“Whole exome sequencing for gene discovery in lethal fetal disorders”

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Overview

My PhD thesis focuses on the delineation of monogenic causes of lethal fetal anomaly phenotypes, supervised by PD Dr. med. Isabel Filges.

Chromosomal anomalies including submicroscopic copy number variations (CNV) account for a significant fraction of about 10% of fetal structural anomalies. For fetal congenital anomaly syndromes with a normal high-resolution karyotype, the monogenic etiology of the phenotypes is poorly understood. Our objective was to use exome sequencing (ES) to identify genes that were not previously reported in a disease-associated context and novel variants in known disease genes to increase our knowledge on genotype-phenotype correlations in fetal anomalies. Ultimately, we aimed at improving clinical care for affected families.

In the first part of the project we used family-based high throughput Next Generation Sequencing (NGS) of families with two or more fetuses affected by a recurrent rare lethal fetal anomaly pattern or a single fetus with a phenotype pattern indicating a defect in a fundamental human developmental pathway. We sequenced the exome of the affected individuals and parents and analyzed the detected variants, regarding their potential to alter protein function, using databases on variant frequencies in healthy and affected individuals, bioinformatic functional prediction and conservation analysis programs, cross-species phenotyping and pathway analysis as well as current knowledge extracted from medical and biological literature. In total 19 families with 26 fetuses participated in the study. In 12 families (63%) a candidate gene was detected (*CENPF, DNHD1, FGFR2, KIF14, KIF4A, MKS1, OTX2, PIGW, PTK7, RYR1, SMAD3* and *TTC28*) and in 6 (50%) of those a definite diagnosis was achieved (*CENPF, FGFR2, KIF14*, *MKS1, OTX2* and *RYR1*).

We studied selected candidate genes (*RYR1*, *KIF4A*, *PTK7* and *SMAD3*) and variants for their consequences on the functional level. For those four genes I designed and implemented mRNA

dosage analysis, for *PTK7* a cell culture model and for *SMAD3* a chicken embryo model in collaboration with the laboratory for regulatory evolution, in order to also elucidate the underlying disease mechanism. Results were returned to the patients within the genetic consultation and counselling of the families regarding recurrence risk for further pregnancies, options for prenatal and preimplantation diagnosis as well as pregnancy management.

Zusammenfassung

Meine Doktorarbeit befasste sich mit der Aufklärung monogener Ursachen von fötal letalen Fehlbildungs- Phänotypen und wurde von Frau PD. Dr. med. Isabel Filges betreut.

Chromosomale Anomalien sind ursächlich für ca. 10% der fötalen Fehlbildungen. Bei Patienten mit angeborenen Fehlbildunssyndromen, welche bei einem hochauflösenden Microarray einen unauffälligen Karyotyp aufweisen, ist die monogene Ursache des Phänotyps selten bekannt. In unserem Projekt führten wir Exom-Sequenzierungen durch, um neue krankheitsverursachende Gene, welche noch nicht als solche erkannt wurden und unbekannte Sequenzvarianten in bekannten Krankheitsgenen zu identifizieren. Der initiale Teil des Projektes umfasste die Hochdurchsatz-Sequenzierung der DNA von Familien mit einem oder mehreren betroffenen Föten, mit wiederkehrenden fötalen Phänotypen oder einem Phänotyp, welcher auf einen Defekt in einem entwicklungsrelevanten Signalweg hindeutet. Wir sequenzierten das Exom, den protein-kodierenden Bereich des Genoms, der betroffenen Föten und deren Eltern und analysierten die detektierten Varianten hinsichtlich ihres Potentials die Proteinfunktion zu beeinträchtigen. Hierfür benutzten wir bioinformatische Prädiktionsprogramme, welche funktionelle Auswirkungen und auch die Konservierung der Variante berücksichtigen, die Häufigkeit der Varianten in Kontrolldatenbanken, den Vergleich mit Tiermodellen, eine Signalweganalyse und Informationen

aus aktueller medizinischer und biologischer Fachliteratur. Insgesamt haben 19 Familien mit 26 betroffenen Föten an dem Projekt teilgenommen. Bei 12 Familien (63%) konnten wir ein Kandidatengen benennen (*CENPF, DNHD1, FGFR2, KIF14, KIF4A, MKS1, OTX2, PIGW, PTK7, RYR1, SMAD3* und *TTC28*). In 6 dieser Familien (50%) konnte eine sichere Diagnose gestellt werden (*CENPF, FGFR2, KIF14*, *MKS1, OTX2* und *RYR1*).

Ausgewählte Kandidatengene und -varianten (*RYR1*, *KIF14*, *PTK7* und *SMAD3*) wurden dann experimentell auf deren molekulare Konsequenzen untersucht. Für diese vier Gene plante und implementierte ich eine mRNA Dosisanalyse, für *PTK7* ein Zellkulturmodell und für *SMAD3* ein Hühner-Embryonen Modell, in Zusammenarbeit mit dem Labor für regulatorische Evolution, um nicht nur die Kausalität zu beweisen, sondern auch den zugrundeliegenden molekularen Mechanismus nachzuvollziehen. In Fällen, in denen eine Diagnose feststand, fanden meine Resultate Eingang in die weitere genetische Beratung der betroffenen Familien. Dies diente dazu das Wiederholungsrisiko für zukünftige Schwangerschaften abschätzen zu können, um eine Pränatal- und Präimplantationsdiagnostik ermöglichen zu können.

1. Introduction

Multiple congenital anomalies (MCA) are detected in about 2-5% of newborns and cause up to 20% of prenatal and perinatal deaths in industrialized countries[1–4]. Through improved high-resolution ultrasound techniques, fetal structural anomalies are detected in increasingly earlier stages of pregnancy[5]. The detection of severe fetal malformations is a calamitous experience for the affected families, raising questions about etiology, diagnosis, prognosis and recurrence risk. Especially the presence of multiple anomalies in the fetus and phenotype recurrence within a family, indicating a genetic etiology for a congenital malformation syndrome. In order to offer appropriate counselling on reproductive choices and options for pregnancy management, including prenatal and preimplantation diagnosis for future pregnancies, the knowledge of the individual genetic cause is needed. Prenatal high-resolution chromosomal microarray analysis achieves a diagnostic yield of approximately 6-10% after the exclusion of frequent aneuploidies[6,7]. Targeted single gene or gene panel testing is indicated if a specific monogenic entity is suspected based on clinical findings[8–10]. The majority of affected families today remains without a definite diagnosis[11].

Next Generation Sequencing (NGS) technologies have revolutionized the detection rate of Mendelian disorders by becoming an affordable and highly effective approach in research and diagnostics to elucidate monogenic causes for developmental disorders[12,13]. Especially exome sequencing (ES), covering the protein-coding regions of the genome (~1%), became a routine method in postnatal diagnostic sequencing. Large-scale projects are ongoing to generate and compile available data on human phenotypes and genetic variants such as Decipher (https://decipher.sanger.ac.uk), the DDD project (Deciphering Developmental Disorders, https://www.ddduk.org) and the 100.000 Genomes project (https://www.genomicsengland.co.uk). Patients, suspected to have a genetic disorder, and their

families are sequenced in order to identify the genetic etiology. So far, these studies were mainly used for the elucidation of postnatal phenotypes, but rarely in severe, often lethal, congenital anomaly phenotypes apparent during pregnancy. For obvious reasons genetic conditions manifesting in viable phenotypes are easier to delineate due to the availability of more patients with the same phenotype as well as the possibility to assess phenotypic data more comprehensively. Whereas the phenotyping in fetuses is restricted to structural anomalies assessed by ultrasound and autopsy, if at all done, postnatal phenotyping includes motor and cognitive abilities as well as appearance of clinical signs over a comparably long developmental period. The fetal phenotype therefore is incomplete and represents a momentaneous picture at a specific human developmental stage.

The elucidation of the genetic etiology of fetal phenotypes received increasing attention including the discussion of introducing ES into standard prenatal care[14–16]. The first ES studies on a limited number of selected fetuses harboring multiple congenital malformations showed a significant fraction where a diagnosis was achieved through ES[17–21], ranging from 6% to 80% highly depending on the selection of the cohort[22]. ES therefore is a promising technique to investigate the monogenic etiology of Mendelian fetal phenotypes[18,20,23–29] However, in 2019, the first large scale study to use ES for prenatal diagnosis in a cohort with unselected variable fetal anomalies as encountered by ultrasound in a prenatal clinic, reports the diagnostic yield at about overall 10%.[30], much lower than for postnatal sequencing studies which yield at about 25% for various monogenic disorders[13].

However, we hypothesize that this discrepancy may be explained in part by the fact that a significant proportion of malformation phenotypes encountered during the antenatal developmental stages may be specific to fetal life since they will lead to embryonic, fetal or perinatal lethality and have escaped etiological research and clinical delineation so far. They may also represent an incomplete or severe allelic presentation of a phenotype described to occur

postnatally, and the diagnosis therefore remains unrecognized at this stage of development. In addition, only about 25% of the genes of the coding exome has been correlated to human phenotypes at all (www.omim.org), functional studies of specific candidate variants confirming pathogenicity is often lacking. Mouse knockout studies support the hypothesis, that about 30% of our genes are specific to embryonic development and a loss-of function causes antenatal death[31]. Lethal phenotypes likely indicate a severe malfunction in early developmental processes, implying major disruption of a crucial developmental pathway component[26,27,32–34]. Genes that play a key role in embryonic development are likely to be highly conserved across species. Thus, cross-species phenotyping is a suitable method to validate causal relationships between candidate variants, identified by ES, and rare human phenotypes[35].

Studying embryonic and lethal fetal phenotypes in humans, but also in animal models, is essential for the understanding of the developmental function of genes for which little to nothing is known. Ultimately, this will increase the clinical utility of ES in a prenatal but also postnatal application as it advances our ability to reliably interpret diagnostic exome data, and to improve counselling and care of the families involved.

2. Research plan and objectives

We aimed at identifying candidate variants in novel genes and novel variants in known disease-genes that cause multiple congenital anomalies in early human development. We will describe novel phenotype-genotype correlations and expand the antenatal phenotype spectrum on rare monogenic disorders

We used ES for identification of disease-genes in deceased fetuses and newborns with rare congenital malformations of unknown etiology. My project focused on selected fetuses where the probability for a monogenic etiology of the phenotype was rather high, e. g. phenotype recurrence in the family is presumably based on a recessive inheritance mode, and/or in-depth phenotyping by autopsy and histological examinations suggested a malformation pattern.

We prospectively recruited 19 families, 26 fetuses (after fetal or perinatal death or from terminated pregnancies with an unfavorable prognosis) and two children that presented severe malformations of unknown etiology after written informed consent for participation was obtained from the parents. Families were included in the study if either i) the fetus showed a pattern of two or more anomalies associated with a high risk for fetal or perinatal lethality that suggested a genetic disorder or ii) there was familial recurrence of the fetal malformation phenotype and if iii) there were detailed clinical fetal ultrasound and/or autopsy data available and iv) high-resolution chromosomal microarray did not show a causal genomic copy number anomaly. Autopsy was performed on affected fetuses of 18 families. We reviewed the fetal phenotypes together with experienced clinical geneticists, fetal- and neuropathologists and maternal-fetal-medicine specialists. We prioritized ES candidate variants and performed extensive literature research, phenotype genotype correlations, cross-species phenotyping and functional studies to prove the

impact of the candidate variants. The complete workflow, from patient recruitment to gene identification, is depicted in Figure 1.

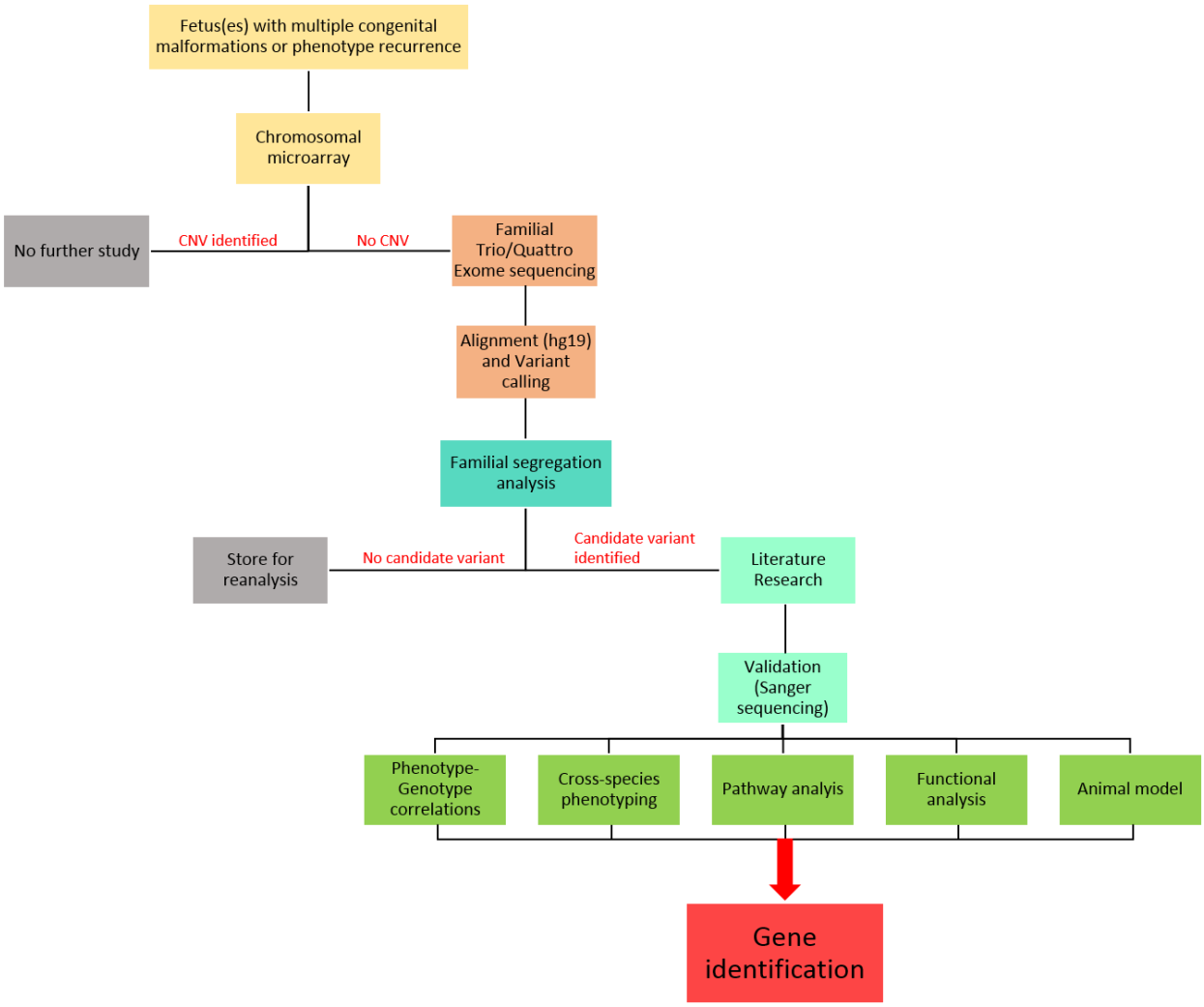

Figure 1: *Schematic experimental workflow for identification of candidate genes in families with lethal fetal anomaly syndromes.* CNV: copy-number variant

