASD) is an incomplete septation of atria in human heart causing circulatory problems. Its frequency is estimated at one per 10 000. Actions of numerous genes have been linked to heart development. However, no single gene defect causing ASD has yet been identified. Incomplete heart septation similar to ASD was reported in transgenic mice with both inactive alleles of gene encoding mammalian zinc metalloprotease a mammalian tolloid-like 1 (*tll1*). Here, we have screened 19 ASD patients and 15 healthy age-matched individuals for mutations in *TLL1* gene. All 22 exons were analyzed exon by exon for heteroduplex formation. Subsequently, DNA fragments forming heteroduplexes were sequenced. In four nonrelated patients, three missense mutations in coding sequence, and one single base change in the 5′UTR have been detected. Two mutations (Met182Leu, and Ala238Val) were detected in ASD patients with the same clinical phenotype. As the second mutation locates immediately upstream of the catalytic zinc-binding signature, it might change the enzyme substrate specificity. The third change, Leu627Val in the CUB3 domain, has been found in an ASD patient with interatrial septum aneurysm in addition to ASD. The CUB3 domain is important for substrate-specific recognition. In the remaining 15 patients as well as in 15 reference samples numerous base substitutions, deletions, and insertions have been detected, but no mutations changing the coding sequence have been found. Lack of mutations in relation to ASD of these patients could possibly be because of genetic heterogeneity of the syndrome.
European Journal of Human Genetics (2009) **17,** 344–351; doi:10.1038/ejhg.2008.175; published online 1 October 2008

Keywords: atrial septal defect; DNA sequencing; gene mutations; heart defects; heteroduplex analysis; mammalian tolloid-like 1

Introduction

Congenital heart defects in general are related to aberrant development of the circulation system in a fetus. The defects might result either from factors damaging the fetus during development or they can be of genetic background. Some environmental factors, drugs, alcohol abuse, and infections contracted by the mother before, or during pregnancy (eg, rubella) may alter heart development.[1–6] However, environmental and maternal factors have been linked to approximately 2% of congenital heart defects.[7] In a significant number of heart abnormalities coexistence of a few anomalies has been shown in different parts of the circulation system indicating genetic factors.[1,8]

***Correspondence: Dr AL Sieron, Department of General and Molecular Biology and Genetics, Medical University of Silesia in Katowice, ul. Medyków 18, Bldg. C-1, 40-752 Katowice, Poland.**
Tel: +48 32 208 8394; Fax: +48 32 208 8382;
E-mail: alsieron@sum.edu.pl
Received 5 May 2008; revised 16 July 2008; accepted 29 August 2008; published online 1 October 2008

Chromosomal anomalies and single gene defects, however, could be attributed to the background of 8% of congenital heart defects.[7,9–11] Thus, the background of most of the congenital heart defects is still poorly understood.

The heart itself and septation of the large vessels begin at the fourth week of fetal development, but the process is not completed until shortly after birth. Septation anomalies are linked to abnormal development of endocardial cushions. They are necessary for completeness of interatrial as well as interventrical septa. Incomplete interatrial septum is a common feature of such genetic disorders as Noonan, Turner, and Holt-Oram syndromes.[10–12]

The condition studied here is called atrial septal defect (ASD) and has some features common with the other just listed heart-related syndromes. Currently, the defects related to ASD are corrected during puberty. However, in the past, when it was untreated, the patients often suffered from numerous severe cardiovascular and pulmonary complications.

In recent years, results of research with the use of transgenic mice models revealed that a gene named tolloid-like 1 (*tll1*) is critical for septa development in the mouse heart.[13] The mice lacking active TLL1 died at mid-gestation because of severe blood circulation failure. The *tll1* gene product is a metalloprotease with domain structure identical to one of the alternatively spliced variants of the *bmp-1* gene product, mammalian tolloid (mTLD). The mTLD was detected in mammals and some other invertebrates.[14–18] The homologous protein BMP-1 was originally reported by Wozney *et al*[19] in a context of ectopic bone formation in rats when protein extracts from demineralized bovine bones were used.[20] Subsequently, based on protein domain structure it was classified in the family of astacin, a group of metalloproteases involved in embryo development of different species.[17,21–23] It is also commonly accepted that BMP-1 is important for numerous physiological activities acting in seemingly unrelated processes, such as extracellular matrix quality control involving formation and activation of numerous factors that act in the regulation of different genetic programs.[17–21,23–32]

An investigation of different mouse model with ventrical spetum defect (VSD) but with active *tll1* gene indicated that a different mechanism must be involved, possibly related to abnormal cell proliferation and differentiation.[33,34] Developmental defects of $tll1^{-/-}$ embryos seemed to be exclusively restricted to the heart.[13] The comparison of the defects with expression of the *tll1* revealed, in normal mice embryo, its elevated expression levels during heart septation. Thus, it is possible that the expression of *TLL1* in humans could also be critical for correct heart positioning and its proper septation.

Here, we have tested 19 adult individuals with clinically confirmed ASD by karyotyping and screening of the *TLL1* gene for mutations by heteroduplex detection followed by DNA sequencing.

