The *OSR1* rs12329305 Polymorphism Contributes to the Development of Congenital Malformations in Cases of Stillborn/Neonatal Death

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Background: Involvement of development-related gene polymorphisms in multifactorial/polygenic etiology of stillborn/neonatal deaths due to malformations has been insufficiently tested. Since these genes showed evolutional stability and their mutations are very rare, we can assume that their polymorphic variants may be a risk factor associated with the occurrence of developmental disorders of unknown etiology or can enhance the phenotypic variability of known genetic disorders.

Material/Methods: To determine the association of 3 polymorphisms involved in the regulation of the early embryonic development of different organs, we conducted an association study of their relation to the particular malformation. We selected 140 samples of archived paraffin tissue samples from deceased patients in which fetal/neonatal autopsy examination had shown congenital abnormalities as the most likely cause of death. The polymorphisms of *OSR1* rs12329305, rs9936833 near *FOXF1,* and *HOXA1* rs10951154 were genotyped using the TaqMan allelic discrimination assay.

Results: After Bonferroni correction for multiple testing, significant allelic association with stillborn/neonatal deaths was observed for rs12329305 ($p=7\times10^{-4}$). In addition, association analysis for the same polymorphism was shown in the subgroup with isolated anomalies (1.25×10^{-5}), particularly in the subgroup of cases with kidney and heart anomalies ($p=4.18\times10^{-5}$, $p=5.12\times10^{-8}$, respectively).

Conclusions: The findings of the present study showed, for the first time, the role of the *OSR1* rs12329305 polymorphism in the development of congenital malformations in cases of stillborn/neonatal death, particularly in those with congenital kidney and heart developmental defects.

MeSH Keywords: **Congenital Abnormalities • Human Development • Polymorphism, Single Nucleotide**

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Background

Congenital anomalies can be defined as a complex and heterogeneous group of embryonic and/or fetal development disorders. In developed countries, the incidence of congenital anomalies is 3–5% among neonates, and they are the main cause of infant mortality [1], in which perinatal autopsy often detects various types of congenital malformations involving 1 or more than 1 system, presenting multisystem malformations. Autopsy examination can certainly detect some potentially underestimated rare malformations of special interest in surgical medicine [2].

Most congenital malformations such as congenital heart disease (CHD), congenital anomalies of the kidney and urinary tract (CAKUTs), anomalies of the gut, lung, cleft lip, and palate have a multifactorial etiology, comprising both genetic and environmental factors [3]. Up to 20% of stillborn children with malformations have chromosomal aberrations [4]. Pathogenesis of congenital malformations in cases of chromosomal disorders is also poorly understood. An imbalance in the normal gene dosage (e.g., 3 copies of chromosome 21 in Down syndrome) itself is not sufficient to cause the most common congenital anomaly – CHD. Additional unidentified genetic variations in the rest of the genome and/or environmental factors probably contribute to the risk of CHD in Down syndrome [5].

By identification of some renal and heart developmental genes, several large studies have recently provided new insight into how the heart and the kidney develop [6,7]. In our study, we hypothesized that polymorphic variants of transcription factors that regulate morphogenesis and differentiation can have significant effects on occurrence of isolated or multiple organ defects.

We selected 3 single-nucleotide polymorphisms (SNPs) of transcription factors genes regulating morphogenesis and differentiation – Odd-Skipped Related 1 (*OSR1*) rs12329305, rs9936833 near Forkhead Box F1 (*FOXF1*), and Homeobox A1 (*HOXA1*) rs10951154 – for this study. *OSR1, FOXF1,* and *HOXA1* have been highly conserved throughout evolution and the knowledge of their function in humans is still limited [8–10]. Mutations in *FOXF1* and *HOX1* have previously been associated with multiple organ defects, suggesting pleiotropic effects and leading to perinatal death, while to our knowledge, mutations in *OSR1* have not been associated with congenital malformations in humans [11–13].The stability of these genes and rarity of gene mutations lead to the assumption that they play fundamental roles in critical developmental processes and we assume their polymorphic variants can be associated with a broad spectrum of isolated or multiple organ defects. The evolutionarily conserved *OSR1*, mapped on chromosome 2p24, encodes a transcriptional regulatory protein with 3 zinc fingers [8]. In mice,

Osr1 is expressed in the mesentoderm and it expression is regulated by the mesenchyme-to-epithelium transition [14]. The early stages of kidney and heart development are critically dependent on *Osr1* expression. Homozygous mice with inactivated *Osr1* die in utero due to heart defects and renal agenesis [15,16]. In humans, *OSR1* expression was shown in mesenchymal stem cells from amnionic fluid and renal progenitor cells from adult kidney, indicating that *OSR1* is essential for nephrogenesis in humans, as it is in mice [14]. *OSR1* mRNA was also detected in the adult colon, small intestine, prostate, testis, and fetal lung in humans [8].

