

Case Report

Array-CGH and quantitative PCR genetic analysis in a case with bilateral hypoplasia of pulmonary arteries and lungs and simultaneous unilateral renal agenesis

Kais Hussein¹, Doris Steinemann², Henrike Scholz¹, Ralf Menkhaus³, Henning Feist⁴, Hans Kreipe¹

¹Institute of Pathology, Hannover Medical School, 30625 Hannover, Germany; ²Institute of Cell and Molecular Pathology, Hannover Medical School, 30625 Hannover, Germany; ³Privat practice for gynaecology, 32427 Minden, Germany; ⁴Institute of Pathology, Diakonissenkrankenhaus Flensburg, Flensburg, Germany; formerly Institute of Pathology, Hannover Medical School, Hannover, Germany.

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Abstract: We describe the clinical course and have characterised anatomically and genetically a unique case of a newborn with bilateral hypoplasia of pulmonary arteries, consecutive extremely hypoplastic lung tissue and associated unilateral renal agenesis. Intrauterine oxygenation by the placenta seemed to have allowed normotrophic body maturity but immediately after delivery, in the third trimester, progressive hypoxemia developed and the newborn succumbed to acute respiratory failure. Genetic analysis by array-based comparative genomic hybridisation and quantitative PCR revealed duplication of 1p21, which, however, might not be the disease causing aberration. This case might represent an extreme form of previously reported, rare cases with simultaneous dysorganogenesis of lungs and kidneys.

Keywords: Array-CGH, quantitative PCR, bilateral hypoplasia of the lung, unilateral renal agenesis

Introduction

Pulmonary hypoplasia and agenesis in humans is rare but occurs frequently ipsilaterally with other associated abnormalities, particularly renal agenesis, which indicates a common underlying aberration as the initial cause [1-3].

During the human embryologic development, lungs and kidneys develop over the same period, starting in the 4th and 5th week of gestation, respectively, and both organs require the induction of mesoderm on the bronchial and the ureteric bud [4-6]. At the start the tracheal and the very early lung bud develops independently of the cardiovascular system in the 4th week followed by invasion of the 6th aortic arch/pulmonary trunk-derived pulmonary arteries at the end of the 4th week. This vascularisation is associated with further development and maturation of the lungs and outgrowth of the capillary plexus and pulmonary veins to the left atrium [4,5]. Similar to the lungs, kidney tissue does

not develop independently but critically requires induction by the ureter, which grows out from the mesonephric duct, and if this is not the case, agenesis of the complete organ results [6].

Conventional cytogenetics had revealed normal karyotypes in some cases but no precise genetic analysis of simultaneous lung and renal dysmorphogenesis had been done, as yet [1-3]. Here, we report on an unusual case of a newborn with excessive hypoplasia of pulmonary arteries, associated consecutive cardiopulmonary malformations and unilateral renal agenesis.

Material and methods

Evaluation of pathological anatomy and immunohistochemistry

Post-mortem examination was performed and representative tissue specimens were formalin

fixed and paraffin embedded (FFPE) for further histological examination (haematoxylin-eosin stained tissue sections).

For analysis of surfactant expression a FFPE lung tissue section was evaluated by immunohistochemistry with a monoclonal anti-surfactant A antibody (Dako, Glostrup, Denmark).

Array-CGH genetic analysis

For evaluation of potential underlying genetic alterations, we used previously established methods for DNA extraction from frozen necropsy (liver tissue with foetal extramedullary haematopoiesis). A DNA chip containing more than 8000 individual BAC/PAC clones (manufactured at the DKFZ, Heidelberg, Germany) was used for BAC/PAC-array comparative genomic hybridization (aCGH) [7]. Clone selection and spotting as well as labelling and hybridization of DNA probes were performed as described previously [7]. Spot quality criteria were set as foreground to background >3.0 and SD of triplicates <0.2 ; 96.3% of clones were normalized according to spot quality criteria and of these 97% showed good quality of DNA hybridization. Image analysis was performed using a dual laser scanner and the GenePix Pro 4.0 imaging software (GenePix 4000 A; Axon Instruments, Union City, CA, USA). Data normalization and analysis were carried out using software packages marray and aCGH from R software3 (<http://www.r-project.org>) as previously described [7,8]. Duplications were defined as log ratio >0.2 and deletions as <-0.2 .