2.1 Data generation, analysis and variant prioritization

Genomic DNA was extracted from remaining prenatal specimen such as chorionic villus sampling, amniocentesis, fresh frozen or formalin fixed paraffin embedded (FFPE) fetal tissue. Parental DNA was extracted from whole blood samples. ES was performed on familial trios (or quattros, if available after recurrence). Sequencing library preparation (Agilent SureSelect^{XT} Library Prep Kit,

Agilent, Santa Clara, CA) and exome capture using the Agilent SureSelect^{XT} Human All Exon V6 (Agilent, Santa Clara, CA) was followed by paired-end read sequencing (2x100 bp read length) on a MiSeq, HiSeq 2500 or HiSeq 4000 platform (Illumina, San Diego, CA) with an average coverage of 100x. Quality estimation of the sequence reads was performed by generating quality control statistics with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Illumina CASAVA (1.8.2) was used to demultiplex the sequencing reads. Adapter trimming was performed with Skewer (Version 0.2.2)[36]. Sequencing reads were mapped and aligned to the reference genome sequence (hg19) with Burrows-Wheeler Aligner (BWA-mem Version 0.7.2)[37]. Alignments with a Phred quality score below 30 for the entire read and potential PCR duplicates were discarded using samtools (Version 0.1.18)[38]. To increase sensitivity, variant calling was performed with samtools and varscan (Version 2.3.5)[39]. Variants with a coverage ≤10X and not supported by at least 4 reads (20%) were excluded. Familial segregation analysis of variants was performed to identify *de novo*, autosomal recessive and X-linked inheritance. For the analysis of the sequencing data, we hypothesized a rare Mendelian disorder and annotated variants as known or novel according to their presence or absence in curated databases (Single Nucleotide Polymorphism Database (dbSNP142), Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD), 1000 Genomes) that display healthy individuals. Heterozygous minor allele frequency (MAF) was set to < 5% for recessive inheritance and < 1% for de novo and X-linked inheritance. We prioritized variants according to their potential to disrupt the protein function using bioinformatic prediction tools (SIFT, Provean, Polyphen2, MutationTaster, Human Splicing Finder v.3.0)[40–43]. We also considered the amino acid conservation (PhyloP, PhastCons) as well as the consequences of the amino acid exchange with Have (y)Our Protein Explained (HOPE)[44]. Further, the guidelines of the American College of Medical Genetics (ACMG)[45] were applied to estimate the pathogenicity of

the detected variants. Candidate variants were confirmed by Sanger sequencing according to standard protocols.

2.2 Genotype-phenotype correlations, cross-species comparison and pathway analysis

Genotype-phenotype correlations were considered critical for the identification of the causal relationship between a candidate variant and the anomaly phenotype. Depending on the relative position of the variant in the protein sequence, the resulting phenotype can be highly variable (pleiotropy). Therefore, existing information regarding gene function and reported phenotypes were compiled from medical literature and databases (PubMed, OMIM, ClinVar, Decipher) and related protein networks and signaling pathways in embryonic development were investigated (Reactome, Uniprot, Ingenuity Pathway Analysis (Qiagen)). We systematically interrogated zebrafish, mouse and drosophila phenotype databases (www.zfin.org; www.informatics.jax.org; www.mousephenotype.org; www.flybase.org) using cross-species phenotype comparison for validation of candidate genes as well as the databases of the International Mouse Phenotyping Consortium (IMPC). IMPC aims to provide a systematic and unbiased characterization of embryonic or perinatal lethal variants at both, the anatomic and transcriptional level.

2.3 Functional investigations of candidate variants

We further examined the functional effect of a candidate variant on a transcriptional level. qPCR on cDNA was done for selected candidate genes (*RYR1*, *DNHD1*, *KIF4A*, *PTK7* and *SMAD3*). These five genes were selected after extensive literature review and therefore held great promise to be related to the observed fetal phenotype. FFPE tissue from affected organs was used against age-matched control fetal FFPE tissue for reverse transcription analysis of the candidate gene to assess

the presence or absence of the transcript. Control tissue was obtained from fetuses without structural anomalies confirmed by autopsy after intrauterine fetal death likely due to asphyxia. For *RYR1* mRNA qPCR showed absence of the affected Exon that might lead to nonsense mediated decay of the transcript. For *KIF4A*, *SMAD3* and *PTK7* mRNA levels were strongly decreased compared to the control fetuses. For *DNHD1* it was not possible to perform qPCR, most likely due to low quality of the available tissue. For *SMAD3* and *PTK7* we developed different experimental models to prove causality for the phenotypes we observed.

In the *SMAD3* gene, a *de novo* missense variant was detected in a fetus with severe agnathia-otocephaly. The SMAD 3 protein acts in the same developmental pathway as OTX2 and PRRX1 as a transcriptional regulator. This pathway is invoved in the development of the first pharyngeal arch in the lower jaw. In addition, there are pathogenic variants described in *OTX2* and *PRRX1* to cause agnathia-otocephaly suggesting genetic heterogeneity. This variant in *SMAD3* was previously reported on ClinVar (SCV000782295.1) and HGMD to cause autosomal dominant Loeys-Dietz syndrome 3 (OMIM: 613795), a connective tissue disorder presenting with arterial aneurysm and tortuosity, neuropathy, cardiac anomalies, cleft palate and significant micro-/retrognathia[46,47]. The etiology is linked to defective components of the transcription factor β (TGFB) pathway[48–50] which is crucial for early developmental regulation and for osteoblastogenesis and bone formation in particular[51]. Proteins of the SMAD family play an important role in the TGFB pathway in transcriptional regulation[52]. The variant we identified is located in the MH2-domain of the SMAD3 protein. It is crucial for the interaction between SMAD3 and other SMAD proteins to trigger their activity as transcription factors[53]. It is also suspected that SMAD3 plays a role in the development of the lower jaw[54]. We hypothesized that agnathia-otocephaly represents the severe end of the Loeys-Dietz syndrome spectrum due to the loss of one of the SMAD3 interactions.

To model the agnathia-otocephaly phenotype we designed a chicken embryo model. We chose a dominant negative expression model, which means we aimed at overexpressing the mutated protein to repress the functionality of the wild-type protein. When establishing this model, we encountered various difficulties to overcome. The cloning of the human cDNA sequence into a shuttle vector and finally to the viral vector was challenging due to the high recombination rate in viral constructs. We overcame this issue by using bacteria that are lacking a recombinase achieving vector stability. We then optimized infection of the embryos at 4 days post fertilization, which revealed insufficient infection of about 45% of the cells (Figure 2) without an abnormal phenotype. The viral vector has an insert size limit of 2.5 kb our insert was 2.3 kb. To enhance the viral spreading in the chicken cells we reduced the insert size of the construct, this was achieved by removing the GFP coding sequence. Further, we changed the viral backbone from RCASBP-A to RCASBP-B as the latter might infect embryonic tissue derived from neural crest cells with a higher efficiency. Additionally, we injected the vector in embryos 24 hrs post fertilization allowing more time for replication. Earlier injection of the embryos resulted in a high loss rate, as well as generally malformed embryos. This might be caused by the high fragility of the embryos at this early stage or by the toxicity of the expressed mRNA resulting in an embryonic lethal phenotype. To reduce the high mortality of the embryos we injected the virus without ink or fast green, to avoid a possible teratogenic effect. A second attempt was to electroporate the viral DNA directly into the neural tube. We also varied the injection time-point. Embryos were injected at Hamburger Hamilton (HH) stage 3 (12-13 hrs post fertilization) to stage 10 (33-38 hrs post fertilization).

Finally, the infection rates of the chicken cells turned out to be very heterogeneous. Electroporation was the transfection method resulting in the lowest loss-rate of embryos and in most efficient infection rate. However, we observed no specific lower jaw phenotype in the chicken

embryos before they died. At that point we decided to no more continue the experiments, due to low chances to mimic the human fetal phenotype in this model.

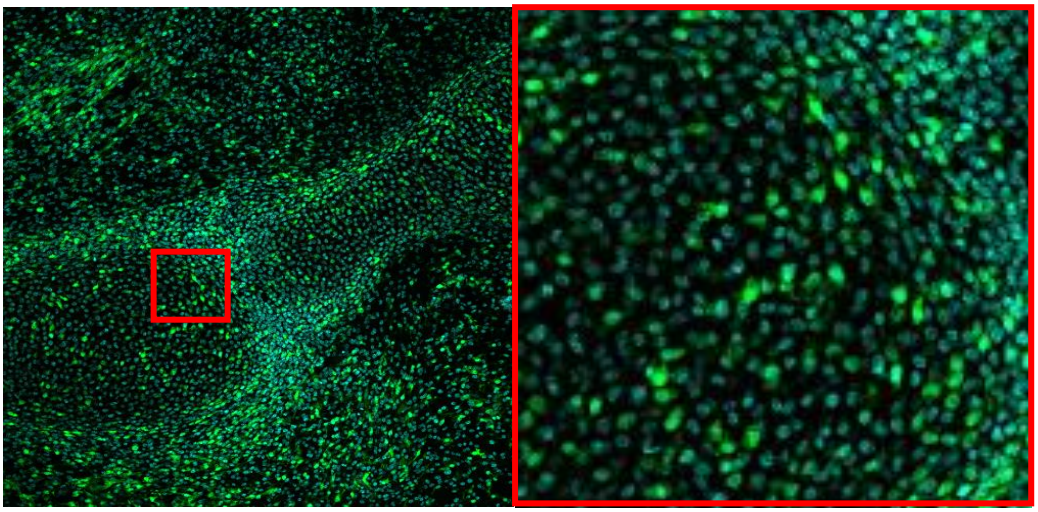

Figure 2: Retroviral transfected chicken embryo
Chicken embryos were transfected with the RCAS viral particles expressing the mutated SMAD3 protein (green=2a-GFP). The picture shows the region where the lower jaw will be formed. Nuclei were stained with DAPI (blue). Not all cells are expressing the viral construct respective the GFP, indicated by lack of GFP-staining.

In parallel colleagues detected a loss-of-function variant in *SMAD3* in a patient with holoprosencephaly[55]. This study concluded that most genes in which variants cause a holoprosencephaly phenotype act in the TGF-β, hedgehog and FGF signaling pathways. This further supports our hypothesis of agnathia-otocephaly being an SMAD3-related phenotype, since agnathia-otocephaly and holoprosencephaly can occur as combined phenotype[56–60] We were not able to identify additional agnathia-otocephaly fetuses with *SMAD3* variants when comparing exome data with international colleagues. There are, however, only few collected families due to the rarity of this phenotype. SMAD3 can also act as an effector in the BMP pathway. Alterations of components in this pathway are associated with different craniofacial abnormalities. Variants in the *BMP4* gene are reported to cause syndromic Microphthalmia (OMIM: 607932) including micrognathia and orofacial clefting (OMIM: 600625) in humans[61–63], and a lack of the

corresponding Alk2 receptor causes micrognathia and cleft palate in mice[64]. Regarding these evidences mechanisms may be much more complicated and will need further study in the future[65].

In the *PTK7* gene two compound heterozygous variants were detected. *PTK7* was not reported so far to be related to human disease phenotypes. The variants we identified are in a transcript, which codes for an alternative protein isoform of PTK7 (isoform e, NM_001270398). The function of this isoform was never assessed before. PTK7 is a transmembrane protein that is active in the planar cell polarity (PCP) and the Wnt signaling pathway[66–68]. Spontaneous mutations in mice resulted in severe neural tube defects (NTDs) with exencephaly and microphthalmia[69,70]. We used overexpression of the uninvestigated isoform of the protein tyrosine kinase 7 (PTK7) in a human embryonic kidney (HEK293) cell culture model in order to confirm a functional impact when altered. With a phosphorylation and a migration assay we aimed at elucidating a deleterious effect of the mutated isoform.