Materials and methods

Patients

Hospital records of more than 100 cardiological patients have been reviewed for ASD cases. Twenty individuals have been identified who fulfill the requirements of this project. All of them were informed about procedures related to the study. The procedures were conducted in accordance with the Institutional Guidelines and Regulations and with the Guidelines for the Conduct of Research Involving Human Subjects approved by the appropriate institutional review board (IRB) Bioethical Committee of the Silesian Medical University in Katowice, Poland, No. L.dz.NN-013-339/03. In addition, appropriate informed consent was obtained from the human volunteers.

Finally, 19 individuals agreed to participate in the study and they all have been surveyed for medical and family health history. On the basis of the survey's results and medical records they were divided into three groups (Table 1).

As a reference, blood samples from 15 age-matching volunteers (11 females and 4 males) were used to isolate DNA for genetic analyses. None of the volunteers have had inherited developmental heart conditions.

Samples collection and handling

From each patient two blood samples were drawn. One 3 ml sample was mixed with heparin and used for cytogenetic diagnosis to exclude possible chromosomal anomalies. The methods for lymphocytes isolation and preparation for karyotyping were the classical methods described before.[35,36] Slides were examined with a Zeiss Axioskop microscope. Images were obtained using a Sony ExwaveHAD digital camera and MultiScanBase software (Computer Scanning System Ltd., PL) with the karyotype option, enabling manual karyotyping. For each patient, a total of 15 metaphases in each of five prepared karyograms were examined to determine the number of chromosomes.

The second sample of blood (5.0 ml) was mixed with EDTA and used for the isolation of genomic DNA.

PCR conditions

The DNA for mutation detection was isolated with the use of commercial blood mini-kit (A&A Biotechnology, Gdansk, Poland) according to supplier instructions. The quality of purified genomic DNA was verified by spectrometry at $A_{260/280}$ and agarose gel electrophoresis.

Fragments of the *TLL1* gene were amplified using pairs of primers designed to complement intronic sequences flanking coding sequences (Table 2). The conditions for amplification PCR were as follows: step (1) denaturation at 95°C for 4 min, step (2) denaturation at 95°C for 30 s,

Table 1 Classification of ASD patients based on their individual clinical symptoms

Patient number	*Sex (F/M)*	*Patient's age (years)*	*Additional defects*
Group 1: ASD without additional defects			
3	M	51	ND
5	M	55	ND
7	M	71	ND
10[a]	M	35	ND
11	M	52	ND
15	F	57	ND
18	F	52	ND
Group 2: ASD with diagnosed aneurysm of interatrial septum and other defects			
1	M	70	Aneurysm, heart attack, cerebral stroke, atrial fibrillation, and cardiac pacemaker implantation
4	M	55	Heart attack
6	M	45	Hypertrophic cardiomyopathy
9	M	72	Aneurysm of interatrial septum sinus bradycardia
12	F	58	Asymptomatic aneurysm of interatrial septum
13	F	48	Aneurysm of interatrial septum
17	F	48	Asymptomatic aneurysm of interatrial septum
19	F	62	Artificial mitral valve implantation
Group 3: ASD with irregular heart beating rhythm			
2	M	52	Atrial fibrillation, cardiac pacemaker implantation
8	M	51	Atrial fibrillation, cardiac pacemaker implantation
14	F	56	Cardiac pacemaker implantation
16	F	67	Atrial fibrillation

ASD, atrial septal defect; F, female; M, male; ND, Not determined.
[a]This patient was only the one who was classified with ASD type I. All the other patients had ASD type II.

step (3) annealing at the appropriate primer pair melting temperature (T_m) (for details see Table 2) for 30 s, step (4) elongation at 72°C for 1 min per each 1250 bp of the amplified DNA fragment. The procedure was repeated 34 times from steps (2) to (4). Following the last annealing, the elongation time at 72°C was extended up to 7 min. The PCR products were purified using method provided in the sequencing manual by Applied Biosystems. The DNA concentration was determined spectrophotometrically at A_{260}. The reference DNA was isolated from the blood of healthy individuals. The DNA reference fragments were obtained with the corresponding primers under the conditions used for patients' samples and subjected to heteroduplex analysis and DNA sequencing, when necessary except for the exons with mutations detected in patients that were sequenced entirely.