Verification of the 1p21 duplication by quantitative PCR

Quantitative gene copy number analysis was performed as a multiplex PCR using the GenomeLab GeXP Genetic Analysis System (Beckman Coulter, Krefeld, Germany) [8]. All loci were analysed in a single well. Primer sequences will be provided upon request. The reaction was performed with 20 nM of each primer, 1 μ l DNA (20-100 ng), 25 mM MgCl₂, PCR buffer and Thermo-Start-polymerase as recommended by Beckman Coulter. After an initial denaturing for 15 min at 95°C, 35 cycles were run at 94°C for 30sec, 55°C for 30 sec, 68°C for 1 min, followed by 5 min at 72°C. Fragment analysis was performed on a CEQ

Sequence Analyzer as recommended by the supplier.

Results

Clinical history

A 20-year-old nullipara Caucasian female was pregnant with a male child. In the 20th week of gestation ultrasonography showed pericardial effusion, dexter renal agenesis and hypoplasia of the thoracic cage (transversal diameter 25.0 mm, sagittal diameter 30.0 mm, thorax circumference 88.0 mm; all three parameters are below the 5th percentile but similar to values of cases with pulmonary hypoplasia and renal anomalies [9-12], **Figure 1A** and **1B**). Follow-up controls confirmed thoracic hypoplasia (35th week of gestation: transverse diameter 50.7 mm, sagittal diameter 58.7 mm, thorax circumference 171.8 mm; <5 th percentile [9]) and revealed extensive hypoplasia of the lungs. Amniocentesis and peripheral blood sampling from the parents were performed in the 21st week of gestation and cytogenetic analysis was performed in another institute and revealed a fetal 46,XYdup(15)(q11.2q11.2)mat karyotype. Both parents were phenotypically inconspicuous.

Immediately after delivery (41st week of gestation) the newborn was cyanotic and progressive hypoxemia developed. Despite endotracheal intubation and then tracheotomy, 20 minutes after birth the newborn succumbed to acute respiratory failure.

Anatomic pathology of thoracic and retroperitoneal organs

Post-mortem examination was initiated and revealed body parameters within the normal ranges of the 41th week of gestation [12]: body weight 3680 g, crown-heel-length 54 cm and foot length 8.5 cm.

Examination of the mediastinum revealed a pulmonary trunk which was connected to the aorta via the *ductus arteriosus* but macroscopically no pulmonary arteries and veins were observed. The *foramen ovale*, caval veins, *ductus venosus*, umbilical cord vessels and all cardiac valves and both ventricles appeared to be normal.