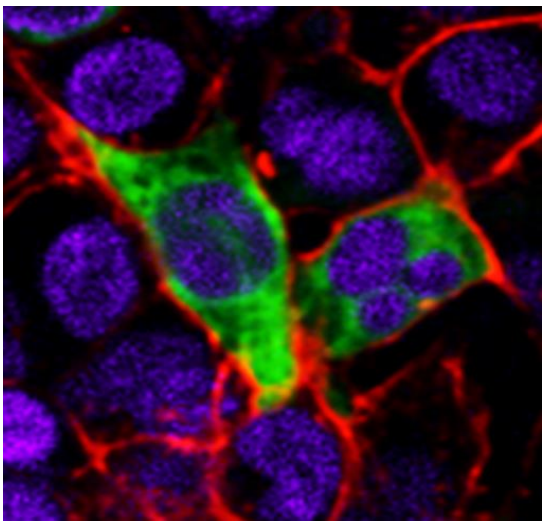

Figure 3: ***Expression of PTK7 isoform e***.
In HEK293 cells isoform e is not located in the cell membrane. Blue: nucleus, Red: cell membrane, Green: PTK7

We showed that PTK7 is expressed in the fetal liver and that the corresponding mRNA is downregulated in the affected fetus (Publication 2, Supplementary Table 3). If isoform a is overexpressed it locates in the membrane of cells and gets translocated if stimulated by canonical

Wnt proteins[66]. However, we showed by overexpression of the protein in HEK293 cells (Figure 3) that the function of isoform e may differ from isoform a as it is not located in the cell membrane. We assessed the impact of the two detected variants in a cell culture model that expresses two plasmids, each carrying one of the variants in the *PTK7* sequence, to mimic a recessive state. We optimized transfection conditions of both plasmids based on the same backbone and carrying different fluorescent proteins. We observed that the plasmid with the red fluorescent mCherry, which was cloned into the original vector, transfected the cells more efficiently than with the GFP. If cells were transfected with a 1:1 mixture of both plasmids more cells were mCherry positive than GFP positive. Rising the amount of the GFP plasmid up to 3-fold of the mCherry resulted in an evenly distribution of cells carrying both plasmids.

Cells were sorted by fluorescence activated cell sorting (FACS), but one day after the sorting cells were not fluorescent anymore. Confocal microscopy showed that the cells are forming endosomal structures, containing both fluorescent proteins, prior to lose fluorescence, which may indicate that they disposed the plasmids. Since this effect repeatedly occurred two days after transfection, the amount of transient overexpressing cells was too low after 48 hrs of cell culture. If the cells were transfected with one plasmid, fluorescence was stable for at least one week. This showed us that the cells are disposing the additional proteins but only if both plasmids carrying the variants are co-transfected. A possible explanation is that the expression of PTK7 isoform e is tissue specific and not naturally expressed in embryonic kidney cells and therefore they are not that tolerant to overexpression of this specific isoform, which means we chose the wrong cell type for the experiments. However, if the cells were not tolerant to the overexpression of both plasmids at the same time, this also may suggest an equivalent to early lethality. An option for further experiments might be to reproduce results on a different cell-line, e.g. hepatic cells or to knockout or knockdown the *PTK7* gene in the cells, to express only the mutated isoform and to prevent a

potentially toxic threshold for protein abundance. In general CRISPR-Cas based animal or cell culture models may be an option for functional investigations in fetal anomaly studies since they have been shown to confirm the phenotypic effect of candidate variants previously.[71,72]. However, several restrictions apply as for example the choice of the model organism based on sequence homology and differences in protein expression between species.

3. Publications

3.1 Publication 1

A novel homozygous splice-site mutation in *RYR1* causes fetal hydrops and affects skeletal and smooth muscle development.

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PRENATAL DIAGNOSIS

RESEARCH LETTER

A novel homozygous splice-site mutation in RYR1 causes fetal hydrops and affects skeletal and smooth muscle development

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Conflicts of interest: None declared

The skeletal ryanodine receptor 1 (RYR1) is a calcium release channel that is essential for excitation–contraction coupling in the sarcoplasmic reticulum of skeletal muscles.[1] Mutations in the *RYR1* gene cause RYR1 protein dysfunction which clinically manifests in a wide spectrum of disease phenotypes of varying symptoms, disease onset and severity. Autosomal dominant mutations cause susceptibility to malignant hyperthermia (MHS) or *RYR1*-related congenital myopathies such as multi-minicore disease, central core disease (OMIM:117000) and core-rod myopathy, with the latter two reported to be lethal.[2,3] Recessively inherited variants have been described to manifest during fetal development. The lethal multiple pterygium syndrome (LMPS, OMIM #253290) presenting with fetal akinesia during pregnancy represents the lethal end of the spectrum of *RYR1* associated phenotypes.[4] The study was approved by the Ethics Commission Northwest Switzerland (EKNZ 2014-174). Written informed consent for participation and publication of clinical data and photos were obtained from the participants.

We report on a non-consanguineous family of Turkish descent who had a reproductive history of one early miscarriage, the birth of two healthy daughters and a fourth pregnancy terminated for fetal hydrops without further investigations. The parents came to our attention after their fifth pregnancy for which the mother reported phenotype recurrence. After she had noticed reduced fetal movement, an ultrasound scan confirmed multiple contractures and hydrops fetalis. The fetus died in utero at the 24th week of gestation. High-resolution chromosomal microarray did not reveal a causal chromosomal abnormality. The post-mortem examination of the fetus confirmed fetal hydrops and bilateral flexion contractures of the joints of the limbs as well as bilateral clubfeet (Figure 1a). Mild pterygia were recognizable in the elbow joints. Autopsy revealed a pronounced generalized hypoplasia of the skeletal muscle tissue including the diaphragm (Figure 1b). Myocardial and esophageal muscles (Figure 1c) were hypoplastic as well. The small intestine showed a 'string of beads' like structure (Figure 1d) with hypertrophy of the walls and alternating hypoplastic segments of thin smooth muscle layers. Due to the finding of a significant generalized hypoplasia of skeletal and smooth muscles, we hypothesized a primary defect of muscle development, leading to the prenatal clinical presentation of arthrogryposis and hydrops in the fetus. Lethality of the condition and potential phenotype recurrence based on the reproductive history suggested an autosomal recessive condition.[5]

In order to delineate the etiology, we performed family based whole exome sequencing (WES) using the trio of fetal DNA extracted from formalin fixed paraffin embedded (FFPE) tissue and parental DNA extracted from blood samples. Tissue or DNA from the previous affected pregnancy was not available.

Library preparation was performed with the Agilent SureSelect^{XT} Library Prep Kit and enriched with Agilent SureSelect^{XT} Human All Exon V6 (Agilent, Santa Clara, California). Paired-end read sequencing (2x100bp read length) was accomplished on a HiSeq 2500 platform (Illumina, San Diego, CA).

Mapping to the reference genome sequence (hg19) and alignment were carried out using Burrows-Wheeler Aligner (BWA-mem Version 0.7.2). Alignments with a Phred quality score below 30 for the entire read and potential PCR duplicates were discarded using samtools (Version 0.1.18). To get a high sensitivity, variant calling was performed with samtools and varscan (Version 2.3.5). Hypothesizing a rare Mendelian disorder, we annotated variants as known or novel according to the presence or absence in curated databases. Variants were filtered on a heterozygous population frequency (GMAF) of <5% in control databases (dbSNP142, ExAC, 1000G, gnomAD) and absence of homozygosity in healthy individuals (ExAC). We prioritized variants according to their potential to disrupt protein function including the use of prediction tools (SIFT, Provean, Polyphen2, Mutationtaster, Human Splicing Finder v.3.0), the amino acid conservation (PhyloP, PhastCons) and the American College of Medical Genetics (ACMG) variant classification guidelines.[6] Prioritized variants were inspected for phenotype–genotype correlations reported in humans and other species, based on the medical literature and public databases including functional and expression data (Pubmed, ClinVar, OMIM, HGMD, Uniprot, UniGene, www.zfin.org;

www.informatics.jax.org;
www.mousephenotype.org).

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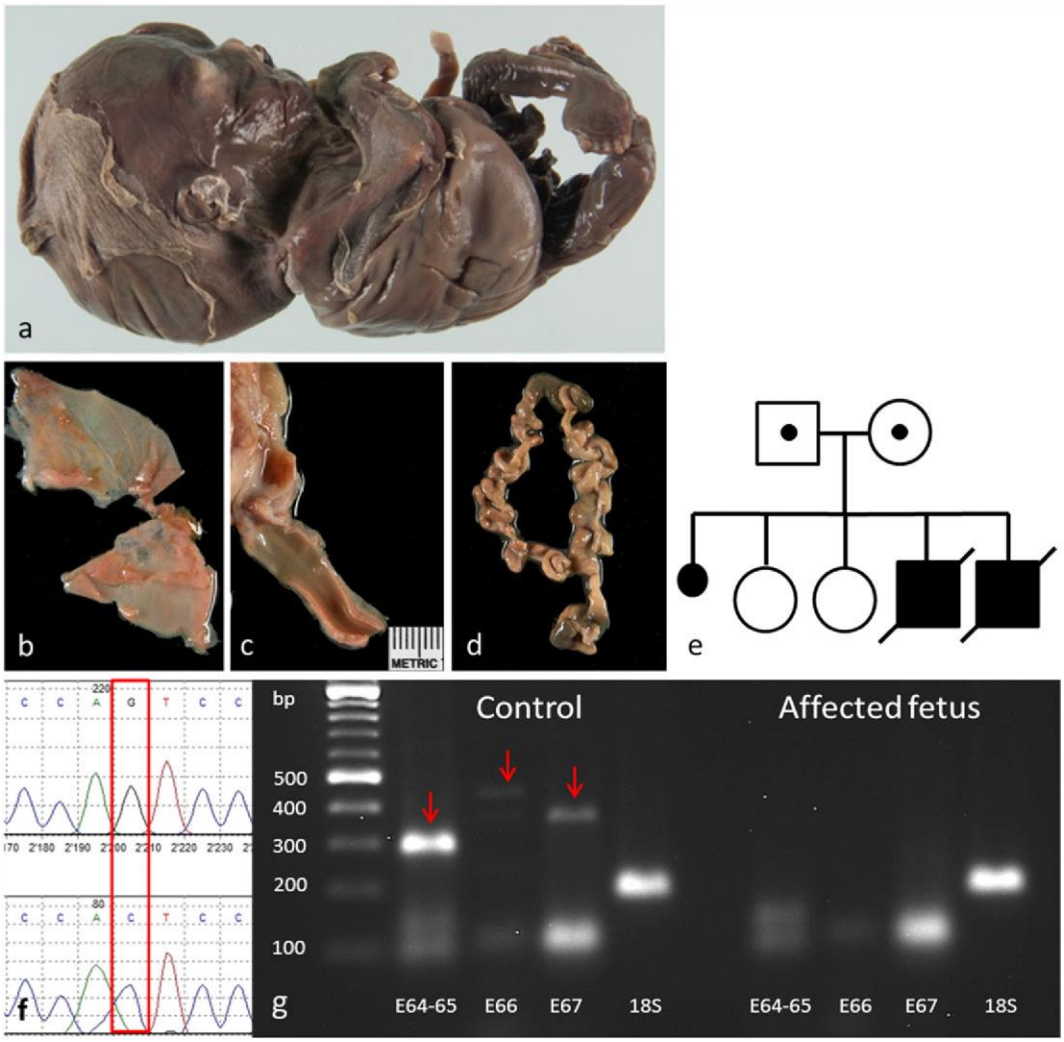

Figure 1: Formalin fixed affected fetus (a). Note flexion arthrogryposis, presence of fluid in the nuchal and abdominal region reminiscent of hydrops and bones visible through skin due to the absence of muscle tissue. Hypoplastic diaphragm (b), esophagus (c) and intestine with a "string of beads" like structure (d). Pedigree (e). Sanger sequencing of the fetal DNA (f) validates the homozygous *RYR1* mutation (c.9686– 1 G > C). PCR products from the cDNA show the presence of exons 64–67 (g) in the control tissue, but their absence in the affected fetus. The bright bands at E67 were identified as primer–dimers. We used 18S RNA primers as quality control.

We prioritized a homozygous splice acceptor-site mutation in the *RYR1* gene at position c.9686-1G > C (NM_001042723.1, chr19:39007998, hg19) as the best candidate out of three homozygous and 28 compound heterozygous mutations. We chose this variant because (1) it was the only truncating mutation, (2) it was absent in any of the databases and therefore novel, (3) it is located in a gene playing a role in muscle development and function and (4) database and literature search identified mutations in the same gene, causing similar phenotypes in humans and other species. Other variants were predicted to be functionally benign, within genes of unknown function without correlation to the phenotype or related pathways. Two homozygous variants in the *COL6A3* gene were reported in a homozygous state in healthy individuals (ExAC database). The novel *RYR1* variant identified is situated in the 3^0 splice-site of exon 66 and is predicted to cause alternative splicing (HSF 3.0, Mutation Taster). According to the ACMG guidelines, the mutation fulfills the criteria for a very strong evidence of pathogenicity (PVS1, PS3, PM2, PP3, PP4).[6] Sanger sequencing using standard polymerase chain reaction (PCR) amplification procedures confirmed homozygosity in the fetus (Figure 1f) and heterozygosity in both unaffected parents. The mutation is located in a 5.37 Mb region of absence of heterozygosity (AOH) in the fetus (ngCGH, Nexus copy number software, Bio discovery). However, the proportion of the autosomal genome (0.9% for stretches of homozygosity >5 Mb) does not indicate consanguinity.[7] In order to confirm the mutational impact on the *RYR1* transcript, whole RNA from skeletal muscle FFPE tissue of the affected fetus, and an age-matched control tissue was extracted following the protocol of the RecoverAll™ Kit (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts). Reverse transcription was performed with the SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts) and accomplished with the Taq DNA Polymerase Kit (Qiagen, Germantown, Maryland). Sanger sequencing confirmed correct exon sequences. Analysis of exons 64–67 of the *RYR1* gene showed their absence in the affected fetus (Figure 1g). Nonsense medicated decay was excluded by the presence of exon 20 and 90 (data not shown) indicating that alternative splicing, leading to an aberrant protein by exon skipping, is the likely mechanism. Because exon 66 encodes amino acids in the α-Solenoid 2 domain of the RYR1 protein which is part of the machinery that reacts to conformational changes to open the receptor for calcium efflux,[8] the α-Solenoid 2 domain is likely misfolded and functionally impaired.