Heteroduplex formation and analysis

Equal amounts (200 ng) of each, the reference and corresponding patients' DNA fragments, in 4 μl were mixed in the final volume of 10 μl supplemented with one-tenth volume of 0.5 M KCl, and nuclease-free water when needed. The samples were handled according to the protocols recommended by the Transgenomic Inc., as follows: melting of the DNA at 95°C for 2 min and then cooling the mixture from 95 to 85°C at the rate 2.0°C per second and from 85 to 25°C at the rate 0.5°C per second. Subsequently, the mixture was subjected to the enzymic assay using Surveyor kit (Transgenomic Inc.). Briefly, the 400 ng of hybridized DNA in the volume 4–15 μl was mixed with 0.5 μl of Surveyor Nuclease S and 0.5 μl Surveyor Enhancer, and water, when needed, and incubated at 42°C for 40 min. The cleavage was terminated by mixing the sample with one-tenth volume of the Stop Solution supplied by the manufacturer in the assay kit. To analyze the Surveyor Nuclease S digestion products by agarose gel electrophoresis one-sixth volume of a 6X loading dye buffer (10 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50% (w/v) sucrose, 0.15% (w/v) bromophenol blue) was added to each sample. For each digestion/loading dye mixture, the entire volume of sample was loaded onto an agarose gel of an appropriate percentage (1.5–1.7%) and separated electrophoretically. The mixture of λ DNA cleaved with *Hin*dIII and ϕX cleaved with *Hae*III (Finnzymes) was used as the DNA length marker. The gels were developed at 5 V/cm until the bromophenol blue had reached two-thirds of the length of the gel. To visualize the bands, an illumination of the gel using a UV transilluminator (250–300 nm) was performed and the pictures were taken.

DNA sequencing

Samples revealing heteroduplex formation with the corresponding fragment of reference DNA were subjected to

Table 2 PCR primers and conditions used for amplification and sequencing of the *TLL1* gene

Exon number	*Primers positions from–to*	*Product length (bp)*	*Primer sequence (length in bp)*	*Primer annealing temperature (°C)*
1	305–1626	1322	CACgTACATCATCTATTAATTgCTgTTTgC (30) TCCCACTggCACAgAACTCTAgTCAACTg (29)	58
Int. 1	644–1626	983	TgCAggCTTTTAAggTCTCgCggCgTAg (28) TCCCACTggCACAgAACTCTAgTCAACTg (29)	62
2	116 547–118 357	1811	ggTAgCTAATATTTgTCCTgTAAC (24) CTCTACTgTCCAgAATgCgTTgTC (24)	60
3	119 808–120 855	1048	gCAgAACAACTTgCTgTCATTCTATgTAg (29) ATgTACTTCAAgCATTTAATTAgTAATCCATC (32)	52
4 and 5	121 553–122 696	1144	gCTTTTgTTCAAgTATgTAATATAACTgAA (29) ACTgTgATCAAAgAATACACATACCTAgTAT (31)	51
6	130 574–131 341	768	ggATACCAgAAACTAgTCATATgTAgTAAT (30) CCTggTATAACACAgATTCCTATggTA (27)	52
7	135 107–135 828	722	CAATATgATggTAgAgTgAAgACAgATgC (29) ACACAgTATAAAgCACAAgTCgCCTAgATT (30)	65 (−0.5°C per cycle)
8	141 561–142 212	652	GAATgTCATCATTgAgTTTATACTAg (26) ATCATgCAACTATTTAATTAATgACTATCA (30)	50
9	152 432–153 304	873	ATTTCTCTTATAATCTTATTCggTgATTCAC (31) TCTAACCTCTTCATATggTCgTCTAg (26)	51
10	166 465–167 014	550	ACTTCTCCTgACATTgTAAAACAgTTA (27) CCTAgTATgTACCTCATAAgTTAAgCTAg (29)	50
11	169 104–169 695	592	CATCTTACTTCTACATATggTAACAATA (28) gATATTATTACATCACTgATACTAgACAA (29)	50
12	170 467–171 296	830	CTACATTACAACCAgTCAACTTTgAT (26) CTgACCCgTCATCTAATAACACAAC (25)	58
13	182 254–183 708	1455	ggATATTACAgTgTACgTTCATgAgAAACA (30) gACAggATACTCCATAATCTCACTCTTCA (29)	51
14	184 178–184 802	625	gCAgCAACATgAATTTgTATACgTTATCCA (30) ATCAgCTgTgACTgTgAATTgAgTCTTACT (30)	52
15	187 056–187 662	607	TCTAAAgATAAgAAAACTgAgACCTAgA (28) TgAgCAgTAAGACAAAgAAAgAATACAATg (30)	50
16	192 768–194 033	1266	TggACAAgCTATATAAAAATCTCTgAACT (29) ACAAACCCTAgACATTgTAAATCTCAT (27)	58
17	201 974–202 547	574	gAggATTACAAgTTATTATCgTAgCg (26) CTAATgCCATCTATgTgCgAAgTAC (25)	53
18	204 748–205 588	841	ATTCAAgTgTATATTTAACTAAgAgAgA (28) CAggATAAgAAACCCTAgAggAgAACAACg (30)	50
19	218 070–218 860	791	AggATAATgTTCAACgggTTCTATgAT (27) CAAAgATCTTTAggTAggATgCTgTATggT (30)	50
20	226 477–227 111	635	CCAgTTgTggAAAACATATCTgACg (25) gAAATTATACCTACTgATTTCATCggT (27)	58
21/22	227 995–231 411	3417	gACAAgTATTCCCAAACATgCgTTAC (26) ACTgTgACATATTAAAATgAgCgACA (26)	57
Int. 21	227 995–229 978	1984	gACAAgTATTCCCAAACATgCgTTAC (26) gTTgTATgCTCATggACAggTAACAgAC (28)	55

Int., primer for starting sequencing PCR inside of exons 1 or 21.