An apparently normal development of pharynx/

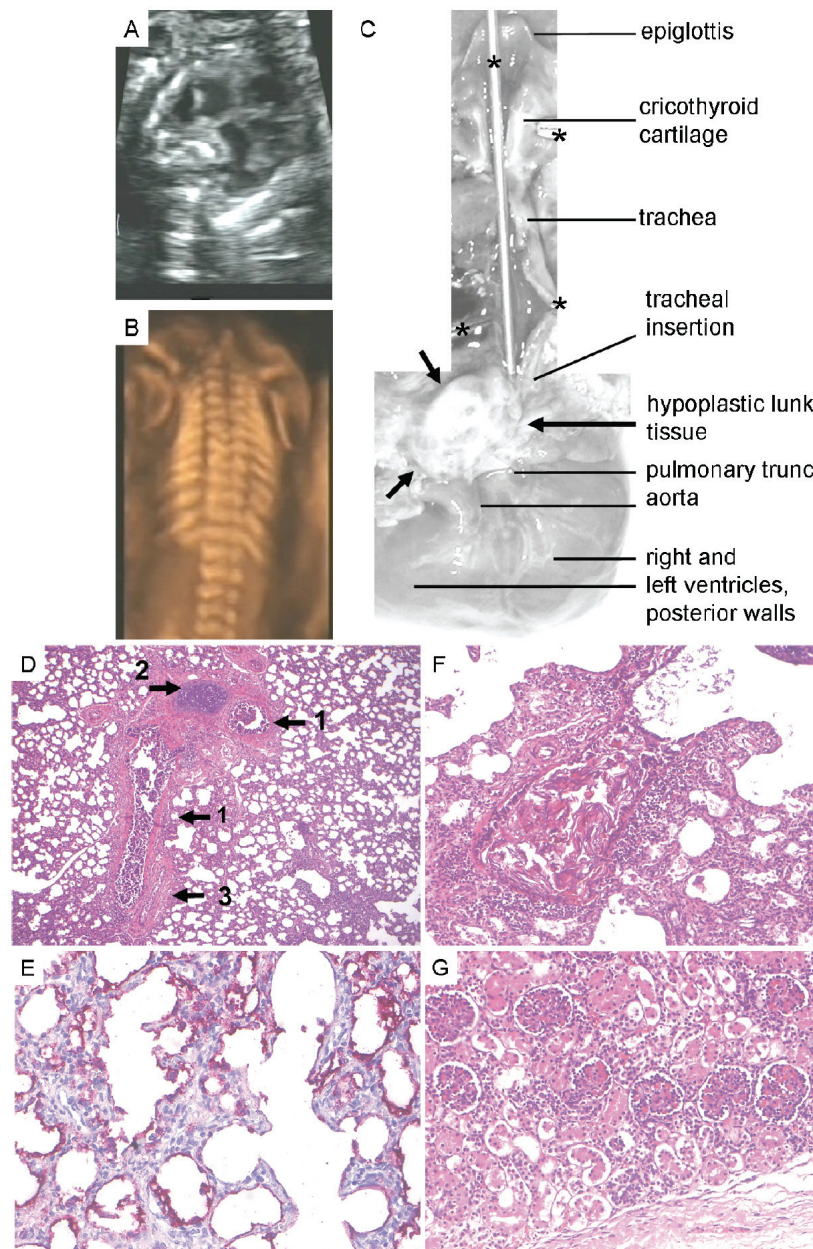


Figure 1 Sonographic and anatomical findings of the thorax and the thoracic organs. A) Sonographic image shows four heart chambers but no lungs (2nd trimester). B) 4D real-time sonography scan of the skeleton is depicted and shows an aberrantly narrow thoracic cage (2nd trimester, images A and B were both produced with a GE Volusion 730 ultrasound device, GE Healthcare, Bedford, UK). C) Autopsy finding: dorsal view of the thoracic situs shows the extremely hypoplastic pulmonary rudiment, which is connected to the apparently normal trachea. Asterisks indicate instruments; note that parts of the cervical connective tissue are covert. D) Haematoxylin-eosin stained formalin-fixed and paraffin-embedded tissue sections of proximal parts of the lung rudiment show bronchial (arrows, 1) and alveolar structures, bronchial cartilage (arrow, 2) and adjacent pulmonary artery-like vessels (arrow, 3); the emphysema is associated with post-natal intubation due to acute respiratory failure (original magnification $\times 200$). E) A representative area is depicted which reveals surfactant covering the alveolar surface (original magnification $\times 200$). F) The distal parts of the lung show focal accumulation of aspirated squamous epidermal cells in a bronchus and some parabronchial lymphoid cells (original magnification $\times 100$). G) Apparently normal glomerular structures of the left kidney (original magnification $\times 100$). Microscopic images were produced with a DP71 Camera (Olympus, Hamburg, Germany) on an Axiophot microscope with Plan-Neofluar objectives (both Zeiss, Jena, Germany) and were processed with Soft Imaging System software (Olympus, Hamburg, Germany).

oesophagus and larynx/trachea could be demonstrated but the lung consisted of a retro-mediastinal longitudinal-oval tissue mass of 30×20×5 mm. No carina or main bronchi were developed and the non-lobulated lung rudiment was connected directly to the distal trachea (**Figure 1C**). Histological examination revealed ventilated bronchial and alveolar structures as well as surfactant production (**Figure 1D-1F**). We observed some pulmonary artery-like vessels paralleled to bronchial structures, indicating extreme hypoplasia, far more than previously reported [13], rather than complete agenesis of pulmonary arteries. Secondary to pulmonary hypoplasia, pleural cavities were empty, but diaphragms on both sides were developed normally.

Retroperitoneal examination confirmed agenesis of the right kidney and revealed agenesis of the corresponding ureter and vessels. The left kidney (**Figure 1G**; 22 g; overweight [12]) and ureter as well as both suprarenal glands (right: 3 g, left: 4g; normal weight [12]), urinary blad-

der and prostate were anatomically inconspicuous. Bilateral testicular descent was completed.