Table 1 **Phenotype genotype correlation of lethal RYR1 phenotypes**

Reference	Mutation	Inheritance	Gestational age	Arthrogryposis	Lung hypoplasia	Cystic hygroma	Hydrops	Hydramnios	Kyphoscoliosis	Amyotrophy	Pterygia	Smooth muscle hypoplasia
This study	c.9686-1G > C	AR	24 wg	+	+	–	+	–	+	+	+	+
2	p.Ile4898Thr	de novo, AD	birth	+	ni	–	–	+	+	+	ni	ni
2	p.Ile4898Thr	de novo, AD	birth	+	ni	–	–	+	+	+	ni	ni
3	p.Arg614Cys, p. Gly215Glu	AR	32 wg	+	ni	ni	ni	+	ni	+	ni	ni
3	p.Gly4899Glu	AD	8 d	ni	ni	ni	ni	+	ni	+	ni	ni
4	p.Arg2242*	AR	12 wg	+	ni	ni	+	ni	ni	ni	+	ni
4	p.Glu699_Gly707del	AR	18 wg	+	ni	+	+	ni	ni	ni	+	ni
4	p.Glu2347del	AR	23 wg	+	+	+	ni	+	+	+	–	ni
4	p.Glu2347del	AR	21 wg	+	+	+	ni	ni	+	+	+	ni
10	c.14130-2A > G, p. Ser3074Phe	AR	termination of pregnancy	+	+	–	+	ni	ni	ni	+	ni
11	p.Trp3284Ter, p. Thr1523Pro	AR	18 wg	+	–	+	+	ni	ni	ni	+	–
12	p.Glu1997Lys, p. Arg819*	AR	birth	+	ni	ni	ni	ni	ni	ni	ni	ni
12	p.Glu1997Lys, p. Arg819*	AR	32 wg	+	ni	ni	+	+	ni	ni	ni	ni
12	p. Arg2241*, p. Thr4882Met	AR	25 wg	+	+	ni	+	ni	ni	+	ni	ni
12	p. Arg2241*, p. Thr4882Met	AR	29 wg	+	+	ni	–	+	ni	+	ni	ni
13	p.Gly705Arg, p. Ser2948Cysfs*58	AR	27 wg	+	+	ni	+	+	ni	+	ni	ni
13	p.Gly705Arg, p. Ser2948Cysfs*59	AR	25 wg	+	+	ni	+	+	ni	+	ni	ni

AR, autosomal recessive; AD, autosomal dominant; wg, week of gestation; f, female; m, male; NI, no information.

Hydrops fetalis is a well-known fetal phenotype, and its etiology is highly heterogeneous, but was – so far – not necessarily linked to congenital disorders of muscle development.[9] We describe a fetus diagnosed with hydrops fetalis for which post-mortem examination identified impaired skeletal and smooth muscle development to be the underlying condition caused by the novel homozygous splice site mutation c. 9686-1G > C in the 3^0splice-site of exon 66 in the *RYR1* gene. Due to the sparsity of the muscle tissue and its autolysis, we were not able to further investigate histopathological signs of core-rod or other *RYR1* related myopathies. Lethal phenotypes caused by recessively inherited mutations in the *RYR1* gene presenting with hypoplasia of skeletal muscles have been rarely documented, and in only half of these patients, a fetal hydrops has been reported. A detailed phenotypic comparison is shown in Table 1.

In all but one of these patients, at least one of the mutations in *RYR1* is truncating, and in only one patient a compound heterozygous splice site mutation has been reported.[10] Mutations in the *RYR1* gene causing the LMPS phenotype have been described in three studies so far.[4,10,11]

The RYR1 receptor is expressed in the skeletal muscle tissue in humans, but its expression in smooth muscle tissue has been demonstrated in rats.[14] For the first time, we report here on the finding of hypoplastic smooth muscle tissue in humans caused by *RYR1* mutations (Table 1 and Figure 1a– d). Thus, dysfunction of the RYR1 protein does not only impair skeletal muscle development but also primarily affects the development of smooth muscle, which previously was often attributed to the absence of movement. In the dyspedic knock-out mouse-model[15] a homozygous mutation in the *RYR1* gene causes a complete loss of function of the protein resulting in a comparable phenotype. Homozygous mice show cystic hygroma and skeletal muscle hypoplasia including hypoplastic diaphragm, leading to perinatal death. The effect on the expression of over 300 genes (up- or down-regulation),[16] including components of the MAPK signaling pathway, the Wnt-signaling pathway as well as Gprotein coupled receptors, illustrates the important role of this receptor in early human development. Twenty-one genes were identified as related to muscle functionality such as muscle force production and contraction, and another 22 were related to structural features of muscle tissue. Because the absence of the RYR1 channel affects many developmental pathways, this protein may be more important to early fetal development and phenotype diversity than currently thought. In the presence of fetal hydrops, a congenital muscle disease, which is often difficult or not possible to assess by ultrasound, should be considered. *RYR1* should be added to the panels of diagnostic sequencing for severe fetal disorders.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Mutations in the RYR1 gene can cause variable phenotypes including autosomal dominant malignant hyperthermia, multiminicore disease, core-rod myopathy and autosomal recessive lethal multiple pterygium syndrome at the severe end of the spectrum.

WHAT DOES THIS STUDY ADD?

- Autosomal recessive mutations in RYR1 cause non-immune fetal hydrops more often than previously thought. These mutations affect skeletal muscle development but also impact smooth muscle tissue in early human development. RYR1 should be added to sequencing panels in prenatal diagnostics.

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3.2 Publication 2

Exome sequencing of fetal anomaly syndromes: novel phenotype-genotype discoveries

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ARTICLE

Exome sequencing of fetal anomaly syndromes: novel phenotype–genotype discoveries

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Abstract

The monogenic etiology of most severe fetal anomaly syndromes is poorly understood. Our objective was to use exome sequencing (ES) to increase our knowledge on causal variants and novel candidate genes associated with specific fetal phenotypes. We employed ES in a cohort of 19 families with one or more fetuses presenting with a distinctive anomaly pattern and/or phenotype recurrence at increased risk for lethal outcomes. Candidate variants were identified in 12 families (63%); in 6 of them a definite diagnosis was achieved including known or novel variants in recognized disease genes (*MKS1*, *OTX2*, *FGFR2*, and *RYR1*) and variants in novel disease genes describing new fetal phenotypes (*CENPF*, *KIF14*). We identified variants likely causal after clinical and functional review (*SMAD3*, *KIF4A*, and *PIGW*) and propose novel candidate genes (*PTK7*, *DNHD1*, and *TTC28*) for early human developmental disease supported by functional and crossspecies phenotyping evidence. We describe rare and novel fetal anomaly syndromes and highlight the diagnostic utility of ES, but also its contribution to discovery. The diagnostic yield of the future application of prenatal ES will depend on our ability to increase our knowledge on the specific phenotype–genotype correlations during fetal development.

Supplementary information The online version of this article (https://doi.org/10.1038/s41431-018-0324-y) contains supplementary material, which is available to authorized users.

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Introduction

Birth defects are the leading cause of perinatal lethality in industrialized countries [1]. With the advance of highresolution ultrasound fetal structural anomalies are now
detected increasingly early during pregnancy, raising questions about diagnosis, etiology, prognosis, and recurrence risk for parents and health care providers, particularly in the presence of more than one fetal anomaly likely indicating a congenital malformation syndrome. Prenatal high-resolution chromosomal microarray analysis will allow a diagnosis of causal copy number variants in up to additional 10% of pregnancies after the exclusion of the frequent aneuploidies, but 80– 90% of families remain without definite diagnosis. Targeted molecular testing may be indicated if a specific monogenic entity is suspected based on clinical signs. The monogenic etiology of phenotypes observed postnatally has been successfully investigated by using genome-wide sequencing technologies [2]. Exome or genome approaches have now become available in routine clinical genetics services for the diagnosis of patients with developmental disorders. Only recently, however, the delineation of fetal anomaly phenotypes received increasing attention including the discussion of introducing exome sequencing (ES) in standard prenatal care. Best et al. [3] discussed its promises and pitfalls including the review of 31 studies with series of five or more fetuses and diagnostic rates varying between 6.2% and 80%. Approaches in such studies are highly heterogeneous ranging from prospective prenatal ES to the study of selected cases with severe fetal anomalies after termination or stillbirth [4]. In general, the diagnostic yield seems to be higher in fetuses with multiple congenital anomalies and in selected series with detailed clinical genetics review [3]. However, a significant proportion of anomaly phenotypes encountered during the antenatal developmental stages may be specific to fetal life since they will lead to embryonic, fetal or perinatal lethality and will have escaped etiological research and clinical delineation so far. They may also represent an incomplete or severe allelic presentation of a phenotype described to occur postnatally, and the diagnosis therefore can remain unrecognized at this stage of development. Filges and Friedman [5] stressed the value of applying genomic sequencing to examine such rare extreme phenotypes despite the challenges to expect when interpreting the clinical significance of variants and proving their causality. Based on these considerations we explored the clinical and molecular diagnosis using ES in a series of 19 families with one or more fetuses with severe structural anomalies. We aimed at identifying causal as well as candidate variants, showing the utility of ES for diagnosis but also discovery, and sought to contribute to the further delineation and etiology of phenotype presentations of known and novel multiple congenital anomaly syndromes in fetal stages intending to increase the yield of future prenatal diagnostic ES.

Patients and methods

The study was approved by the Ethics Commission Northwest Switzerland (EKNZ 2014-174). After

written informed consent for participation was obtained from the parents, we prospectively recruited 19 families with 26 fetuses (after fetal or perinatal death or from terminated pregnancies) and one child—that presented with severe anomalies of unexplained etiology initially identified by ultrasound scanning. Families were included in the study if (i) the fetus showed a pattern of two or more anomalies associated with a high risk for fetal or perinatal lethality that suggested a genetic disorder or (ii) there was familial recurrence of the fetal anomaly phenotype and if (iii) there were detailed clinical fetal ultrasound and/or autopsy data available and (iv) high-resolution chromosomal microarray did not show a causal chromosomal anomaly or copy number variant. Families were recruited through the Medical Genetics Clinic at the University Hospital Basel, Switzerland. The fetal phenotypes were reviewed by experienced clinical geneticists, fetal and neuropathologists, and maternal-fetalmedicine specialists. Autopsy was performed in affected fetuses of 18 families.

Exome sequencing (ES) and variant prioritization

Genomic DNA was used from previous prenatal specimen extracted from chorionic villus sampling, amniocentesis or extracted from fresh frozen or formalin fixed paraffin embedded (FFPE) fetal tissue. Parental DNA was extracted from whole-blood samples. ES was performed in familial trios (or quattros, when available after recurrence). Library preparation (Agilent SureSelect^{XT} Library Prep Kit) and exome capture using the Agilent SureSelect^{XT} Human All Exon V6 (Agilent, Santa Clara, CA) was followed by paired-end read sequencing (2 × 100 bp read length) on a HiSeq 2500 or HiSeq 4000 platform (Illumina, San Diego, CA) with an average coverage of ×100. Quality estimation of the sequence reads was performed by generating quality control statistics with FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Illumina CASAVA (1.8.2) was used to demultiplex the sequencing reads. Adapter trimming was performed with Skewer (Version 0.2.2). Sequencing reads were mapped and aligned to the reference genome sequence (hg19) with Burrows-Wheeler Aligner (BWAmem Version 0.7.2). Alignments with a Phred quality score below 30 for the entire read and potential PCR duplicates were discarded using samtools (Version 0.1.18). To increase sensitivity, variant calling was performed with samtools and varscan (Version 2.3.5). Variants with a coverage $\leq$10X and not supported by at least 4 reads (20%) were discarded. Familial segregation of variants was performed to identify de novo, autosomal recessive and Xlinked inheritance. Variants were filtered on a heterozygous population frequency (GMAF) of <5% in control databases (dbSNP142, 1000G, gnomAD), and absence of homozygosity in healthy individuals (ExAC) and classified as known or novel according to their presence or absence in curated databases. Variants were prioritized according to their potential to disrupt protein function using standard prediction tools (SIFT, Provean, Polyphen2, Mutationtaster, Human Splicing Finder v.3.0), amino acid conservation assessment across species (PhyloP, PhastCons) and the American College of Medical Genetics and Genomics (ACMG) variant classification guidelines [6]. In addition, we applied the HOPE protein prediction program (http://www.cmbi.ru.nl/hope/) which combines structural information including the 3D structure of the protein, if available, to analyze the specific mutational effect on the protein structure. Putative candidate variants were visually reanalyzed in each family using the Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/) and confirmed by Sanger sequencing (primer sequences are available on request). Genotype-phenotype correlations were considered critical for identification of the causal relationship between a candidate variant and malformation anomaly phenotype. Existing information was compiled from medical literature and databases (Pubmed, OMIM, ClinVar, Decipher) and for related protein networks and signaling pathways in embryonic development (Reactome, Uniprot, Ingenuity pathway analysis (Qiagen)). We systematically interrogated zebrafish, mouse and drosophila phenotype databases (www.zfin.org;www.informatics.jax.org; www.mousephenotype.org; www.flybase.org) using cross-species phenotype comparison for validation of

Table 1 Fetal phenotypes and disease-associated and candidate variants identified

