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HSPA6: A new autosomal recessive candidate gene for the VATER/VACTERL malformation spectrum

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Abstract

Background —The VATER/VACTERL association refers to the non-random co-occurrence of at least three of the following component features (CFs): vertebral defects (V), anorectal malformations (ARM) (A), cardiac defects (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R), and limb defects (L). Patients presenting with two CFs have been termed VATER/VACTERL-like phenotypes.

Methods —We surveyed the exome for recessive disease variants in three affected sib-pairs. Sib-pair 971 consisted of two brothers with ARM and additional hydronephrosis in one brother. Sib-pair 1098 consisted of two sisters with ARM. In family 1346 the daughter presented with ARM and additional hypoplasia of both small fingers and ankyloses. Her brother presented with unilateral isolated radial hypoplasia. Sib-pair 971 and 1346 resembled a VATER/VACTERL-like phenotype.

Results —We detected a novel maternally inherited missense variant (c.1340G>T) and a rare paternally inherited deletion of the trans-allele in *HSPA6* in both siblings of family 1346. *HSPA6* belongs to the heat shock protein (HSP) 70 family. Re-sequencing of *HSPA6* in 167 patients with VATER/VACTERL and VATER/VACTERL-like phenotypes did not reveal any additional bi-allelic variants.

Conclusions—Until now, only TNF-receptor associated protein 1 (*TRAP1*) had been reported as an autosomal recessive disease-gene for the VATER/VACTERL association. *TRAP1* belongs to the heat shock protein 90 family (HSP90). Both *Hsp70* and *Hsp90* genes have been shown to be important embryonic drivers in the formation of mouse embryonic forelimb tissue. Our results suggest *HSPA6* as a new candidate gene in VATER/VACTERL-like phenotypes.

Keywords

HSPA6, anorectal malformations; VATER/VACTERL association; whole-exome sequencing; autosomal recessive inheritance

Introduction

Anorectal malformations (ARM) are rare disorders of the embryonic distal hindgut. They occur in about 1 in 4.000 live births (Cuschieri, 2001; Bartels et al., 2012). They are caused by defective differentiation of the primitive hindgut (Matsumaru et al., 2015). ARM present with a broad phenotypic spectrum ranging from perineal fistula to severe cloacal malformations. For phenotypic classification of ARM, the Krickenbeck classification system is usually applied (Holschneider et al., 2005). Around 60% of ARM patients present with co-occurring congenital malformations, most of which belong to the congenital anomaly spectrum of the VATER/VACTERL association. The VATER/VACTERL association refers to the non-random co-occurrence of at least three of the following component features (CFs): vertebral defects (V), anorectal malformations (ARM) (A), cardiac defects (C), tracheoesophageal fistula with or without esophageal atresia (TE), renal malformations (R), and limb defects (L) (Belloni et al., 2000; Kohlhase, Wischermann, Reichenbach, Froster, & Engel, 1998; Solomon et al., 2014). Patients presenting with two CFs have been termed VATER/VACTERL-like phenotypes. ARM in combination with major upper limb malformations as defined by van den Hondel et al. occurs in about 6% of ARM patients (van den Hondel et al., 2016).

After the detection of causative copy number variations in patients with ARM and ARM as part of their VATER/VACTERL association (Bartels et al., 2011; Dworschak et al., 2013; Schramm, Draaken, Bartels, Boemers, Aretz, et al., 2011; Schramm, Draaken, Bartels, Boemers, Schmiedeke, et al., 2011; Schramm, Draaken, Tewes, et al., 2011) Saisawat et al. (2014) were the first who identified a recessive disease-gene, namely *TRAP1* (TNF-receptor associated protein 1), in patients with VATER/VACTERL association and CAKUT (congenital anomalies of the kidney and urinary tract) (Saisawat et al., 2014). *TRAP1*, located on 16p13.3, belongs to the heat shock protein 90 family (HSP90) (Chen et al., 2005).