DNA sequencing.[37] Sequencing PCR was conducted using reagents supplied by Applied Biosystems under conditions recommended by the manufacturer. Briefly, the PCR products were purified by ethanol precipitation. The precipitated DNA was solubilized in the Hi-Di Formamide (Applied Biosystems) denatured at 95°C for 5 min, cooled and analyzed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The sequence obtained was verified using the ChromasPro software. Sequence alignment was obtained with the use of MegAlign software in DNA-Star package v. 5.0, Lasergene. The reference sequence for *TLL1* used here was AC097502.3.1.168254 (GenBank).

Results

As no chromosomal anomalies were detected by G-banding karyotype analysis in any of the ASD patients, further analyses focused on heteroduplex detection and subsequent DNA sequencing.

Heteroduplex formation frequency

All amplified PCR fragments were subjected to the DNA heteroduplex formation analysis. In two fragments containing exons 3 and 11, no heteroduplex formation was detected in any of the ASD individuals as well as in all 15 reference samples.

In all patients, mismatches were detected in gene fragments containing exons 1, 5, 12, 14, 19, and 20. The DNA fragments containing exons 2, 4, 6–10, 13, 15–18, 21, and 22 revealed various changes regarding the number of formed heteroduplexes as well as the number of mismatches in a single fragment. However, in 122 (34%) heteroduplexes found in patients, as well as in all heteroduplexes detected in reference samples, their sequencing excluded mismatches, indicating that the heteroduplex detection method also generated false-positive results. The remaining 66% of the heteroduplexes detected in patients resulted from changes located mostly in the intronic sequences. Some changes were located within the distance of 50 bp from the putative RNA splicing sites. Finally, 30 changes in the introns were confirmed by sequencing. They were substitutions: A to G (13), G to A (14), T to G (1), A to T (1), and C to T (1). The remaining changes detected in the heteroduplexes could not be verified by sequencing, as they were probably located in regions out of readable sequences.

Potential polymorphic changes in ASD patients

In the DNA gene fragment containing the exon 19 in six patients from group 1, in three patients from group 2, and in two patients from group 3 as well as in control sample, a polymorphic change A to G at the position 218 697 was detected. Another polymorphic change of T to C at position 218 734 of the same fragment was found in four patients from group 1, in three individuals from group 2, in three patients from group 3, and in the control sample. In addition, a polymorphic change in two patients, one from group 1 and the other one from group 2 as well as in the reference sample was identified at the position 218 441 (T to A). There was also a single base deletion (T del. 218 443) in four patients from group 1, in two individuals from group 2, and in two patients from group 3. Double-base insertion (TA ins. 218 328–9) has been detected in four patients from group 1, in two patients from group 2, and in two patients from group 3.

Mutations changing sense of codons

In all examined exons 4 changes detected by heteroduplex analysis were confirmed by DNA sequencing (Figure 1).

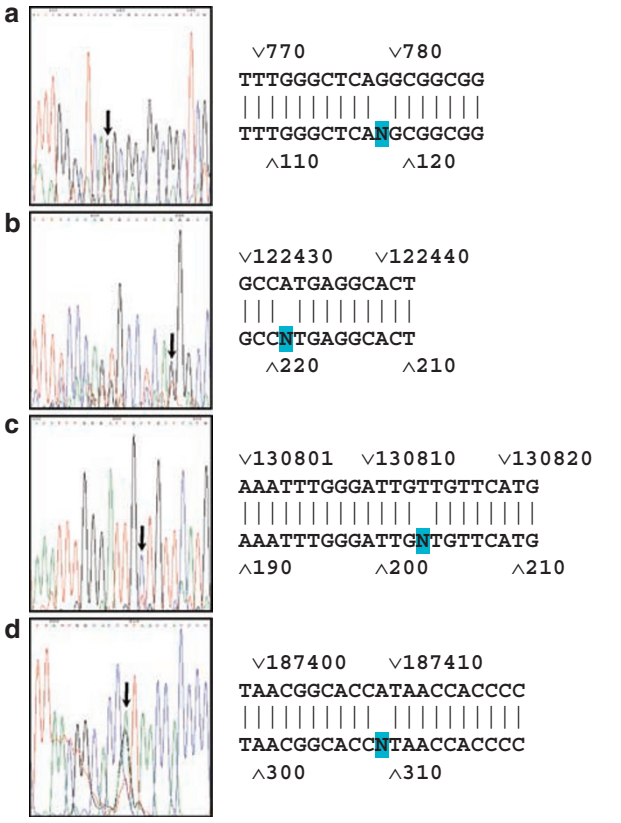

Figure 1 Heterozygous mutations detected in ASD patients. (**a**) A change G:G/T at position 779 detected in patient no. 17 from group 2 with ASD, aneurysm of interatrial septum and other defects. (**b**) A change A:A/C at position 122 433 (1191 in cDNA) detected in patient no. 10 from group 1 with ASD symptoms alone. In the presented case, the sequence was obtained with the use of reverse primer to sequence the fragment starting at the position 121 553 (exons 4 and 5). (**c**) A change T:C at position 130 814 (1360 in cDNA) detected in the patient no. 1 from group 2 with ASD, aneurysm of intersinal septum and other defects. (**d**) A change A:A/G at position 187 409 (1885 in cDNA) detected in the patient no. 9 from group 2 with ASD, aneurysm of intersinal septum and other defects. Arrow in each panel indicates the detected changes.