Affected individual	Gestational age and sex	Phenotype	Gene	Variants	Type of variant	Inheritance	Variant classification[a]
Family 1, II.1	14 GA male	Meckel-Gruber syndrome	*MKS1* OMIM: 609883	NM_017777.3: c.[417G>A];[417G>A], p.E139 = MAF: 1.9×10^{-4}	Splicing affected	Autosomal recessive	Affects function, Pathogenic (II)
Family 2, II.1	Birth male	Agnathia-Otocephaly complex	*OTX2* OMIM: 600037	NM_001270523.1: c.[746delG], p.(G249Vfs*45)	Frameshift	De novo	Affects function, Pathogenic (Ia)
Family 3, II.3	22 + 3 GA male	Corpus callosum agenesis, small brain, mesocardia, syndactylies on hands and feet, Apert syndrome	*FGFR2* OMIM: 176943	NM_000141.3: c.[755C>G], p.(S252W) MAF: 9×10^{-6}	Missense	De novo	Affects function, Pathogenic (Ia)
Family 4, II.4	24 + 5 GA, male	Hydrops, skeletal and smooth muscle hypoplasia	*RYR1* OMIM: 180901	NG_008866.1(NM_001042723.1): c.[9686-1G>C];[9686-1G>C]	Splice site	Autosomal recessive	Affects function, Pathogenic (Ia)
Family 5, BII-2 Filges et al., 2016	32 + 2 GA female	Microphthalmia, Xiphoid cleft, Hydronephrosis, duodenal atresia and «apple peel» atresia	*CENPF* OMIM: 600236	NM_016343.3: c.[1744G>T];[9280C>T], p.(E582*);(R3094) MAF: 2.5×10^{-5}	Nonsense	Autosomal recessive	Affects function, Pathogenic (Ia)
BII-3 Filges et al., 2016	22 GA male	Xiphoid cleft, bilateral renal hypoplasia, duodenal atresia type III, jejunal atresia, string of beads appearance	*CENPF* OMIM: 600236	NM_016343.3: c.[1744G>T];[c.9280C>T] p.(E582*);(R3094*) MAF: 2.5×10^{-5}	Nonsense	Autosomal recessive	Affects function, Pathogenic (Ia)
Family 6, Fetus 1 Filges et al., 2014	21 + 4 GA female	Cerebral hypoplasia, cerebellar hypoplasia, agenesis of occipital lobes, bilateral renal agenesis, ureteral agenesis, uterine hypoplasia	*KIF14* OMIM: 611279	NM_014875.2: c.[1750_1751delGA];[1780A>T], p.(E584Ifs*16);(R594*)	Frameshift, Nonsense	Autosomal recessive	Affects function, Pathogenic (Ia)
Fetus 2 Filges et al., 2014	18 + 5 GA female	Corpus callosum agenesis, cerebral hypoplasia, arhinencephaly, bilateral renal hypoplasia and cystic dysplasia, ureteral hypoplasia, uterine hypoplasia, vaginal atresia	*KIF14* OMIM: 611279	NM_014875.2: c.[1750_1751delGA];[1780A>T], p.(E584Ifs*16);(R594*)	Frameshift, Nonsense	Autosomal recessive	Affects function, Pathogenic (Ia)
Family 7, II.1	20 GA male	Agnathia-Otocephaly complex	*SMAD3* OMIM: 603109	NM_005902.3: c.[860G>A], p.(R287Q)	Missense	De novo	Probably affects function, Likely pathogenic (II)
Family 8, II.1	22 + 3 GA male	Hydrocephalus internus	*KIF4A* OMIM: 300521	NM_012310.4: c.[2096T>A], p.(V699E)	Missense	X-linked recessive	VUS
Family 9, II.1	18 GA male	Dandy Walker malformation, hydronephrosis, genital hypoplasia	*PIGW* OMIM: 610275	NM_178517.3: c.[106A>G];[106A>G], p.(R36G) MAF: 2×10^{-4}	Missense	Autosomal recessive	Probably affects function, Likely pathogenic (II)
II.2	12 GA female	Dandy Walker malformation, dysplastic kidneys, hydronephrosis, diaphragmatic hernia	*PIGW* OMIM: 610275	NM_178517.3: c.[106A>G];[106A>G], p.(R36G) MAF: 2×10^{-4}	Missense	Autosomal recessive	Probably affects function, Likely pathogenic (II)
Family 10, II.1	22 GA female	Brain malformations, unilateral anophthalmia, hepatomegaly, bile duct atresia, Mullerian duct agenesis	*PTK7* OMIM: 601890	NM_001270398.1: c.[19G>A];[1238A>G], p.(G7R);(N413S) MAF: 0.007, MAF: 3×10^{-4}	Missense	Autosomal recessive	VUS
Family 11, II.1	22 + 5 GA female	Hypoplastic left heart, persisting left cardinal and vena cava with estuary in the left atrium, atrial septum defect type II, fused lung lobes, absent extrahepatic bile ducts, intestinal stenosis and pouch	*DNHD1* OMIM: 617277	NM_144666.2: c.[6109A>G];[6109A>G], p.(S2037G) MAF: 0.003548	Missense	Autosomal recessive	VUS
Family 12, II.2	33 + 1 GA male	Duodenal atresia III, jejunal and ileal atresia	*TTC28* OMIM: 615098	NM_001145418.1: c.[3638A>G];[794A>C], p.(D1213G);(K165T)	Missense	Autosomal recessive	VUS

GA gestational age in weeks, *MAF* Minor allele frequency (gnomAD), *VUS* variant of unknown significance

[a]Nomenclature and variant classification according to HGVS Version 15.11 (https://varnomen.hgvs.org) and ACMG guidelines [6]

candidate genes. GeneMatcher (genematcher.org) was queried for similar cases. We used the HGVS nomenclature Version 15.11 to describe variant effects (https://varnomen.hgvs.org/). All variants identified were submitted to the LOVD database (https://databases.lovd.nl/shared/individuals 00181103-09, 00181141, 00181143-49).

Functional studies

In 3 fetuses, FFPE tissue from affected organs was used against age-matched control fetal FFPE tissue for reverse transcription analysis of the candidate gene to assess the presence or absence of the transcript. Control tissue was obtained from fetuses without structural anomalies confirmed by autopsy after intrauterine fetal death likely due to asphyxia. RNA from FFPE tissue specimens was isolated with the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts). Reverse transcription (RT) of mRNA was per- formed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). 18S RNA primers served as quality control. In 2 fetuses, additional qPCR was used to confirm the lower concentration of the transcript. qPCR was performed with the SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA). Expression levels of the transcripts were normalized to *GUSB* expression.

Results

Phenotypes and causal or candidate variants, if identified, are summarized in Table 1. Supplementary Table S1 shows the unsolved cases. The frequency of congenital anomalies in different organ systems is displayed in Supplementary Table S2. We identified variants that would cause or potentially explain the fetal anomaly phenotype in 12 out of 19 families (63%). A definite diagnosis was achieved in 50% of those (6 families), while in the remaining 6 families variants had to be classified as variants of unknown significance according to the ACMG guidelines [6]. Variants in 12 different genes were identified (*RYR1*, *MKS1*, *FGFR2*, *PIGW*, *CENPF*, *KIF14*, *SMAD3*, *KIF4A*, *PTK7*, *DNHD1*, *TTC28*, and *OTX2*). Variants that were classified to affect or likely affect function were detected in 8 different genes (*RYR1*, *MKS1*, *FGFR2*, *PIGW*, *CENPF*, *KIF14*, *SMAD3*, and *OTX2*). The variants identified are categorized into the following groups: (1) a priori diagnostic, since they were known or novel in known disease genes explaining the fetal anomaly phenotype, (2) confirmed to be disease-associated by the identification of additional families and/or functional analysis, and thus led to the description of a new disease gene and related phenotype, (3) likely disease-associated by clinical and functional review in (a) a known disease gene or (b) a new candidate gene.

Known or novel variants in disease genes explaining fetal anomalies

We identified variants that affect or likely affect protein function in *MKS1*, *OTX2*, *FGFR2*, and *RYR1*. The homozygous variant in *MKS1* was previously reported to affect splicing [7] and explained a recurrent Meckel-Gruber syn- drome-like phenotype in family 1. Variants in *OTX2* are reported to cause agnathia-otocephaly in rare instances [8]. Although novel, the de novo frameshift variant (c. [746delG], p.(G249Vfs*45)) was considered to affect function, causing the typical phenotype of agnathia-otocephaly in family 2. In a fetus with bilateral syndactyly of hands and feet and corpus callosum agenesis (family 3), the molecular diagnosis of Apert syndrome was made by identifying a known pathogenic de novo variant in *FGFR2* [9]. For family 4, we confirmed previously that a novel homozygous splice-site variant in the skeletal ryanodine receptor 1 gene (*RYR1*) causes lethal hydrops and severe skeletal and smooth muscle hypoplasia [10].

Variants in novel genes confirmed to be causal for new fetal phenotypes

In family 5, previously reported by Filges et al. [11], novel compound heterozygous truncating variants in *CENPF* were identified in a fetus with a suggested clinical diagnosis of Strømme syndrome and a sibling with a severe malformation phenotype reminiscent of a ciliopathy phenotype (c.[1744G>T];[c.9280C>T], p.(E582*);(R3094)). The identification of variants in the same gene in unrelated families and functional zebrafish results confirmed causality and a variable phenotype syndrome to a lethal fetal ciliopathy [11, 12].

Novel compound heterozygous truncating variants were identified in *KIF14* (c.[1750_1751delGA];[1750A>T], p. (E584Ifs*16);(R594*)) in two fetuses with a ciliary phenotype not compatible with any described syndrome in family 6 [13]. This was the first report of a human phenotype caused by bi-allelic truncating variants in *KIF14*. The authors suggested *KIF14* to be a candidate gene for allelic viable phenotypes including isolated microcephaly, which was now confirmed in several patients harboring disease associated variants in *KIF14* as well [14].

Candidate variants—likely disease-associated by clinical and functional review

In 6 families potential candidate variants were identified in genes for which other variants are known to cause a postnatal phenotype that, however, significantly differs from the phenotype observed in the fetus (*SMAD3*, *KIF4A*, *PIGW*) or that are currently not known to cause a human phenotype at all (*TTC28*, *PTK7*, *DNHD1*). They were prioritized due to their role in distinct pathways and/or similar phenotypes in animal models.

Genes in which novel candidate variants may cause a fetal phenotype different from the postnatal phenotype

In family 7, fetal ultrasound identified agnathia-otocephaly complex with complete absence of the mandible and a submandibular position of the ears, cleft palate, and aortic isthmus stenosis. No variants were identified in *OTX2* and *PRRX1* through ES and additional Sanger sequencing, in which disease-causing variants were previously reported to cause the agnathia-otocephaly phenotype [8, 15]. The novel de novo missense variant in SMAD3 was prioritized (c. [860G>A], p.(R287Q)) out of four remaining candidates (*KRT86*, *PRICKLE1*, *MYH3*, *SMAD3*) since SMAD3, OTX2 and PRRX1 are reported to act in the same signaling pathway implicated in the development of the branchial arches [16]. Variants in *SMAD3* were previously reported to cause autosomal dominant Loeys-Dietz syndrome 3 (OMIM: 613795), a connective tissue disorder, presenting with aortic aneurysm, cardiac anomalies, cleft palate and significant micro-/retrognathia. Proteins of the SMAD family play an important role in transcriptional regulation. The variant we identified is located in the MH2-domain of *SMAD3* important for the interaction between the different SMAD proteins (Uniprot). According to the HOPE prediction, the mutated residue may disturb the binding properties of the MH2 domain since it cannot form hydrogen bonds and salt bridges. Further functional studies on chicken embryos to prove causality are ongoing.

Among 3 variants identified in different genes (*OSGIN1*, *KIF4A*, *BCAT1*) potentially disease-causing according to prediction algorithms we considered the X-linked novel hemizygous missense variant in the *KIF4A* gene (c. [2096T>A], p.(V699E)) likely to be associated with the isolated hydrocephalus in the male fetus of family 8. The variant was also present in the healthy mother, consistent with X-linked recessive inheritance. Variants in *KIF4A* are reported to cause X-linked intellectual disability (OMIM: 300923) [17]. However, KIF4A is a motor protein that translocates PRC1, a cytokinesis protein, to the ends of the spindle microtubules during mitosis [18], regulates the PARP1 activity in brain development and the survival of neurons [19] and is a member of the L1CAM recycling pathway. Variants in *L1CAM* are well known to cause X-linked isolated and syndromic hydrocephalus [20]. The novel variant is in the highly conserved PRC1 interacting domain and is predicted to disrupt interaction. qPCR confirmed a significant reduction of *KIF4A* mRNA in brain tissue of the affected fetus (12%) compared to FFPE brain tissue of an age-matched control (Supplementary Figure S1). The low mRNA levels indicate nonsense-mediated decay (NMD) and a likely loss-of-function mechanism.

In family 9 both fetuses presented with a Dandy-Walker malformation, hydronephrosis, dysplastic kidneys, and genital hypoplasia, and an additional diaphragmatic hernia in one fetus, suggesting a clinical diagnosis of Fryns- or Fryns like syndrome. In total, variants in 6 genes were considered (*ABCA1*, *AIM1L*, *CTDSP2*, *NOP16*, *RSU1*, and *PIGW*). A homozygous missense variant in *PIGW* (c.[106A>G];[106 A>G], p.(R36G) that segregated in both fetuses was prioritized because phenotypes caused by variants in *PIGV* and *PIGN* genes of the PIG family show a phenotypic overlap with Fryns syndrome

[21] or are described to be causal for the Fryns phenotype [22]. The presence of PIGW is required in the early steps of GPI anchor biosynthesis, and bi-allelic variants in genes encoding components of the GPI anchor biogenesis pathway have been suggested to be a rare cause of variable developmental and malformation phenotypes [23]. So far, variants in *PIGW* were reported to cause a glycosylphosphatidylinositol biosynthesis defect 11 (OMIM 610275) presenting with developmental delay, intellectual disability, and seizures [24]. The variants in patients described are closer to the 3′-end of the gene than the one in the fetuses with an anomaly phenotype we identified. This variant is located in the transmembrane domain and the mutant amino acid is predicted to disturb the transport activity of the protein (HOPE).