Here, we aimed to identify rare autosomal recessive disease-genes for isolated ARM or ARM as part of VATER/VACTERL-like phenotypes. For this purpose, we performed whole exome sequencing (WES) in three affected sib-pairs and their healthy parents and re-sequenced the top candidate gene in 167 patients with VATER/VACTERL and VATER/VACTERL-like phenotypes.

Materials and Methods

PATIENTS, CONTROLS, AND DNA ISOLATION

Patients and families were recruited through the German network for congenital uro-rectal malformations (CURE-Net, www.cure-net.de) and the great-consortium (Genetic risk of esophageal atresia, www.great-konsortium.de). Written informed consent was obtained from every family. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (Lfd. Nr. 073/12 & 146/12). Genomic DNA was isolated from whole blood using the Chemagic DNA Blood Kit special (Chemagen, Baesweiler, Germany). Genomic DNA from saliva samples was isolated with the Oragene DNA Kit (DNA Genotek Inc., Kanata, Canada).

Our study included three affected sib-pairs and their healthy parents. In family 971, two affected brothers (971_II.1, 971_II.2) presented with ARM and additional hydronephrosis in 971_II.1 and ARM in form of a perineal fistula in 971_II.2. Sib-pair 1098 consisted of two affected sisters (1098_II.1, 1098_II.2) presenting with ARM in form of a perineal fistula. In family 1346 the affected daughter (1346_II.1) presented with ARM in form of a perineal fistula and additional hypoplasia of both small fingers and ankyloses. Her brother (1346_II.2) presented with unilateral isolated radial hypoplasia. Hence, sib-pairs 971 and 1346 resemble the phenotypic spectrum of a VATER/VACTERL-like phenotype (Table 1).

For re-sequencing of the top candidate gene, we chose 167 patients presenting with VATER/VACTERL and VATER/VACTERL-like phenotypes.

WHOLE-EXOME SEQUENCING (WES) AND DATA ANALYSIS

WES was performed at the Next Generation Sequencing Laboratory of the Institute of Human Genetics of the University of Bonn. For the enrichment of exonic and adjacent intronic sequences from genomic DNA the NimbleGen SeqCap EZ Human Exome Library v2.0 enrichment kit was used. WES was performed on an Illumina HiSeq2500 sequencer using a 100bp paired-end read protocol, following the manufacturer's recommendations. Data analysis and filtering of mapped target sequences was accomplished with 'Varbank' exome and genome analysis pipeline v.2.1 (<https://varbank.ccg.uni-koeln.de>). We especially filtered for high-quality (coverage of more than six reads, a minimum quality score of 10, VQSLOD greater than -8) and rare (allele frequency < 0.5%) autosomal variants. In sib-pair 971 with two affected brothers, we also filtered for X-chromosomal recessive variants. A detailed description of filter criteria has been described elsewhere (Zhang et al., 2016).

CONFIRMATION OF VARIANTS DETECTED BY WES

Variants identified by WES and called to be possibly causative were amplified from the DNA by polymerase chain reaction (PCR). Automated sequence analysis was carried out using standard procedures. From the UCSC Human Genome Browser (<http://genome.ucsc.edu/>) and Ensembl (<http://www.ensembl.org/index.html>) genomic and cDNA sequences were obtained to compile primers. PCR products were subjected to direct automated BigDye Terminator sequencing (3130XL Genetic Analyzer, AppliedBiosystems, FosterCity, California, USA). Both strands from each amplicon were sequenced and

presence of the variants in each family was confirmed by sequencing the respective PCR product (primer sequences are available upon request).

RE-SEQUENCING OF CANDIDATE GENE *HSPA6*

For the amplification of DNA PCR primers were designed for exon 1 of *HSPA6*. The resultant PCR products were referred to direct automated sequencing with the 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA).