The DNA fragment containing the exon 1 (305–1625) from one patient from group 2 revealed one mismatch (G/T) in the 5′UTR at position 779 (Figure 1a). The change, however, is not located within the Kozak consensus sequence and it was earlier annotated as SNP (GenBank – AC097502.3.1.168254).

In the fragment containing exon 5, a heterozygous base substitution A:C at position 122 433 (Figure 1b) (1191 in the cDNA; NM_012464) changing a codon for Met to codon for Leu at position 182 was detected. Another mutation was detected in the fragment containing exon 6 in one patient from group 2. It was a change from T to C at position 130 814 changing valine 238 to alanine (Figure 1c). The last change detected during study reported here was a mutation in the gene fragment covering exon

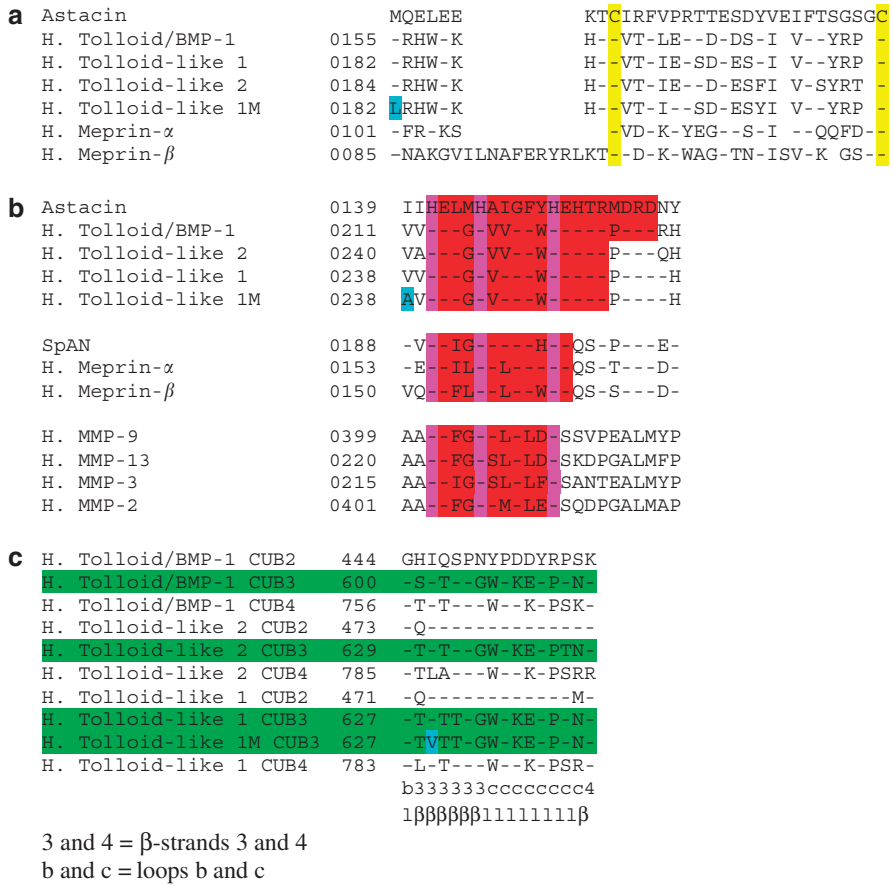

Figure 2 Comparison of conserved amino acid sequence domains mutated in TLL1 with the corresponding domains in selected metzincins. (**a**) *N*-terminal end of the catalytic domain, (**b**) active centers of selected metalloproteases from astacin family and other selected metzincins. (**c**) *N*-terminal end of the CUB3 domain. Symbols: H, amino acid sequence of human polypeptides; M, mutated TLL1; dashes, identical residues as in the polypeptide aligned at the top of the alignment. Colors: yellow, conserved cysteines; pink, histidines coordinating zinc in the active center of the enzyme; red, amino acid sequence in extended catalytic center; green, CUB3 domains in different enzymes; blue, mutations detected in ASD patients.

15 in one patient from group 2 at position 187 409 (2532 in cDNA; NM_012464) changing A to G and resulting in a codon 629 for isoleucine being substituted to codon for valine (Figure 1d).