Novel candidate genes

The structural anomalies of the fetus of family 10, including multiple brain anomalies, spina bifida, unilateral anophthalmia, bile duct atresia, agenesis of the Müllerian ducts, hepatomegaly and bilateral cleft palate was not specific for any previously described congenital anomaly syndrome. From the two genes with potentially disease-associated biallelic variants (*MTHFD1L*, *PTK7*) we selected compound heterozygous missense variants in *PTK7* (c.[19G>A]; [c.1238 A>G], p.(G7R);(N413S)) as the best candidates since two PTK7 loss of function mouse models show severe neural tube defects, cystic kidneys, micrognathia, and unilateral anophthalmia [25, 26]. No human phenotype has been described so far, but PTK7 deficiency is suspected to play a role in neural tube defects [27], being a key protein in the embryonic Wnt and PCP signaling pathways. Furthermore, PTK7 regulates the outgrowth of the Wolffian duct [28], which is essential for the formation of the Müllerian duct [29]. One of the detected variants (c.19G>A) is located in a transcript (NM_001270398.1), that is coding for the protein isoform e which has an alternative exon 1 that lacks a signal peptide sequence for localization in the cell membrane. Currently, functional properties of this isoform are investigated. RT-PCR on RNA extracted from liver FFPE tissue of the affected fetus and tissue of an age matched control showed a significant signal decrease in the affected fetus. qPCR of the same samples confirmed a decrease of mRNA expression to 6% (Supplementary Figure S2). These findings are indicative of NMD of the *PTK7* mRNA due to aberrant splicing, at least in the liver and for this isoform.

In family 11, the fetus had a complex heart defect with a hypoplastic left heart and aorta ascendens, subtotal mitral valve atresia, persistent cardinal vein and atrial septal defect, fused lung lobes, gallbladder agenesis, and reduced intrahepatic bile ducts, incomplete intestinal rotation and a pouch-like extension of the proximal jejunum. The malformation pattern was suggestive of a heterotaxy/ciliopathy phenotype. ES detected potentially disease-associated variants in three different genes (*CACNA1A*, *PLCH2*, *DNHD1*). A homozygous missense variant in *DNHD1* (c.[6109 A>G];[6109 A>G], p.(S2037G)) was prioritized due to its function in the dynein heavy chain, although no consanguinity was reported. Variants in other genes such as *DNAL1* (OMIM: 610062), *DNAI1* (OMIM: 604366) and *DNAH11* (OMIM: 603339), which are coding for dynein compartments, are known to cause situs inversus-like phenotypes. Little is known about the exact function of *DNHD1*. It was reported as a candidate gene for intellectual disability [30]. Family 12 presented with recurrence of intestinal atresia. DNA was only available from one child for trio analysis. ES detected compound heterozygosity in 4 different genes (*CASZ1*, *PLEKHM2*, *COL5A1*, and *TTC28*), but only variants in *TTC28* (c.[3638 A>G];[c.794 A>C], p.(D1213G);(K265T)) were predicted disease associated and were highly conserved across species. We prioritized *TTC28* because variants in *TTC7A* (OMIM: 609332), a member of the same gene family, causes autosomal recessive gastrointestinal defects, and variants in *TTC21B* (OMIM: 612014) are reported in human ciliopathies.

Discussion

The overall primary detection rate of disease-associated variants in known disease genes explaining the fetal phenotype is 21% in our highly selected series of fetuses with structural anomalies. For those families counseling to inform about

prognosis and recurrence risk as well as reproductive choices, such as prenatal diagnosis or preimplantation genetic diagnosis, in further pregnancies is available. Other studies report detection rates of 10–21% [4, 31–33], however, inclusion criteria and approaches in all studies including ours are highly heterogeneous and therefore restrict comparability. Our detection rate increases after having ascertained disease-association for the variants in the novel candidate genes *KIF14* and *CENPF* by the investigation of additional unrelated affected patients as well as functional and animal model studies [11, 13]. Whereas clinical diagnoses such as agnathia-otocephaly and Meckel-Gruber syndrome were obvious, the presentation of Apert syndrome was atypical since craniosynostosis, the key clinical sign in the corresponding postnatal phenotype, cannot be appreciated at that early developmental stage and therefore hampered targeted molecular genetic testing. This reverse phenotyping highlights the importance of appreciating fetal phenotypes as a variable of developmental timing. Candidate variants identified in the additional 6 families do not have an immediate clinical impact but may guide the future search for further affected patients and functional studies for confirmatory evidence. Particularly, the suspected causal relationships between variants in *PIGW* for a Fryns syndrome phenotype, KIF4A as a player in the L1CAM pathway for X-linked hydrocephalus and variants in *SMAD3* which may cause agnathia-otocephaly as the severe end of the Loeys-Dietz spectrum emphasize the importance of clinical and developmental genetics review in addition to formal variant assessment. Similarly, the phenotype of about one-third of the fetuses examined, including family 10, 11, and 12, is reminiscent of some type of ciliopathy compatible with the fundamental role of pathways implied in ciliogenesis and ciliary function in embryonic development. Nine of the twelve conditions identified are likely following autosomal recessive inheritance, which may in part reflect recruiting bias including families with phenotype recurrence. However, embryonically lethal mouse models suggest that autosomal recessive inheritance may play an important role in phenotypes with early lethality. The identification of *KIF14* variants causing a lethal ciliary anomaly pattern highlights the significance of investigating such extreme phenotypes for the primary description of a human disease gene, representing the severe phenotypic spectrum of an allelic viable postnatal disorder. Large-scale projects such as the International Mouse Phenotyping Consortium (IMPC) producing knockout mouse lines will be an important resource to support the characterization of novel human disease genes. In four of the seven families in which we were not able to delineate a compelling candidate gene, fetuses presented with recurrence of an isolated anomaly (Supplementary Table S1). This supports the observation of others as well as previous prenatal microarray studies for CNVs that a monogenic etiology is more likely identified in the presence of multiple anomalies. Besides the multiple limitations in fetal phenotyping and exome analysis, multifactorial or polygenic inheritance or epigenetic mechanisms remain a possible explanation. Much more research is needed to explore the utility of genome-wide sequencing in isolated anomalies, particularly when there is phenotype recurrence, potentially also suggestive of a recessive disorder with incomplete phenotype presentation in the antenatal period. The recruitment of additional unrelated fetuses presenting with the same phenotype is one of the most important recognized challenges in elucidating pathogenicity of candidate variants in the prenatal field despite an arising awareness of the importance to investigate such families. Prenatal phenotypes may be imprecise, and autopsy often not done for various reasons impairing phenotype–genotype comparisons and the collection of a larger number of similarly affected fetuses. Functional analysis of potential candidate variants and the generation of appropriate variant specific animal models demands expertise and significant resources. The current diagnostic yield of prenatal ES, however, may remain limited when the interpretation of variants continues to rely on experiences with postnatal patients exclusively. Recruiting patients in a perinatal setting may raise high parental expectations, and a thorough discussion of the current limitations in the interpretation of private variants is required. However, using ES as a discovery tool integrating the delineation of precise

fetal phenotypes, we contribute to further understand altered developmental pathways specific to fetal life. Recruitment of additional families and studies of variant effects will be mandatory to confirm causal relationships and understand disease mechanisms. Besides the gain of knowledge on biological mechanisms in early human development, this will allow increasing the utility of prenatal ES in a long-term perspective, and much more families will benefit from diagnostic certainty in this sensitive field.

Supplementary information regarding this article is available at (https://www.nature.com/ejhg/)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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3.3 Publication 3

Expanding the phenotypic spectrum of *SMAD3*-related phenotypes to agnathia-otocephaly

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CLINICAL REPORT

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Expanding the spectrum of *SMAD3*-related phenotypes to agnathia-otocephaly

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Abstract

Background: Agnathia-otocephaly is a rare and lethal anomaly affecting craniofacial structures derived from the first pharyngeal arch. It is characterized by agnathia, microstomia, aglossia, and abnormally positioned auricles with or without associated anomalies. Variants affecting function of *OTX2* and *PRRX1*, which together regulate the neural crest cells and the patterning of the first pharyngeal arch as well as skeletal and limb development, were identified to be causal for the anomaly in a few patients. **Methods:** Family-based exome sequencing (ES) on a fetus with severe agnathiaotocephaly, cheilognathopalatoschisis, laryngeal hypoplasia, fused lung lobes and other organ abnormalities and mRNA expression analysis were performed.

Results: Exome sequencing detected a de novo *SMAD3* missense variant in exon 6 (c.860G>A) associated with decreased mRNA expression. Variants in *SMAD3* cause Loeys–Dietz syndrome 3 presenting with craniofacial anomalies such as mandibular hypoplasia, micro- or retro-gnathia, bifid uvula and cleft palate as well as skeletal anomalies and arterial tortuosity. The SMAD3 protein acts as a transcriptional regulator in the transforming growth factor β (TGFB) and bone morphogenetic (BMP) signaling pathways, which play a key role in the development of craniofacial structures originating from the pharyngeal arches.

Conclusion: Agnathia-otocephaly with or without associated anomalies may represent the severe end of a phenotypic spectrum related to variants

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in genes in the interacting SMAD/TGFB/BMP/SHH/FGF developmental pathways.

KEYWORDS

agnathia-otocephaly, exome sequencing, prenatal, SMAD3

1 | INTRODUCTION

Agnathia-otocephaly (OMIM 202650) is a rare congenital anomaly pattern with an estimated incidence of less than 1 in 70,000 births. The anomaly is characterized by mandibular hypoplasia, severe micrognathia or agnathia and variable

ventromedial auricular malposition and/or fusion and microstomia with microglossia or aglossia. Holoprosencephaly is the most commonly identified associated anomaly, but cyclopia, uni-/bilateral microphthalmia/anophthalmia, cleft palate, pharyngeal and laryngeal hypoplasia, skeletal, genitourinary, and cardiovascular anomalies, and situs inversus have been

reported. The disorder is almost always lethal. It is considered a defect of blastogenesis, resulting in the variable dysmorphogenesis of structures developing from the first pharyngeal arch derivatives or a failure of neural crest cells to migrate into the first and second pharyngeal arches (Faye-Petersen et al., 2006; Opitz, Zanni, Reynolds, & Gilbert-Barness, 2002).

Craniofacial development during embryo- and feto-genesis requires complex interactions of several developmental molecular pathways. Among the pertinent pathways probably involved in the etiology of agnathia-otocephaly, Sonic Hedgehog (SHH) signaling, bone morphogenetic (BMP) and fibroblast growth factor (FGF) signaling, WNT and glycosylphosphatidylinositol-anchoring pathways have been suggested (Gekas, Li, & Kamnasaran, 2010). However, pathogenic variants in only two genes, *PRRX1* (OMIM 167420) and *OTX2* (OMIM 600037) were reported to be causal for the agnathia-otocephaly phenotype in a few patients so far, suggesting further genetic heterogeneity. *PRRX1* encodes a homeobox gene, which functions as transcriptional regulator (Kern, Argao, Birkenmeier, Rowe, & Potter, 1994), is expressed in undifferentiated human embryonic cranial neural crest cells and most abundantly in cardiac, skeletal, and smooth muscle tissues in adults (Çelik et al., 2012). It functions together with other proteins such as PRRX2 and also OTX2, encoded by a homeobox family gene expressed in the developing head, to regulate neural crest cells and the patterning of the first pharyngeal arch as well as skeletal and limb development.

Here we report on non-consanguineous healthy parents of Albanian descent with a reproductive history of one early miscarriage but an otherwise unremarkable family history. Written informed consent for participation and publication of clinical data and pictures was obtained from the participants. The family was part of a larger study (Meier et al., 2019) with approval of the Ethics Commission Northwest Switzerland (EKNZ 2014-174).

The family came to our attention during the second pregnancy at 19 + 2 weeks of gestation when severe micrognathia was detected by ultrasound and otocephaly suspected (Figure 1a,b). Fetal postmortem autopsy revealed an agnathia-otocephaly complex with severe hypoplasia of the mandible and maxilla, ventral median position of the auricles (Figure 1c– d), cheilognathopalatoschisis, microglossia, larynx hypoplasia and esophageal hypoplasia. In addition, the fetus had an aortic isthmus stenosis, fused left lung lobes, a right unilateral rudimental 4th thoracic rib and a cake kidney positioned in the left pelvis with tubular ectasias and megalocytosis of the adrenal cortices. There was no additional brain or eye anomaly. We performed Sanger sequencing for all exons and intron boundaries for both *PRRX1* and *OTX2* but detected no variants.