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Quantitative PCR (qPCR) was performed on a Roche Light Cycler 480 II (Roche, Basel, Switzerland) with Roche Light Cycler Sybr Green I Master Mix. Each assay included the respective DNA specimen at a final concentration of 10 ng/μl in triplicate. Reaction mixtures (10 μl) contained 0.2 mmol of each primer and 5 μl of SYBR Green Master Mix (Roche) with cycling conditions as follows: initiation 50°C for 2 min, denaturation 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and a combined annealing and extension step at 64°C for 60 s. The threshold cycle (C_t) values were normalized using the C_t value of three reference genes (*BNCL*, *CFTR* and *RNaseP* subunit p38) for each DNA sample. Relative quantification was done using the comparative C_t method normalizing to the mother as a control sample, since she was found to be heterozygous for variant c.1340G>T and therefore could not carry a deletion of this region (primer sequences are available upon request).

IN SILICO PREDICTION OF VARIANTS

To predict the effect of the variants on the function and structure of the encoded proteins, we used seven online prediction tools (SIFT [<http://sift.jcvi.org/>], Mutation Assessor 3 (<http://mutationassessor.org/r3/>), fathmm (<http://fathmm.biocompute.org.uk/>), PROVEAN (<http://provean.jcvi.org/index.php>), Mutation Taster [<http://www.mutationtaster.org/>], PolyPhen-2 [<http://genetics.bwh.harvard.edu/pph2/>] and the CADD-PHRED-score [<http://cadd.gs.washington.edu/home>]).

Results

Using WES, we investigated three affected sib-pairs and their healthy parents for autosomal recessive disease variants. In the case of affected male brothers, we also surveyed the exome for X-chromosomal disease variants (Table 1). Analysis of the WES-data revealed two candidate genes in family 971 and family 1346. No X-chromosomal disease variant was found in sib-pair 971. No autosomal recessive disease variants were found in sib-pair 1098.

Sib-pair #971

We detected two possible disease-causing variants in *ITSN2*, suggesting a compound heterozygous background in the affected brothers 971_II.1 and 971_II.2 (c.1442T>A and c.4367A>G, according to ENSEMBL GRCh37/hg19 transcript ENST00000355123). The variant c.1442T>A (p.Ile481Asn, ENSP00000347244) was predicted to be deleterious by five out of seven in silico prediction tools (deleterious in SIFT (score: 0), medium functional impact by Mutation Assessor (FI-score: 2.175), tolerated in fathmm (score: 0.08), deleterious in PROVEAN (score: -5.80), disease-causing in Mutation Taster (p-value: 1), probably

damaging by PolyPhen-2 (humvar score 0.996), CADD score of 25.4). In contrast, for the second variant c.4367A>G (single nucleotide polymorphism [SNP]: rs139986826; p.Asn1456Ser (ENSP00000347244)), the amino acid change was predicted to be deleterious by two out of the seven in silico prediction tools (tolerated in SIFT (score 0.64), low functional impact in Mutation Assessor (score: 1.01), tolerated in fathmm (score: -0.26), deleterious in PROVEAN (score: -3.67), disease-causing in Mutation Taster (p-value: 0.926), benign in PolyPhen-2 (humvar score 0.009), CADD-PHRED-score of 17.3). The zebrafish (*danio rerio*) and the green spotted pufferfish (*tetraodon nigroviridis*) present with a Serine at the homologous position. Previously, high-throughput sequencing revealed recessive mutations of *ITSN2* as causing nephrotic syndrome (NS) in humans (Ashraf et al., 2018). Neither of the two brothers (971_II.1 and 971_II.2) presented with symptoms of NS. Of course, we cannot exclude that the two brothers will manifest NS in the future. However, none of the initially reported patients with NS and recessive disease variants in *ITSN2* exhibited any CF of the VATER/VACTERL association spectrum. Hence, we assume that the variants in *ITSN2* are probably not disease-causing for the development of ARM in an autosomal recessive context in our patients. Screening for X-linked variants did not reveal any disease variant for the two brothers.