FOXF1 is located on the long arm (q) of chromosome 16 at position 24 and belongs to a family of genes known as *FOX* (forkhead box) [9]. FOX proteins are transcription factors essential for the normal development of the gastrointestinal tract in mice [17]. The mice homozygous for *Foxf1* mutations die in utero with variable phenotype including lung defects, narrowing of the esophagus and trachea, esophageal atresia, and tracheal-oesophageal fistula [18]. High perinatal mortality was also found in humans with heterozygous mutations in *FOXF1* gene. *FOXF1* mutations were observed in patients with alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV), always accompanied by other organs malformations (e.g., cardiac, intestinal, and genitourinary tract malformations) [11].

HOXA1 is located on human chromosome 7p15.3 and encodes a DNA-binding transcription factor that may regulate gene expression, morphogenesis, and differentiation [10,19,20]. The vertebrate nervous system is the main location of *HOX* expression and function. A mutation in *HOXA1*, a member of the *HOXA* cluster, leads to numerous developmental defects, including hindbrain deficiencies, abnormal skull ossification, and defects of the inner ear [21]. A recent study found severe cardiovascular malformations in patients carrying a homozygous truncating mutation in *HOXA1* (Bosley-Salih-Alorainy Syndrome [BSAS]) [12,13].

The aim of this study was to investigate the role of the *OSR1* rs12329305 silent mutation located in exon2 at position 19353152, rs9936833 intergenic/unknown mutation near *FOXF1,* and *HOXA1* rs10951154 mis-sense, transition substitution, and intragenic mutation located in the 5'near gene region at position 27095695 in cases of stillborn/neonatal deaths due to different organs malformations in comparison with a control group of healthy children. We assume that the 3 selected polymorphisms represent genetic susceptibility factors or 1 of the possible factors involved in multifactorial/polygenic etiology of stillborn/neonatal deaths due to malformations. They can have functional effects on genes involved in the regulation of early embryonic development or can enhance the phenotypic variability of known genetic disorders.

Material and Methods

Samples/patients

We selected total of 140 archived paraffin tissue samples in which fetal/neonatal autopsy examination had shown congenital abnormalities as the most likely cause of death. All perinatal deaths were at ≥28 weeks of gestation or ≤28 days at birth and were collected at the Department of Pathology, University Hospital Split, during a 12-year period from 2000 to 2011. Macerated stillborns; recognizable single-gene inherited disorders; and disorders caused by maternal diseases, nutritional deficiencies, or drug consumption were excluded from the study. All clinical data for 140 cases of stillborn/neonatal death were obtained from clinical medical records and autopsy examination reports performed or supervised by a specialist neonatologist and perinatal pathologist. Until recently, autopsy examination was obligatory in such cases in Croatia. Karyotypes for the most common chromosomal disorders were analyzed prenatally from amniotic fluid or immediately after delivery from the blood of the neonates. Sex distribution among cases was 55 (39.3%) females and 85 (60.7%) males. The control group consisted of 200 children who visited the hospital for follow-up medical examination after acute respiratory infection. Sex distribution among healthy controls was 98 (49.0%) males and 102 (51.0%) females.

Twenty of all 140 cases (24.3%) had the most common chromosomal disorders, and the remaining 120 (85.7%) cases had 1 or more malformations. Proportion of stillbirths and neonatal death was 30 (21.4%) versus 110 (78.6%), respectively. Neonatal death was defined as death in the first 4 weeks of life in liveborn neonates.

A group of cases (120) of stillborn/neonatal deaths due congenital malformations with an unknown genetic background was classified into the 2 following subgroups of cases: isolated and multiple. The first subgroup, with isolated anomalies, included 63 cases, while the second subgroup, with multiple anomalies, included 57 cases. Cases in the second subgroup had 2 or more malformations. In this group we included some recognizable malformation/dysplasia syndromes such as: VATER and VACTERL association (non-random co-occurrence of birth defects, vertebral anomalies, anal atresia, cardiac defects, tracheoesophageal fistula and/or esophageal atresia, renal and radial anomalies, and limb defects), different types of Potter's syndrome, and prune belly syndrome (Table 1).