In order to further delineate the etiology of this phenotype we performed family-based exome sequencing using the trio of fetal DNA extracted from formalin fixed paraffin embedded (FFPE) tissue and parental DNA extracted from blood samples. Library preparation was performed with the Nextera© Rapid Capture Kit for the TruSight One panel (Illumina). Paired-end read sequencing (2 × 100 bp read length) was accomplished on an MiSeq platform (Illumina). Quality estimation of the sequence reads was performed by generating quality control statistics with FastQC (http://www. bioin forma tics.bbsrc.ac.uk/proje cts/fastqc). The Genome Analysis Toolkit (GATK, www.broad instit ute.org/gatk) was used to perform variant calling. Variants with a coverage ≤10X and not supported by at least four reads (20%) were discarded. Familial segregation of variants was performed to identify de novo, autosomal recessive and X-linked

recessive inheritance. Hypothesizing a rare Mendelian disorder, we annotated variants as known or novel according to presence or absence in curated databases. Variants were filtered on a heterozygous population frequency (GMAF) of <5% in control databases (dbSNP150, ExAC, 1000G, gnomAD) and absence of homozygosity in healthy individuals (ExAC, gnomAD). We prioritized variants according to their potential to disrupt protein function including the use of prediction tools (SIFT, Provean, Polyphen2, Mutationtaster, Human Splicing Finder v.3.0), the amino acid conservation (PhyloP, PhastCons) and the American College of Medical Genetics (ACMG) variant classification guidelines (Richards et al., 2015). Prioritized variants were inspected for phenotype–genotype correlations reported in humans and other species, based on the medical literature and public databases including functional and expression data (Pubmed, ClinVar, OMIM, HGMD, Uniprot, UniGene, www.zfin.org; www.infor matics.jax.org; www. mouse pheno type.org). We also focused on genes within the craniofacial developmental pathways including interaction partners of O*TX2* and *PRRX1*.

In the list of *de novo* variants we prioritized a *SMAD3* (OMIM 603109) missense variant in exon 6 (c.860G>A, (p.R287Q), LOVD #0000405920) out of a total of four candidate variants (including variants in *KRT86*, *PRICKLE1*, *MYH3*). This variant was previously described as likely pathogenic in a patient with Aneurysms-osteoarthritis syndrome (AOS) and a patient with Loeys–Dietz-Syndrome type 3 (LDS3) (Aubart et al., 2014; Schepers et al., 2018).

Sanger sequencing confirmed the variant in the fetus and its absence in the parents. In silico prediction tools rated the variant as truncating (SIFT, PolyPhen2, Mutationtaster), the HOPE protein prediction (http://www.cmbi.ru.nl/hope/) predicted a loss of hydrogen bonds and salt bridges in the protein, due to the amino acid exchange. According to the ACMG guidelines (Richards et al., 2015) the variant was classified as likely pathogenic (PM1, PM2, PP3, PP5). SMAD3 is a direct mediator of transcriptional activation by the TGF-beta receptor. *SMAD3* consists of nine exons that code for an MH1 and MH2 domain, to interact with other SMAD proteins and signal transducing receptors, that are connected through a linker. The variant is located in the MH2 domain of the protein that is highly conserved across species.

To confirm the functional consequence of the variant, mRNA was extracted following the protocol of the RecoverAll™ Kit (Ambion) from fetal FFPE liver and kidney tissue of the affected fetus and an age-matched control fetus without structural anomalies. Kidney and liver were chosen based on tissue availability and the expression data in the Unigene database. Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). SYBR™ Green PCR Master Mix (Applied Biosystems) was used for qPCR. Expression levels were normalized to *GUSB* expression. The results showed a significant decrease of *SMAD3* mRNA (Figure 1f) up to 44% in kidney and 20% in liver tissue, confirming that the *SMAD3* variant affects the expression of mRNA. The variant c.860G>A (p.R287Q), we detected in *SMAD3* in the fetus presenting with agnathia-otocephaly, was previously reported to cause LDS3 (Aubart et al., 2014; Schepers et al., 2018), an autosomal dominant connective tissue disorder initially described as AOS (van de Laar et al., 2011). These patients present with generalized arterial tortuosity, arterial aneurysms and an increased risk for dissections, in particular for the ascending aorta, craniofacial anomalies including hypertelorism, micro-/ retro-gnathia, bifid uvula and cleft palate, skeletal and cutaneous anomalies, and early-onset osteoarthritis (van de Laar et al., 2012).

Because of the many overlapping clinical features of LDS type 1 and 2 patients, including the craniofacial anomalies, arterial tortuosity and widespread aneurysms, and harboring autosomal dominant variants in the disease-responsible genes *TGFBR1/2* and *TGFB2/3* in the same TGF-β signaling pathway, AOS is now usually classified as LDS type 3 (MacCarrick et al., 2014). Schepers and colleagues extensively reviewed known and novel variants in TGFB2/3 ad SMAD2/3 causing a broad phenotypical LDS spectrum (Schepers et al., 2018). We suggest, to extend the phenotypic spectrum to agnathia-otocephaly which may represent the lethal or severe end of the TGFBR/SMAD-pathway associated phenotypes.

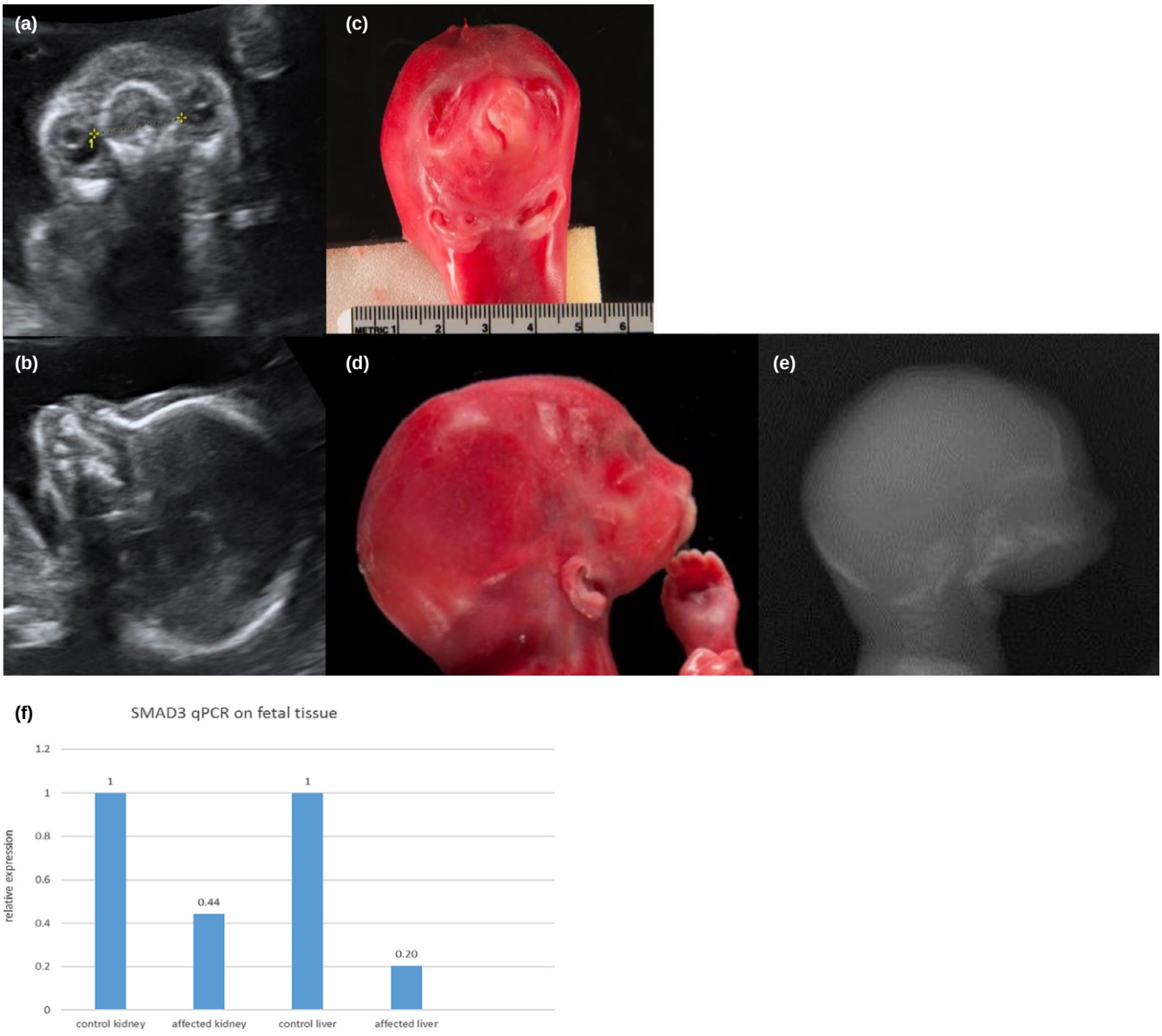

FIGURE 1 (a–e) The affected fetus at 19 + 2 weeks of gestation in prenatal ultrasound (a and b) and post mortem (c–e) showing cheilognathopalatoschisis, microstomia, absent mandible and ventral median positioned auricles. (f) Relative *SMAD3* mRNA expression in kidney and liver tissue of an age-matched control fetus and the affected fetus (f). RNA was extracted from FFPE tissue, the expression of *SMAD3* was normalized to *GUSB* expression. FFPE, formalin fixed paraffin embedded

The SMAD family member three gene (*SMAD3*; OMIM 603109) encodes the SMAD3 protein which belongs to the receptor-activated (R)-SMAD family. These proteins are intracellular effectors of the canonical transforming growth factor-β (TGF-β) signaling pathway including bone morphogenetic proteins (BMPs) that belong to the TGF-β superfamily (Hata & Chen, 2016; Massagué, 2012). The SMAD3 protein responds to TGF-β and regulates TGF-β-mediated chondrocyte and osteoblast differentiation (Wu, Chen, & Li, 2016). Several studies have shown that SMAD3 deficiency leads also to enhanced BMP signaling and accelerated chondrocyte differentiation which causes osteoarthritis (Li et al., 2006).

Furthermore, the SMAD3 protein acts in the same developmental pathway as *OTX2* and *PRRX1* as a transcriptional regulator. This pathway is involved in the development of the first pharyngeal arch in the lower jaw (Gekas et al., 2010). Knockout mice show the typical signs of an AOS but also micrognathia (Yang,

1999). A knockdown of *SMAD3* in zebrafish resulted in a truncated anterior head and neuronal maldevelopment. However, absence of the lower jaw was not specifically mentioned (Casari et al., 2014).

SMAD3 acts in both the TGF-β and BMP pathway that are crucial for embryonic development. Figure 2 depicts the complexity of the SMAD3-related downstream processes. The BMP pathway, where SMAD3 acts as an intracellular effector, is also involved in bone formation (Rahman, Akhtar, Jamil, Banik, & Asaduzzaman, 2015). A knockout mouse model of *Alk2*, a BMP receptor, resulted in mice with a hypoplastic mandible, missing jugal bones and enlarged fontanels due to lack of ossification (Dudas, Sridurongrit, Nagy, Okazaki, & Kaartinen, 2004). In humans, variants in *BMP4* cause anophthalmia, microphthalmia with brain and digit anomalies (Bakrania et al., 2008).

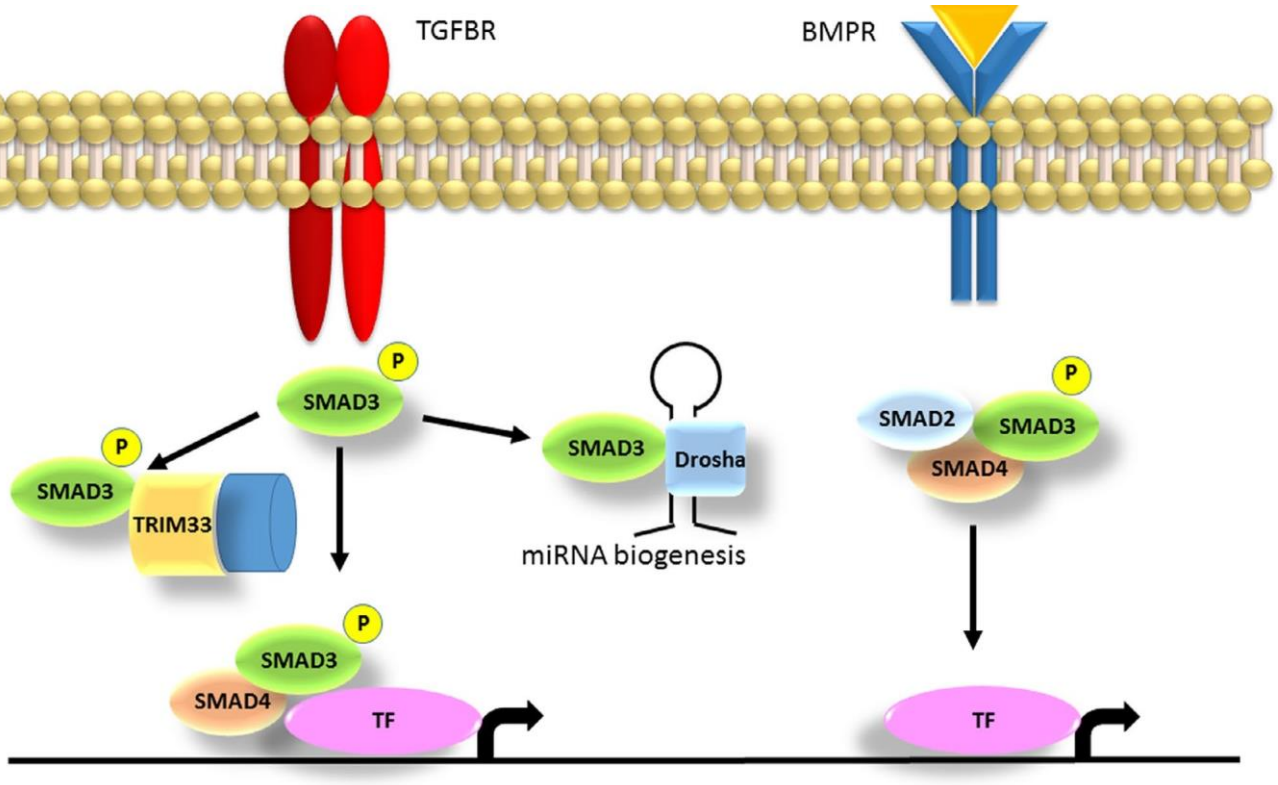

FIGURE 2 Roles of SMAD3 in the TGF-β and the BMP pathway. The SMAD3 protein gets activated through TGF-β signaling and can act in different downstream pathways. If SMAD3 binds to TRIM33, a chromatin reading and remodeling protein, the complex opens the histone to allow other TFs to enter the DNA. Complexes of SMAD3 and SMAD4 get recruited to different genes, and the transcription of a specific gene is determined by varying partner TFs. In those effector pathways SMAD3 is involved in chondrocyte and osteoblast maturation (Massagué, 2012). If SMAD3 binds to DROSHA it regulates the processing of different miRNA precursors. SMAD3 forming a complex with other SMAD proteins driven by BMP signaling also activates transcription via other TFs, regulating various bone formation processes (Rahman et al., 2015). TGF-β, transforming growth factor-β; TFs, transcription factors

Interestingly, a loss of function variant in *SMAD3* was recently described as a possible candidate for a patient with holoprosencephaly (Roessler et al., 2018). This study concluded that most genes in which variants cause a holoprosencephaly phenotype act in the TGF-β, hedgehog and FGF signaling pathways. This further supports our hypothesis of agnathia-otocephaly being a *SMAD3*-related phenotype, as agnathia-otocephaly and holoprosencephaly can occur as a combined phenotype (Faye-Petersen et al., 2006; Ozden, Fiçicioğlu, Kara, Oral, & Bilgiç, 2000; Puvabanditsin et al., 2006; Rodriguez et al., 2019; Wai & Chandran, 2017). Agnathia-otocephaly with or without associated anomalies is clinically and genetically heterogeneous and likely represents the lethal or severe end of a phenotypic spectrum related to variants in genes in the interacting SMAD/TGFB/BMP/SHH/FGF developmental pathways. In order to understand the disease mechanisms and the phenotypic variability, it will be necessary to further study genotype–phenotype correlations as well as the function of genes and their variants in these pathways.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in LOVD at https://databases.lovd.nl, reference number #0000405920.