Sib-pair #1346

In sib-pair 1346 WES revealed a novel apparent homozygous mutation in *heat shock protein family A (HSP70) member 6 (HSPA6 or HSP70B*, here referred to as *HSPA6*) (c.1340G>T, according to ENSEMBL GRCh37/hg19 transcript ENST00000309758) in exon 1. Polyphen2, SIFT, Mutation Taster, Mutation Assessor and PROVEAN predicted the mutation to be damaging (table 2). With a CADD-score of 25.5 it is amongst 1% of the most deleterious variants in the human genome. The *HSPA6* p.Gly447Val mutation (single nucleotide polymorphism [SNP]: rs56366425) alters a highly conserved amino acid. It is conserved throughout the species to *Saccharomyces cerevisiae* with no orthologue in mice (T. K. C. Leung, Hall, Rajendran, Spurr, & Lim, 1992) (table 2). Segregation analysis revealed a heterozygous variant in the mother. The father presented with a wildtype allele and qPCR revealed a deletion of *HSPA6* on the trans-allele. Both children inherited variant c.1340G>T from their mother. They also inherited a microdeletion harboring *HSPA6* from their father on the trans-allele (figure 1). In our in-house control cohort, one out of 1.320 healthy individuals carried a deletion at the *HSPA6* locus (data not shown). Copy number variations (CNVs) in our in-house controls were identified via the human Infinium Omni 2.5 BeadChip (Illumina, San Diego, California) and evaluated with the Illumina Genome Viewer (IGV) program. The frequency of 1 in 1.320 (≈ 0.0008) of the observed deletion in our population-based control cohort is clearly below the population-based frequency of ARM and ARM as part of the VATER/VACTERL association in a recessive disease-model (1 in 4.000 and 1 in 40.000 live births, respectively; Cuschieri, 2001; Bartels et al., 2012). *HSPA6* is a member of the HSP70 protein family coding for heat shock proteins throughout the human organism (T. K. Leung, Rajendran, Monfries, Hall, & Lim, 1990). It contains only one exon of 2.731 base pairs (according to ENSEMBL GRCh37/hg19 transcript ENST00000309758), a conserved N-terminal nucleotide binding domain (NBD) as well as a C-terminal peptide substrate binding domain (SBD) (Wisniewska et al., 2010).

To follow up on this finding, we re-sequenced *HSPA6* in 167 patients with VATER/VACTERL or VATER/VACTERL-like phenotypes resembling the phenotypes of patients 1346_II.1 and 1346_II.2 (ARM and limb malformation).

Sanger sequencing revealed four exonic heterozygous missense variants with minor allele frequencies (MAF) > 0.08. Additionally, we found three synonymous rare exonic variants (MAF < 0.08) and two exonic synonymous common single nucleotide polymorphisms (SNPs) were detected. In the untranslated region three common SNPs as well as one rare variant (MAF unknown) were detected. Overall, we did not detect any further recessive disease variants in the investigated cohort.

Discussion

Our WES analysis identified compound heterozygous variants in an affected sib-pair with congenital anomalies of the VATER/VACTERL association spectrum. Both carried a novel missense variant c.1340G>T in *HSPA6*, inherited from their healthy mother and a deletion of *HSPA6* on the trans-allele inherited from their healthy father. The variant was absent in the gnomAD browser beta (<http://gnomad.broadinstitute.org/>, (Lek et al., 2016) or in the exome variant server database (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: <http://evs.gs.washington.edu/EVS/>) [12/2018]). In the ExAC Browser (Beta) (<http://exac.broadinstitute.org/>, (Lek, et al., 2016) the allele count for this variant was 36 in 121098 alleles without any homozygous individuals listed and with an allele frequency of about 0,0003. Fifteen studies listed in the database of genomic variants describe the loss of genome sequences in the region of *HSPA6* (<http://dgv.tcag.ca/dgv/app/home?ref=>; (MacDonald, Ziman, Yuen, Feuk, & Scherer, 2014)) without mentioning population-based frequencies. Here, we investigated a healthy control cohort of 1.320 individuals and found one of these 1.320 healthy individuals to carry a deletion at the *HSPA6* locus revealing a population-based frequency clearly below the population-based frequency of ARM and ARM as part of the VATER/VACTERL association in a recessive disease-model (1 in 4.000 and 1 in 40.000 live births, respectively; Cuschieri, 2001; Bartels et al., 2012). Screening 167 patients with VATER/VACTERL or VATER/VACTERL-like phenotypes resembling the phenotypes of patients 1346_II.1 and 1346_II.2 (ARM and limb malformation) did not reveal any recessive disease variants.