In addition, we classified in detail isolated malformation in the 4 following different subgroups: CHD; CAKUTs; gastrointestinal tract abnormalities (GTA); and central nervous system (CNS), including neural tube defects (NTDs), craniofacial structures, and others (Table 1).

The study was approved by the Ethics Committee of University Hospital Split, and informed consent was obtained from the patient's parents prior to blood sampling in the control group.

DNA extraction and genotyping

Genomic DNA was extracted from the formalin-fixed paraffin-embedded (FFPE) tissues (different tissues were used) in cases and from peripheral blood leukocytes in the control group.

We extracted genomic DNA from FFPE using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany, cat. no. 56404) and DNA from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany, cat. no. 51104).

Genotyping of the 3 SNPs – rs12329305, rs9936833, and rs10951154 – within *OSR1*, near *FOXF1*, and within *HOXA1*, respectively, was performed with real-time PCR using the ABIPRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, USA) and pre-developed TaqMan assay reagents, C_25994108_20 VIC/FAM, CCGCAGTGCCGCACCTGTGGTCTCG [C/T] AGGTGGTCTTGCCTCCGGAAGGCTT for rs12329305 SNP, C_1937647_20 VIC/FAM, TTTAACAAAACAGAAGTCAAAAGCA [C/T] TGCCACTCTCTACCACCCTCCTAGA for rs9936833 SNP and C_27262818_10 VIC/FAM, and CTGGTAGGTAGCCGGCTGGGGGTGG [C/T] GATGGTGGTGGTGGTGGTGGTGGTG for rs10951154 SNP. PCR reaction was performed according to the manufacturer's protocol.

Genotype call rate for SNP genotyping was 91.8%. After repeated genotyping with higher DNA concentrations, call rate was 95.6%. Call rate was limited because DNA isolated from FFPE tissue is often fragmented and cross-linked and is therefore difficult to genotype.

Statistical analysis

Prior to association analysis, we performed quality control of the obtained genotypes. We tested genotyping rate, Hardy-Weinberg equilibrium (HWE), and minor allele frequencies (MAFs) for all samples using HaploView 4.1. MAFs of the 3 SNPs were compared with the National Center for Biotechnology Information SNP database (NCBI dbSNP) MAFs for the central European (CEU) population (www.ncbi.nlm.nih.gov/projects/SNP/). Association analyses were performed using implementations of the case-control single-point association test in HaploView 4.1 [22]. Haplotype frequencies were estimated using the expectation-maximization algorithm implemented in HaploView 4.1. After Bonferroni correction for multiple testing, p-values less than 0.0166 were considered nominally significant.

Calculations of 80% power study at α=0.05 were performed using Quanto. The results are expressed as OR. When OR>1, genotype confers sensitivity to the effects of exposure [23].

Table 1. Classification of cases with stillborn/neonatal deaths due to malformations/genetic disorders according autopsy examinations.

	Cases	Neonatal deaths (%)		Stillborns (%)	
	No	No	(%)	No	(%)
Malformations	**140**	**110**	**(78.6)**	**30**	**(21.6)**
Gender					
Males	85	68	(80.0)	17	(20.0)
Females	55	42	(76.3)	13	(23.7)
GA					
Full-term	38	30	(78.9)	8	(21.1)
Preterm	102	80	(78.4)	22	(21.6)
Chromosomal disorders	**20**	**16**	**(80.0)**	**4**	**(20.0)**
Trisomy 21	10	8	(80.0)	2	(20.0)
Trisomy 18	7	5	(71.4)	2	(28.6)
Trisomy 13	2	2	(100)	0	(0.00)
Duplication 7q	1	1	(100)	0	(0.00)
Unknown genetic background	**120**	**94**	**(78.3)**	**26**	**(21.7)**
Multiple malformations	**57**	**45**	**(78.9)**	**12**	**(21.1)**
Isolated malformations	**63**	**49**	**(77.7)**	**14**	**(22.3)**
CHD	14	12	(85.7)	2	(14.3)
CNS anomalies, craniofacial anomalies, NTDs	10	8	(80.0)	2	(20.0)
Gastrointestinal tract – anomalies	15	12	(80.0)	3	(20.0)
CAKUTs	16	10	(62.5)	6	(37.5)
*Others	8	7	(87.5)	1	(12.5)

GA – gestational age; Preterm <37 weeks gestational age; CHD – congenital heart disease; CAKUTs – congenital anomalies of the kidney and urinary tract; CNS – central nervous system; NTDs – neural tube defects: *Other included – 3 cases with abdominal wall defect (3 neonatal deaths) + 4 cases with Hand- Foot-Clubfoot (2 neonatal deaths + 1 stillborn) + 1 case with congenital cystic adenomatoid unilateral lung malformation (1 neonatal death).