No other abnormalities were observed.

Genetic analysis reveals duplication of a small chromosome 1p segment

Necropsy-derived DNA showed good quality (NanoPhotometer, Implen, Munich, Germany): A260/A280 ratio = 1.881 and A260/A320 ratio = 2.029. Alterations were considered to be true chromosomal aberrations only if at least two independent methods confirmed duplication or deletion. A male karyotype with a duplication of 1p21 was detected by aCGH as well as qPCR but no chromosome 15 aberrations (**Table 1**, **Figure 2**).

Discussion

From the embryological point of view, the mediastinal organ dysgenesis sheds light on the complex sequential organogenesis of cardiovas-

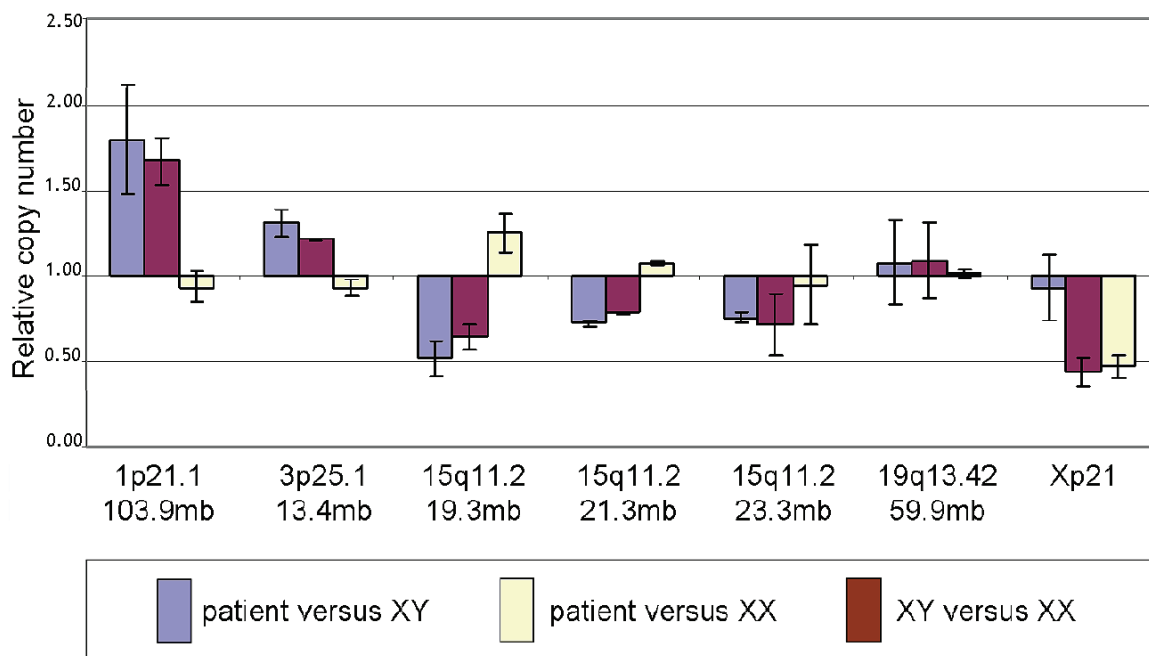


Figure 2. Verification of the 1p21 duplication by quantitative PCR. Fluorescence ratios of the individual loci of interest against a control locus (a pool of four loci showing unaltered copy number state according to aCGH) were calculated in the patient probe as well as in a male and a female reference sample to obtain the relative copy number (the quotient of the patient ratio versus the control ratio) shown on the y-axis. As an additional control, the Xp21 locus was chosen to demonstrate normal copy number when the patient's Xp21 copy number is compared to the Xp21 copy number in the XY reference sample, but loss of the X-locus when compared to the XX reference. Also, the reference XY sample shows a loss of one X copy, when compared to the XX sample, as postulated.