The highly conserved *HSPA6* gene (Daugaard, Rohde, & Jäättelä, 2007; Gupta & Golding, 1993) belongs to the multigene heat shock protein family A (HSP70). Little is known about the function of *HSPA6*, a gene present in the human genome but absent from mouse and rat (Deane & Brown, 2018). To our knowledge, there are no animal models for functional screening of the orthologous gene although it is present in other vertebrates and species such as frog, fruitfly or yeast (table 2). Emerging research suggests the HSPA6 protein to be involved in apoptotic response and cellular differentiation and to contribute to protection of differentiated human neuronal cells from cellular stress (Deane & Brown, 2018). Only when induced after severe stress events (Parsian et al., 2000) it functions as a secondary responder to proteotoxic stress and maintains cellular survival by directly binding to Apaf-1 and inhibiting the assembly of a functional apoptosome (Beere et al., 2000; Ej Noonan, Place, Giardina, & Hightower, 2007). The involvement of HSP70s in regulation of the cell cycle

and apoptotic pathways is essential for human embryonal development as they protect embryonal cells from cell stress such as hyperthermia or chemical teratogens (Luft & Dix, 1999). This is supported by the identification of *HSPA6* in context of preterm birth in genetic association studies (Ryckman et al., 2010). Noonan et al. demonstrated that a knockdown of *HSPA6* in human colon cell lines leads to cell death induced by proteasome inhibition (Noonan, Fournier, & Hightower, 2008). Furthermore, expression patterns of members of the HSP70 family and of the HSP90 family during embryo development in mice show their involvement and protective role in limb formation (Zhu et al., 2012). Interestingly, different HSPs show a characteristic correlation with the developmental phases in mice embryos. As bi-allelic mutations in the *TRAP1* gene (member of the HSP90 family) cause CAKUT and malformations of the VATER/VACTERL association spectrum, one might assume a similar effect of mutations in *HSPA6* being part of a tight interaction network of HSPs during chaperone activity and transcriptional regulation (Mayer & Bukau, 2005; Prodromou, 2016).

In summary, our results suggest that bi-allelic recessive mutations in *HSPA6* contribute to congenital anorectal and limb malformations as part of the VATER/VACTERL association spectrum. Our analysis focused on exome-variants generated by WES in three affected sib-pairs and their healthy parents. Our finding of a paternally inherited deletion of *HSPA6* in sib-pair 1346 may be further explored by using CNV analysis. This may help detecting autosomal recessive microdeletions or -duplications in sib-pair 1098. Further studies are needed to explore how *HSP70s* influence the development of ARMs and features of the VATER/VACTERL association.

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References

- Ashraf Z, Kudo H, Rao J, Kikuchi A, Widmeier E, Lawson JA, ... Hildebrandt F (2018). Mutations in six nephrosis genes delineate a pathogenic pathway amenable to treatment. *Nature Communications*, 9(1), 1960 *doi:* 10.1038/s41467-018-04193-w
- Bartels E, Draaken M, Kazmierczak B, Spranger S, Schramm C, Baudisch F, ... Reutter H (2011). De novo partial trisomy 18p and partial monosomy 18q in a patient with anorectal malformation. *Cytogenetic and genome research*, 134(3), 243 *doi:* 10.1159/000328833 [PubMed: 21709416]
- Bartels E, Jenetzky E, Solomon BD, Ludwig M, Schmiedeke E, Grasshoff-Derr S, ... Zwink N (2012). Inheritance of the VATER/VACTERL association. *Pediatric surgery international*, 28(7), 681–685. 10.1007/s00383-012-3100-z [PubMed: 22581124]
- Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, ... Green DR (2000). Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nature Cell Biology*, 2, 469 *doi:* 10.1038/35019501 [PubMed: 10934466]