Discussion

Recently, numerous reports described mutations at the level of single genes, including *NKX 2.5* and *GATA4* in relation to heart developmental anomalies.[38] Therefore, the lack of structural and numerical chromosomal abnormalities observed here is not surprising. Lack of mutations in exons 3 and 11 indicates that the structural parts of the protein encoded by these exons must be vital for the development. Indeed, the exon 3 encodes amino acids building central portion of the latency propeptide. Thus, we postulate that any change in this part of the peptide could alter the structure by the mean of its miss folding, which in turn could lead to serious problems in specific activation of the enzyme by putative furins.[14] Retention of the latency peptide would be a reason for the lack of activity of TLL1.

The exon 11 encodes amino acids 421–460 being a part of the CUB1 domain. For proteinases homologous to TLL1, such as mTld and BMP-1, it has been shown that the CUB1 domain is critical for their secretion from the cells.[39,40] Lack of this domain or shuffling it into a different part of the recombinant protein led to the retention of such an aberrant product in the cytoplasm.[39] Therefore, it is possible that mutations in the exon 11 would cause a complete lack or at least poor secretion of TLL1 in a human and to a natural abortion of the embryo because of the developmental abnormalities as has been reported for transgenic mice null for *tll1*.[13]

The mutation in codon at position 182 may affect conversion of the zymogen to the active enzyme. The methionine 182 is the first residue in the catalytic domain, which is conserved among all the astacins including maprins α and β (Figure 2a). It is also the first amino acid in the sequence with positions conserved for two cysteines

in all known members of the astacin family (Figure 2a). Therefore, mutation of the hydrophobic residue such as Met182 to an aliphatic Leu residue, which occupies place in the first α helix of the catalytic domain[41] might have a consequence in altering the structure of catalytic domain and affect the activity of TLL1 on its native substrate, chordin.

Another mutation caused a change of residue at position 238, which is located within the catalytic domain, just before the zinc-binding signature (Figure 2b). The structure of this region is critical for substrate accommodation of the catalytic cleft. This cleft in all tolloids is deeper than the cleft in astacin.[42,43] A deeper cleft limits substrates that may fit into the active center of the enzyme to hydrolyze the specific peptide bound.[42,43] The conserved two residues preceding the zinc-binding signature in proteases classified in astacin family are always isoleucine or valine at first position and isoleucine, alanine, valine, glutamic acid or aspartic acid at second position. In other metzincins, such as MMPs, the two corresponding residues located immediately upstream of the zinc-binding signature are alanines. Substitution of the two Ile residues in astacin and mTld by valine and alanine in both TLLs might be related to change in substrate specificity of these metalloproteases. Except for MMPs the alanine is always present in the second position after the valine. In MMPs that have a broad spectrum of substrates, the two alanines, that are less bulky than valines, do not interfere with the accommodation of various substrates in the cleft of the active center. Thus, the change in the substrate specificity of TLL1 resulting from substitution of the bulkier valine by smaller alanine could allow competition for the binding of nonspecific substrates to the catalytic cleft. This, in turn, could result in lower enzymic efficacy of the mutated TLL1 in the regulation of heart septation during embryonic and fetal development, which would finally lead to the permanent ASD condition.

The CUB3 domain is the only domain present in all *BMP-1* gene products as well as in both TLLs. A comparison of the sequence flanking the mutated residue in TLL1 shows almost complete conservation of the motif GX(I/L)T(S/T)PGWPKEYPNK (Figure 2c). This signature is also highly conserved among CUB2, 3, and 4 domains in mTld and TLLs with Ile preserved in all the CUBs except in CUB4 of TLL2. Such conservation indicates for functional importance of the peptide in the protein structure. The significance of the change in CUB3 of Val for Ile needs to be verified by obtaining such mutated enzyme and assaying its substrate specificity. Earlier reports indicated that intact structure consisting of CUB3 with both flanking EGF-like motifs are required in mTld for maintaining its activity on specific substrate.[39,40]

The analysis of the *TLL1* is, at present, time and labor consuming. The gene consists of 231 784 bp divided into 22 exons. The cDNA is 6654 bp long but the ORF consists only 3042 bp and it encodes a polypeptide of 1013 amino acids long. The 5′ upstream sequence of 647 bp is a large 5′UTR with four methionine codons of an unclear function. The STOP codon is followed by a 230 bp sequence reach in A and T stretches. Most of the coding sequence of this gene is enclosed in two large exons, E1 (816 bp) and E21/22 (3100 bp). The remaining sequence of 2738 bp is divided into 19 short exons of similar length. There is also a large intron 1, covering more than half of the gene sequence (116 123 bp). Its role has yet to be clarified.

Our observations also require, further epidemiological studies on larger and clinically more uniform group of ASD patients to determine the population frequency of mutations in TLL1 gene. The reason that we did not detect defects in all our ASD patients could be explained by the fact that some patients might have mutations in different genes known to be involved in heart development. In addition, familial cases of ASD with *TLL1* mutations are yet to be detected in larger population studies that already have begun.