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4. Discussion

We recruited in total 19 families with different ethnical backgrounds, one family with proven consanguinity. In total, we detected a candidate variant in 12 out of 19 families (63%) in 12 different candidate genes. Our detection rate of variants in 6 known disease-causing genes (31%) is comparable to previous studies[15,23,25,28,29,73] but potentially biased by our strict selection criteria focusing on rare phenotypes including phenotype recurrence. In 8 of the 12 families (67%), we confirmed autosomal recessive inheritance, in 3 a dominant *de novo* (25%), in one family X-linked recessive (8%). Concordant with spontaneous recessive mouse models, Recessive inheritance may indeed play a significant role in human lethal fetal anomaly syndromes as well, even if no phenotype recurrence was reported. This may affect clinical approaches to detect disease-associated variants by sequencing the potential carrier parents alone and detect potentially truncating variants without using limited fetal DNA, or even further in terms of carrier screening for prevention[74,75]. Nevertheless, disease-associated *de novo* variants represent the second most frequent cause in our cohort. Those variants were all located in known disease-associated genes, two with either novel or incomplete phenotypes (*FGFR2, SMAD3*) as compared to phenotypes in postnatal studies for variants in the same genes. In families with recessive inheritance, we detected three novel candidate genes that are not linked to a human phenotype so far (*PTK7, DNHD1, TTC28*), but in two of them (*PTK7* and *TTC28*) variants were suspected to be associated with other specific conditions[68,76]. In one case we detected a maternally inherited X-linked candidate variant in *KIF4A* likely causing severe hydrocephalus, because of its interaction with *L1CAM*, and intellectual disability and mild ventriculomegaly was reported previously to be associated with a variant in *KIF4A* in one patient[77]. All results are are displayed and discussed in detail by Meier et al., 2019[78].

One major finding were unexpected phenotype-genotype correlations and the importance of fetal autopsies in phenotype assessment which is often underestimated. We showed that variants in the *RYR1* gene can also affect smooth muscle tissue [79]. As an example of an incomplete or different phenotype as compared to what we know from postnatal ascertainment, craniosynostosis, a hallmark clinical sign for Apert syndrome, was not present at the developmental stage in a fetus with an *FGFR2* variant (Publication 2, Table 1).

The families in which we were not able to identify a causal or a candidate variant showed phenotype recurrence, but the affected fetuses mostly had a single major organ anomaly. In fetuses with a multiple anomaly phenotype and a detailed phenotyping by additional post-mortem autopsy we were more likely able to detect a monogenic etiology of the anomaly syndrome.

However, there is an increasing number of monogenic etiologies identified also for isolated major anomalies. Particularly in the fetal period they can be part of a more complex phenotype that is not recognizable by ultrasound or autopsy only, since all functional aspects of phenotyping as well as development over time cannot be assessed. Standard bioinformatic pipelines may mislead when e.g. disregarding benign and synonymous variants since they may alter splicing. In the case of Meckel syndrome (OMIM: 249000) we identified a synonymous variant in *MKS1* (OMIM: 609883) that was previously described as disease-associated and has been proven to alter splicing of the transcript[80]. Inherent to the technology, exome sequencing may miss variants in genes which are subject to incomplete or low coverage, small (e.g. exonic) copy number variants or variants in regulatory elements that are in non-coding regions of the genome. Low-level mosaicism may escape detection or mosaicism in general when the causal variant is present only in the affected tissue[22] . Multifactorial or polygenic etiologies, however, may account for a certain proportion when the anomaly is truly isolated,

and family-based exome or genome-wide sequencing cannot identify such causes. Epigenetic influences may be considered as well [81]. In the future some of the variant detection issues will be probably overcome by genome-wide sequencing technologies, providing a more even coverage and amenable to small copy number variant detection. Several technical challenges, however, including low input DNA protocols for fetal samples as well as bioinformatic issues will need to be solved.

One of the major challenges of our and other exome sequencing studies for gene identification is to prove that a candidate variant affects gene function and is therefore causal for the phenotype. This is particularly relevant if there are only one or two affected individuals, which is often the case in the very rare phenotype presentations including fetal anomalies. Despite the effort of international databases such as genematcher it is notoriously difficult to find additional patients. In addition, depending on the properties of the respective gene, such as its specific function and its conservation in orthologs and the position of the variant to be investigated, functional approaches and experiments as well as the choice of the appropriate animal model must vary. Since these demand significant resources and time, such functional evidence is often lacking in current candidate gene identification studies. Our functional experiments, however, also showed the challenges to model a severe or lethal fetal phenotype. We were able to show decreased RNA expression in tissue of the affected fetuses in comparison with age-matched controls, but even after multiple experimental optimization we failed to model agnathia-otocephaly in the chicken embryo. Early lethality occurred before we were able to assess the phenotype, and lower dosage of the non-functional protein did not generate the specific phenotype. Besides the possibility of off-target toxicity the dosage dependent early lethality also points to a direct major impact on early development by the

altered protein. In the future, a more specific knockout model of *SMAD3,* eventually using CRISPR/Cas9 of *SMAD3,* may hold promise, however bearing its proper challenges.

6. Conclusion and Outlook

Exome sequencing is on the verge of being implemented in routine prenatal diagnosis for congenital anomalies suggestive of a monogenic disorder. There are, however, a number of challenges which restricts its current clinical utility, in particular our still limited knowledge on how to interpret the numerous variants as to their causality which is specifically true for specific fetal phenotypes. In our work we contributed to fill this gap as we successfully used family-based exome sequencing to identify known and novel variants as well as candidate genes in fetal anomaly syndromes and discovered novel aspects in genotype-phenotype correlations. Anomalies which we would expect from postnatal phenotype description may not be apparent or may not yet have manifested in the fetal period at the time of assessment. We also explored the developmental pathways involved in such malformation patterns, cross-species phenotyping as well as the possibility of functional studies to prove variant causality. For those families where we found a monogenic etiology and thus a definite diagnosis, clinicians were able to provide recurrence risk and counsel them regarding reproductive choices such as pregnancy and perinatal management and prenatal and preimplantation diagnostic options for further pregnancies.

With further improvement of the NGS techniques, the availability of genome-wide sequencing and development of more sophisticated bioinformatic algorithms and functional protein prediction, we believe that the etiology of even more cases of our and other patient

cohorts can be solved. More research, however, is needed to explore the utility of genome-wide sequencing in isolated anomalies, particularly when there is familial recurrence.

An important challenge remains the study of the functional impact of a specific candidate variant, but we need developmental research, even if unsuccessful sometimes, to improve experimental strategies and to understand the biological pathways and mechanisms which lead to the specific fetal anomaly phenotypes.

Ultimately, the knowledge of specific genotype-phenotype correlations will increase the utility of prenatal ES in a long- term perspective, and much more families will benefit from diagnostic certainty in this sensitive field.

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81 Guo W, Zhu X, Yan L, Qiao J. The present and future of whole-exome sequencing in studying and treating human reproductive disorders. *J Genet Genomics* 2018; **45**: 517–525.

Curriculum Vitae

Personal data:

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Born: May 26th 1987
Born in: Freiburg im Breisgau, Germany
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Working experience

2019/02-on going: **Synlab MVZ Humangenetik Freiburg**

Scientific associate

- Introduction of Exome-sequencing to routine diagnostics
- NGS library preparation and sequencing
- Introduction of new analysis pipelines
- Collaboration with MDs
- Responsible for Illumina MiSeq and NextSeq desktop sequencer
- NGS data analysis
- Rating of sequence variants according to ACMG guidelines
- Preparation of clinical reports
- Training of technical assistants in NGS-library preparation
- Training of colleagues in handling analysis software
- Design and introduction of SOPs

2015/09 – on going: **Medical Genetics University Hospital Basel, group PD Dr. med. I. Filges**

PhD candidate

- Management of patient samples
- NGS library preparation and sequencing
- NGS data analysis
- Molecular genetics
- Clinical genotype-phenotype correlations
- Identification of new disease candidate genes
- Cloning of bacterial and mammalian vectors
- Functional experiments
- Planning of animal models
- Collaborative work with other labs
- Confocal microscopy
- Recruitment of external funding

2014/11 - 2015/5: **TPL Path Labs Freiburg**

Medical technical assistant

- histological treatments of animal and human tissues under GLP/GCLP
- initial training of new employees
- staff training in first aid measures
- generation of a risk assessment for the lab
- customer care (BASF, Novartis, Roche)

2014/8 – 2014/11: **Institute for Biology III, University of Freiburg, group Prof. Hess**

Biological technical assistant

- Taking care of the cyanobacterial stocks
- Genetic and biochemical analyses
- Recombinant protein expression in *E. coli*
- design and implementation of experiments
- Statistical and graphical evaluation of experimental results

2013/5 – 2014/9: **Bioss centre, University of Freiburg, group Dr. Pyrowolakis**

Research assistant

- Taking care of the *Drosophila melanogaster* main stocks
- Recombinant protein expression in *Drosophila melanogaster*

University education

2015/09 – on going: **Institute for Medical Genetics and Pathology,**

University Hospital Basel

PhD in Medical Genetics

Focus: Human Genetics and Embryology

Title of the thesis: "Whole exome sequencing for gene discovery in lethal fetal disorders"

2012/10 – 2014/08: **Albert-Ludwigs-University Freiburg**

Master of Science in Biology

Focus: Genetics and Developmental biology

Title of the Master thesis: *"Activity of RNase E under UV-light stress"and "Two-Hybrid-Screen on interaction partners of RNase E in Prochlorococcus marinus"*

2008/10 – 2011/09: **Albert-Ludwigs-Universität Freiburg**

Bachelor of Science in Biology

Focus: Genetics

Genetics of eucaryotes

Immunology

Title of the Bachelor thesis: *„Labeling parts of the trancriptional and translational complex of Sinorhizobium meliloti with EGFP and analysis of the cellular localization of the marked proteins"*

Scientific Publications

1. Meier N, Bruder E, Filges I. A novel homozygous splice-site mutation in *RYR1* causes fetal hydrops and affects skeletal and smooth muscle development. Prenat Diagn. 2017;37:720–4.
2. Filges I, Meier N, Lapaire O, et al. Gene Identification in fetal malformation phenotypes. Prenatal Diagnosis 2016, 36(Suppl. 1), 23–84
3. Meier, N., Bruder, E., Lapaire, O., Hoesli, I., Kang, A., Hench, J., … Filges, I. (2019). Exome sequencing of fetal anomaly syndromes: Novel phenotype-genotype discoveries. *European Journal of Human Genetics*, *27*(5), 730–737. https://doi.org/10.1038/ s41431-018-0324-y
4. Meier N, Bruder E, Miny P, Tercanli S, Filges I. Expanding the spectrum of *SMAD3*-related phenotypes to agnathia-otocephaly. *Mol Genet Genomic Med* 2020; : 1–6.

Formal education

1997/09 – 2006/07: Goethe Gymnasium Emmendingen
(matriculation standard)

1993/09 – 1997/07: Carl-Friedrich Meerwein Elementary school, Emmendingen

Internships

2013/09: Four-week internship in the clinical microbiology in the lab of Prof. Dr. Georg Häcker at the University Hospital Freiburg, Focus: *Chlamydia trachomatis* infections in human cells.

2010/08: Workshop in forensic entomology with Dr. Mark Benecke in Cologne.

2005/08: Two-week internship in the forensic anthropology in the lab of Prof. Dr. Ursula Wittwer-Backofen

Other skills and qualifications

Computer applications:

- MS-Office (Word, Excel, Powerpoint)
- Image J
- Illumina NGS-data analysis
- SOPHiA Genetics platform

- VarVis Platform, Limbus MedTec
- SeqPilot, JSI
- CLC Bio Genomics Workbench
- IPA Pathway analysis (Qiagen)

Languages:

- German (native language)
- English (fluent, incl. technical terms)
- French (basic knowledge)
- Spanish (basic knowledge)

Other matters:

- Paramedic
- Canoeing guide
- Skiing instructor

Basel, 22.06.2020

Nicole Meier