- Belloni E, Martucciello G, Verderio D, Ponti E, Seri M, Jasonni V, ... Scherer SW (2000). Involvement of the HLXB9 Homeobox Gene in Currarino Syndrome. *American Journal of Human Genetics*, 66(1), 312–319. doi: 10.1086/302723 [PubMed: 10631160]
- Chen B, Piel WH, Gui L, Bruford E, Monteiro A (2005). The HSP90 family of genes in the human genome: insights into their divergence and evolution. *Genomics*, 86(6), 627–37. doi: 10.1016/j.ygeno.2005.08.012. [PubMed: 16269234]
- Cuschieri A (2001). Descriptive epidemiology of isolated anal anomalies: A survey of 4.6 million births in Europe. *American Journal of Medical Genetics*, 103(3), 207–215. doi: 10.1002/ajmg.1532 [PubMed: 11745992]
- Daugaard M, Rohde M, & Jäättelä M (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Letters*, 581(19), 3702–3710. doi: 10.1016/j.febslet.2007.05.039 [PubMed: 17544402]
- Deane CAS & Brown IR (2018). Knockdown of HSPA6 (Hsp70B') and HSPA1A (Hsp70-1) Sensitizes Differentiated Human Neuronal Cells to Cellular Stress. *Neurochemical Research*, 43(2), 340–350. doi: 10.1007/s11064-017-2492-z
- Dworschak GC, Draaken M, Marcelis C, De Blaauw I, Pfundt R, Van Rooij IALM, ... Reutter H (2013). De novo 13q deletions in two patients with mild anorectal malformations as part of VATER/VACTERL and VATER/VACTERL-like association and analysis of EFNB2 in patients with anorectal malformations. *American Journal of Medical Genetics Part A*, 161(12), 3035–3041. doi: 10.1002/ajmg.a.36153
- Gupta R, & Golding G (1993). Evolution of HSP70 gene and its implications regarding relationships between archaebacteria, eubacteria, and eukaryotes. *J Mol Evol*, 37(6), 573–582. doi: 10.1007/bf00182743 [PubMed: 8114110]
- Holschneider A, Hutson J, Peña A, Beket E, Chatterjee S, Coran A, ... Kunst M (2005). Preliminary report on the International Conference for the Development of Standards for the Treatment of Anorectal Malformations. *Journal of Pediatric Surgery*, 40(10), 1521–1526. doi: 10.1016/j.jpedsurg.2005.08.002 [PubMed: 16226976]
- Kohlhase J, Wischermann A, Reichenbach H, Froster U, & Engel W (1998). Mutations in the SALL1 putative transcription factor gene cause Townes-Brocks syndrome. *Nature Genetics*, 18, 81 doi: 10.1038/ng0198-81 [PubMed: 9425907]
- Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, ... MacArthur DG (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature*, 536(7616). doi: 10.1038/nature19057
- Leung TKC, Hall C, Rajendran M, Spurr NK, & Lim L (1992). The human heat-shock genes HSPA6 and HSPA7 are both expressed and localize to chromosome 1. *Genomics*, 12(1), 74–79. doi: 10.1016/0888-7543(92)90409-L [PubMed: 1346391]
- Leung TK, Rajendran MY, Monfries C, Hall C, & Lim L (1990). The human heat-shock protein family. Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA. *The Biochemical journal*, 267(1), 125 doi: 10.1042/bj2670125 [PubMed: 2327978]
- Luft J, & Dix D (1999). Hsp70 expression and function during embryogenesis *Cell Stress Chaperones* (Vol. 4, pp. 162–170). [PubMed: 10547065]
- MacDonald JR, Ziman R, Yuen RKC, Feuk L, & Scherer SW (2014). The Database of Genomic Variants: a curated collection of structural variation in the human genome. *Nucleic Acids Research*, 42(Database issue), D986–D992. doi: 10.1093/nar/gkt958 [PubMed: 24174537]
- Matsumaru D, Murashima A, Fukushima J, Senda S, Matsushita S, Nakagata N, ... Yamada G (2015). Systematic stereoscopic analyses for cloacal development: The origin of anorectal malformations. *Scientific Reports*, 5, 13943 doi: 10.1038/srep13943 [PubMed: 26354024]
- Mayer MP, & Bukau B (2005). Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences*, 62(6), 670–684. doi: 10.1007/s00018-004-4464-6 [PubMed: 15770419]
- Noonan E, Fournier G, & Hightower L (2008). Surface expression of Hsp70B' in response to proteasome inhibition in human colon cells. *Cell Stress and Chaperones*, 13(1), 105–110. doi: 10.1007/s12192-007-0003-3 [PubMed: 18347947]