Finally, we have encountered serious problems in obtaining good quality DNA sequence data from the first half of exon 1 and from the large 3′ region of exon 21/22. Thus, it is still possible that in some patients there could be mutations resulting in frame shift and/or nonsense codon mutations. In addition, the promoter and other regulatory regions that we did not sequence in this study could contain mutations affecting gene expression levels. However, here, we have shown for the first time that mutations in *TLL1* are linked to the same human syndrome, which was originally generated in transgenic animals by knocking out the gene.[13] Thus, our results indicate that in some patients with ASD, the genetic causes of the syndrome are heterozygous mutations in *TLL1*.

Acknowledgements

The study was supported in part by the Grant no. 2P05D 059 27 from the Ministry of Sciences and Education, and by institutional funds NN-1-006/03, NN-4-042/04 and NN-1-014/06.

References

1 Kahler RL, Braunwald E, Plauth Jr WH, Morrow AG: Familial congenital heart disease: familial occurrence of atrial septal defect with A-V conduction abnormalities, supravalvular aortic and pulmonic stenosis, and ventricular septal defect. *Am J Med* 1966; **40**: 384–399.
2 Boughman JA, Berg KA, Astemborski JA *et al*: Familial risks of congenital heart defect assessed in a population based epidemiologic study. *Am J Med Genet* 1987; **26**: 839–849.
3 Van Mierop LHS: Embryology of the atrioventricular canal region and pathogenesis of endocardial cushion defects; in Feldt RH, McGoon DC, Ongley PA, Rastelli GC, Titus JL, Van Mierop LHS (eds): *Atrioventricular Canal Defects*. Philadelphia, PA: WB Saunders, 1976, pp 1–12.
4 Hoffman JI: Congenital heart disease: incidence and inheritance. *Pediatr Clin North Am* 1990; **37**: 25–43.