Table 1. Table 1 Array-CGH and quantitative PCR analysis revealed duplication of 1p21

Chromosome region	aCGH-derived karyotype 46,XY	Log ratio (+/-0.2)	Confirmation by qPCR
1p21	arr cgh 1 (RP5-1108M17>RP11-508C1) × 3	0.345	Yes
3p25	arr cgh 3 (RP11-488M6>RP11-512I22) × 3	0.28	No
4p16	arr cgh 4 (CTC-36P21>RP11-572017) × 3	0.205	No
5p15.3	arr cgh 5 (RP11-117 B23>CTD-2265D9) × 3	0.275	No
7p22	arr cgh 7 (CTB-164D18>RP11-106E3) × 3	0.215	No
15q11.2	arr cgh 15(RP11-2F9-RP11-446P9) × 2	0.103	No
17p11.2	arr cgh 17(RP11-416I2>RP1-37N7) × 3	0.203	No
19q13.4	arr cgh 19 (RP5-1060P11>CTB61M7) × 1	- 0.309	No

cular and pulmonary structures [4,5]. Apparently, the 4th and 6th aortic arches have been differentiated into normal left ventricle-derived aorta, right-ventricle-derived pulmonary trunk and the *ductus arteriosus*, but the branching of pulmonary arteries from the pulmonary trunk has occurred in a very hypoplastic manner. In an embryologic murine model, lungs were capable of branching without blood vessel supply *ex vivo* [14], which demonstrates the capability of an independent but limited lung development. In this given case, without appropriate induction by pulmonary arteries, apparently the initially independently developed lung tissue did not generate matured bilateral organs. However, intrauterine oxygenation, supplied by the placenta, seemed to have allowed normotrophic body maturity (placenta was not sent in for examination). Furthermore, certain features of normal lung development were recapitulated, including peripheral alveolar structures and surfactant production, which is expected by the 24th weeks of gestation [4]. In summary, these anatomical findings strongly suggest an initially parallel and independent cardiovascular and laryngo-tracheal development depending on adequate vascularization by pulmonary arteries around the 4th week of gestation, as soon as the pulmonary tissue reaches the upper mediastinum.

Several genetic aberrations were detectable by aCGH analysis and we subsequently re-evaluated these results by qPCR and could confirm the duplication of 1p21. Of note, neither

aCGH nor qPCR could confirm the cytogenetically assessed duplication of the pericentric region of chromosome 15. Thus, we consider the duplication of 1p21 as the sole verified chromosomal aberration in this patient. However, there is no specific disease phenotype which has been associated to this aberration. We reviewed our computational archive for similar anatomical and/or chromosomal aberrations (2000-2008, 111 paediatric post-mortem examinations, ~19,900 cytogenetic analysis) but found none, thus reflecting the rarity of this malformation. Utkas et al. have summarized seven cases with duplications of chromosome 1 including at least parts of segments of 1p21 [15]. These patients had a variety of musculoskeletal abnormalities but no renal or lung dysmorphogenesis, or, in a mother and her son, even no phenotypical disorders [15]. In fact, a growing number of genomic copy number variations in healthy individuals is known [16-18] while their functional significance is lacking. In the catalogue of genomic variants, the duplcon 1p21 is mentioned many times (<http://projects.tcag.ca/variation>). It has been shown that a duplication of 1p21 can be associated with a higher body stature in males [19]. The known genes mapping to the duplicated chromosomal region code for amylase enzymes [20] and collagen type XI alpha 1 [21] but none of the important growth and transcription factors which are involved in pulmonary and renal morphogenesis [6,22]. Furthermore, this type of collagen is not specific for the lung [21]. Therefore, this makes a causal role of increased 1p21 gene dosage in the

pathogenesis of these complex morphological abnormalities unlikely, which highlights the importance of the correlation of genetic findings with clinical and anatomical data to assess the relevance of any chromosomal alteration.

In conclusion, i) the bilateral hypoplasia of pulmonary arteries and the lung with associated unilateral renal agenesis might be an extreme variant of previously described simultaneous dysmorphogenesis of the pulmonary and renal system, and ii) these phenotypical abnormalities in the presence of duplication 1p21 might be caused by genetic/epigenetic defects other than the duplication.

Please address correspondence to: Kais Hussein, MD, Institute of Pathology, Medizinische Hochschule, Hannover, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany, Tel. 0049-511-532-4501, Fax: 0049-511-532-5799, E-Mail: Hussein.Kais@MH-Hannover.de

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Bilateral hypoplasia of the lung with unilateral renal agenesis

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