- Noonan E, Place R, Giardina C, & Hightower L (2007). Hsp70B ' regulation and function. *Cell Stress & Chaperones*, 12(3), 219–229. *doi:* 10.1379/csc-278.1 [PubMed: 17915554]
- Parsian AJ, Sheren JE, Tao TY, Goswami PC, Malyapa R, Van Rheeden R, ... Hunt CR (2000). The human Hsp70B gene at the HSPA7 locus of chromosome 1 is transcribed but non-functional. *Biochimica et biophysica acta*, 1494(1–2), 201. [PubMed: 11072087]
- Prodromou C (2016). Mechanisms of Hsp90 regulation. *Biochemical Journal*, 473(16), 2439–2452. *doi:* 10.1042/bcj20160005 [PubMed: 27515256]
- Ryckman KK, Morken N-H, White MJ, Velez DR, Menon R, Fortunato SJ, ... Jacobsson B (2010). Maternal and Fetal Genetic Associations of PTGER3 and PON1 with Preterm Birth (Genetics of Preterm Birth). *PLoS ONE*, 5(2), e9040 *doi:* 10.1371/journal.pone.0009040 [PubMed: 20140262]
- Saisawat P, Kohl S, Hilger AC, Hwang D-Y, Gee HY, Dworschak GC, ... Hildebrandt F (2014). Whole exome resequencing reveals recessive mutations in TRAP1 in individuals with CAKUT and VACTERL association. *Kidney international*, 85(6). *doi:* 10.1038/ki.2013.417
- Schramm C, Draaken M, Bartels E, Boemers TM, Aretz S, Brockschmidt FF, ... Reutter H (2011). De novo microduplication at 22q11.21 in a patient with VACTERL association. *European Journal of Medical Genetics*, 54(1), 9–13. *doi:* 10.1016/j.ejmg.2010.09.001 [PubMed: 20849991]
- Schramm C, Draaken M, Bartels E, Boemers TM, Schmiedeke E, Grasshoff-Derr S, ... Reutter H (2011). De novo duplication of 18p11.21–18q12.1 in a female with anorectal malformation. *American journal of medical genetics. Part A*, 155A(2), 445 *doi:* 10.1002/ajmg.a.33820 [PubMed: 21271671]
- Schramm C, Draaken M, Tewes G, Bartels E, Schmiedeke E, Märzheuser S, ... Ludwig M (2011). Autosomal-dominant non-syndromic anal atresia: sequencing of candidate genes, array-based molecular karyotyping, and review of the literature. *Eur J Pediatr*, 170(6), 741–746. *doi:* 10.1007/s00431-010-1332-2 [PubMed: 21042811]
- Solomon BD, Baker LA, Bear KA, Cunningham BK, Giampietro PF, Hadigan C, ... Warren-Mora N (2014). An Approach to the Identification of Anomalies and Etiologies in Neonates with Identified or Suspected VACTERL (Vertebral Defects, Anal Atresia, Tracheo-Esophageal Fistula with Esophageal Atresia, Cardiac Anomalies, Renal Anomalies, and Limb Anomalies) Association. *The Journal of Pediatrics*, 164(3), 451–457.e451. *doi:* 10.1016/j.jpeds.2013.10.086 [PubMed: 24332453]
- van den Hondel D, Wijers CHW, van Bever Y, de Klein A, Marcelis CLM, de Blaauw I, ... Ijsselstijn H (2016). Patients with anorectal malformation and upper limb anomalies: genetic evaluation is warranted. *European Journal of Pediatrics*, 175, 489–497. *doi:* 10.1007/s00431-015-2655-9 [PubMed: 26498647]
- Wisniewska M, Karlberg T, Lehtiö L, Johansson I, Kotenyova T, Moche M, & Schüler H (2010). Crystal Structures of the ATPase Domains of Four Human Hsp70 Isoforms: HSPA1L/Hsp70-hom, HSPA2/Hsp70-2, HSPA6/Hsp70B', and HSPA5/BiP/GRP78. *PLoS ONE*, 5(1), e8625 *doi:* 10.1371/journal.pone.0008625 [PubMed: 20072699]
- Zhang R, Thiele H, Bartmann P, Hilger AC, Berg C, Herberg U, ... Reutter H (2016). Whole-Exome Sequencing in Nine Monozygotic Discordant Twins 19(1), 60–65. *doi:* 10.1017/thg.2015.93
- Zhu Y, Zhou H, Zhu Y, Wan X, Zhu J, & Zhang T (2012). Gene expression of Hsp70, Hsp90, and Hsp110 families in normal and abnormal embryonic development of mouse forelimbs. *Drug and Chemical Toxicology*, 2012, Vol.35(4), p.432–444, 35(4), 432–444. *doi:* 10.3109/01480545.2011.640683