5 Fyler DC: Atrial septal defect secundum; in Fyler DC (ed): *Nadas Pediatric Cardiology*. Boston, MA: Mosby Year Book Inc., 1992, pp 513–524.
6 Brand A, Keren A, Branski D, Abrahamov A, Stern S: Natural course of atrial septal aneurysm in children and the potential for spontaneous closure of associated septal defect. *Am J Cardiol* 1989; **64**: 996–1001.
7 Zetterqvist P, Turesson I, Johansson BW, Laurell S, Ohlsson NM: Dominant mode of inheritance in atrial septal defect. *Clin Genet* 1971; **2**: 78–86.
8 Pease WE, Nordenberg A, Ladda RL: Familial atrial septal defect with prolonged atrioventricular conduction. *Circulation* 1976; **53**: 759–762.
9 Li Volti S, Distefano G, Garozzo R, Romeo MG, Sciacca P, Mollica F: Autosomal dominant atrial septal defect of ostium secundum type. Report of three families. *Ann Genet* 1991; **34**: 14–18.
10 Benson DW, Basson CT, MacRae CA: New understandings in the genetics of congenital heart disease. *Curr Opin Pediatr* 1996; **8**: 505–511.
11 Johnson MC, Hing A, Wood MK, Watson MS: Chromosome abnormalities in congenital heart disease. *Am J Med Genet* 1997; **70**: 292–298.
12 Basson CT, Cowley GS, Solomon SD *et al*: The clinical and genetic spectrum of the Holt-Oram syndrome (heart-hand syndrome). *N Engl J Med* 1994; **330**: 885–891.
13 Clark TG, Conway SJ, Scott IC *et al*: The mammalian Tolloid-like 1 gene, Tll, 1 is necessary for normal septation and positioning of the heart. *Development* 1999; **126**: 2631–2642.
14 Takahara K, Brevard R, Hoffman GG, Suzuki N, Greenspan DS: Characterization of a novel gene product (mammalian tolloid-like) with high sequence similarity to mammalian tolloid/bone morphogenetic protein-l. *Genomics* 1996; **34**: 157–165.
15 Dumermuth E, Sterchi EE, Jiang W *et al*: The astacin family of metalloendopeptidases. *J Biol Chem* 1991; **26**: 21381–21385.
16 Mullins MC: Holy Tolloido: Tolloid cleaves SOG/Chordin to free DPP/BMPs. *Trends Genet* 1998; **14**: 127–129.
17 Scott IC, Blitz IL, Pappano WN *et al*: Mammalian BMP-1/Tolloid-related metalloproteinases, including novel family member mammalian Tolloid-Like 2, have differential enzymatic activities and distributions of expression relevant to patterning and skeletogenesis. *Dev Biol* 1999; **213**: 283–300.
18 Ge G, Fernández CA, Moses MA, Greenspan DS: Bone morphogenetic protein 1 processes prolactin to a 17-kDa antiangiogenic factor. *Proc Natl Acad Sci USA* 2007; **104**: 10010–10015.
19 Wozney JM, Rosen V, Celeste AJ *et al*: Novel regulators of bone formation: molecular clones and activities. *Science* 1988; **242**: 1528–1534.
20 Wang EA, Wozney JM: Identification of transforming growth factor 13 family members present in bone-inductive protein purified from bovine bone. *Proc Natl Acad Sci USA* 1990; **87**: 9843–9847.
21 Suzuki N, Labosky PA, Furuta Y *et al*: Failure of ventral body wall closure in mouse embryos lacking a procollagen C-proteinase encoded by BMP1, a mammalian gene related to Drosophila tolloid. *Development* 1996; **122**: 3587–3595.
22 Yan L, Pollock GH, Nagase H, Sarras Jr MP: A 25.7×10^3 Mr hydra metalloproteinase (HMP1), a member of the astacin family, localizes to the extracellular matrix of Hydra vulgaris in a head-specific manner and has a developmental function. *Development* 1995; **121**: 1591–1602.
23 Childs SR, O'Connor MB: Two domains of the tolloid protein contribute to its unusual genetic interaction with decapentaplegic. *Develop Biol* 1994; **162**: 209–220.
24 Mott JD, Thomas CL, Rosenbach MT, Takahara K, Greenspan DS, Banda MJ: Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. *J Biol Chem* 2000; **275**: 1384–1390.
25 Goodman SA, Albano R, Wardle FC, Matthews G, Tannahill D, Dale L: BMP1-related metalloproteinases promote the development of ventral mesoderm in early Xenopus embryos. *Develop Biol* 1998; **195**: 144–157.
26 Pappano WN, Scott IC, Clark TG, Eddy RL, Shows TB, Greenspan DS: Coding sequence and expression patterns of mouse chordin and mapping of the cognate mouse Chrd and human CHRD genes. *Genomics* 1998; **52**: 236–239.
27 Piccolo S, Agius E, Lu B, Goodman S, Dale L, De Robertis EM: Cleavage of chordin by xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* 1997; **91**: 407–416.
28 Marquès G, Musacchio M, Shimell MJ, Wünnenberg-Stapleton K, Cho KW, O'Connor MB: Production of a DPP activity gradient in the early Drosophila embryo through the opposing actions of the SOG and TLD proteins. *Cell* 1997; **91**: 417–426.
29 Panchenko MV, Stetler-Stevenson WG, Trubetskoy OV, Gacheru SN, Kagan HM: Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C-proteinase. *J Biol Chem* 1996; **271**: 7113–7119.
30 Takahara K, Kessler E, Biniaminov L *et al*: Type I procollagen COOH-terminated proteinase enhancer protein: identification, primary structure, and chromosomal localization of the cognate human gene (PCOLCE). *J Biol Chem* 1994; **269**: 26280–26285.
31 Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS: Bone morphogenetic protein-l: the type 1 procollagen C-proteinase. *Science* 1996; **271**: 360–362.
32 Li SW, Sieron A, Fertala A, Hojima Y, Arnold WV, Prockop DJ: The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1. *Proc Natl Acad Sci USA* 1996; **93**: 5127–5130.
33 Rossant J: Mouse mutants and cardiac development: new molecular insights into cardiogenesis. *Circ Res* 1996; **78**: 349–353.
34 Olson EN, Srivastava D: Molecular pathways controlling heart development. *Science* 1996; **272**: 671–676.
35 Barch MJ, Lawrence HJ, Arsham MS: Peripheral blood culture; in Barch MJ (ed): *The ACT Cytogenetics Laboratory Manual, 2nd edition*. New York: Raven Press, 1991, pp 17–30.
36 Henegariu O, Heerema NA, Lowe Wright L, Bray-Ward P, Ward DC, Vance GH: Improvements in cytogenetic slide preparation: controlled chromosome spreading, chemical aging and gradual denaturing. *Cytometry* 1991; **43**: 101–109.
37 Church GM, Gilbert W: Genomic sequencing. *Proc Nat Acad Sci USA* 1984; **81**: 1991–1995.
38 Doevendans PA, van Bilsen M: Transcription factors and the cardiac gene programme. *Int J Biochem Cell Biol* 1996; **28**: 387–403.
39 Hartigan N, Garrigue-Antar L, Kadler KE: Bone morphogenetic protein (BMP-1): Identification of the minimal domain structure for procollagen C-proteinase activity. *J Biol Chem* 2003; **278**: 18045–18049.
40 Garrigue-Antar L, Francois V, Kalder KE: Deletion of epidermal growth factor-like domains converts mammalian tolloid into a chordinase and effective procollagen C-proteinase. *J Biol Chem* 2004; **279**: 49835–49841.
41 Gomis-Rüth FX, Stöcker W, Huber R, Zwilling R, Bode W: Refined 1.8 Å X-ray crystal strucutre of astacin, a zinc-endopeptidase from the crayfish *Astacus astacus* L. Structure determinantion, refinement, molecular structure and comparison with thermolysin. *J Mol Biol* 1993; **229**: 945–968.
42 Stöcker W, Ng M, Auld DS: Fluorescent oligopeptide substrates for kinetic characterization of the specificity of Astacus protease. *Biochemistry* 1990; **29**: 10418–10425.
43 Garrigue-Antar L, Barker C, Kadler KE: Identification of amino acid residues in bone morphogenetic protein-1 important for procollagen C-proteinase activity. *J Biol Chem* 2001; **276**: 26237–26242.