Figure 1. Pedigrees of the families.

A) 1098_II.1 and 1098_II.2 presented both with ARM in form of a perineal fistula. B) 971_II.1 presented with ARM and left hydronephrosis. 971_II.2 presented with ARM in form of a perineal fistula. C) 1346_II.1 presented with ARM in form of a perineal fistula, hypoplasia and ankylosis of both small fingers. 1346_II.2 presented with isolated radial hypoplasia. The presence or absence of genetic variants detected by whole exome sequencing and (quantitative) polymerase chain reaction are indicated. Members affected with anorectal or skeletal malformations are shown in black, while unaffected members are shown in white. Males and females are represented in squares and circles, respectively. *HSPA6*, heat shock 70kD protein 6; *ITSN2*, intersectin 2; wt, wild type; del, deletion

Table 1

Phenotypic features of the patients of family 1098, 971 and 1346.

Family number	Patient	Anorectal malformation	Other features
1098	II.1	Anorectal malformation in form of a perineal fistula	
	II.2	Anorectal malformation in form of a perineal fistula	
971	II.1	Anorectal malformation	Hydronephrosis, left
	II.2	Anorectal malformation in form of a perineal fistula	
1346	II.1	Anorectal malformation in form of a perineal fistula	Hypoplasia and ankyloses of both small fingers
	II.2		Radial hypoplasia

Table 2

Mutations in *HSPA6* (NM_002155.3) in patients 1346_II.1 and 1346_II.2 with anorectal malformations and limb deformities.

Nucleotide alteration/deletion (zygosity)	Exon	Amino Acid Change	MutT	PP2	SIFT	MutA	FATHMM	PROVEAN	CADD v1.3	Segregation/. state of alleles	CP	Gg	Xt	Dr	Ci	Ce	Dm	Sc
c.1340G>T (h)	1	G447V	D.C.	1.000	D	4.085	T	D	25.5	Maternal inheritance	G	G	G	G	G	G	G	G
Deletion (h)	1	-	-	-	-	-	-	-	-	Paternal inheritance	-	-	-	-	-	-	-	-

Abbreviations: *Cp*, *cavia porcellus*; *Gg*, *gallus gallus*; *Xt*, *xenopus tropicalis*; *Dr*, *danio rerio*; *Ci*, *ciona intestinalis*; *Ce*, *caenorhabditis elegans*; *Dm*, *drosophila melanogaster*; *Sc*, *Saccharomyces cerevisiae*; MutT, mutation taster prediction class; PP2, PolyPhen2 humvar score; SIFT, sorting intolerant from tolerant prediction class; MutA, Mutation Assessor 3 functional impact score; FATHMM, Functional Analysis through Hidden Markov Models (v2.3); PROVEAN, Protein Variation Effect Analyzer; CADD v1.3, Combined Annotation Dependent Depletion v1.3; PHRED like scaled C-score; D, deleterious; D.C., disease causing; T, tolerated; h, heterozygous; G, Glycine; V, Valine.