Table 2. Allelic association of *HOXA1* rs10951154, *OSR1* rs12329305, and near *FOXF1* rs9936833 single nucleotide polymorphisms (SNPs) with stillborn/neonatal death due to malformations/genetic disorders. (No of cases: 140; No of controls: 200).

SNP (gene)	Base change	Location	MAF*-cases	MAF*-controls	χ^2	p-value
rs10951154 (*HOXA1*)	A>G**	7p15	0.134	0.166	1.267	0.2603
rs12329305 (*OSR1*)	C>T**	2p24	0.134	0.056	**11.454**	**7×10^{-4}**
rs9936833 (near *FOXF1*)	T>C**	16q24	0.434	0.385	1.462	0.2266

*MAF – minor allele frequency; ** risk alleles; Values of p were adjusted taking into account the structured ancestral distribution. p<0.0166 was considered statistically significant.
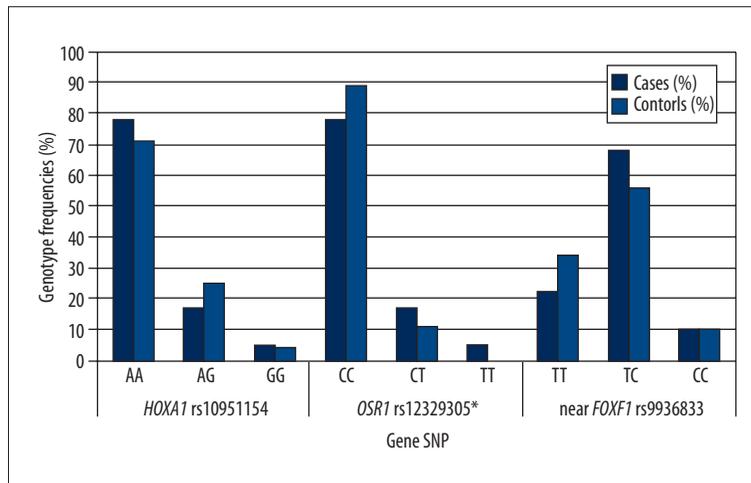
Figure 1. Genotype association of *HOXA1* rs10951154, *OSR1* rs12329305, and *FOXF1* rs9936833 single-nucleotide polymorphisms (SNPs) with stillborn/neonatal death due to malformations/genetic disorders (140 cases, 200 controls) (*p=0.0013).

Table 3. Allelic association of *OSR1* rs12329305 polymorphism and specific subgroups of stillborn/neonatal death malformations/genetic disorders according to autopsy examinations.

Number of participants	Total No	Allele C	Allele T	χ^2	p
Controls	195	368	22		
Cases	119	206	32	11.454	0.0007
Chromosome disorders	16	25	7	12.178	5×10^{-4}
Multiple malformations	47	90	4	0.286	0.5927
Isolated malformations	56	91	21	19.092	1.25×10^{-5}
CHD	13	17	9	29.671	5.12×10^{-8}
CNS anomalies, craniofacial structure, NTDs	8	15	1	1.3	0.254
Gastrointestinal tract anomalies	14	26	2	0.109	0.7414
CAKUTs	16	24	8	16.784	4.18×10^{-5}
Others*	5	9	1	0.342	0.5588

CHD – congenital heart disease; CAKUTs – congenital anomalies of the kidney and urinary tract; CNS – central nervous system; NTDs – neural tube defects. * Others included: 4 cases with abdominal wall defect + 1 case with congenital cystic adenomatoid unilateral lung malformation.

Results

Genotyping

Genotyping was performed on 140 cases and 200 control subjects. Genotype in cases and controls fit HWE, except for rs9936833, which deviated from HWE in cases of stillborn/neonatal death (p=4×10^{-6}).

Allelic testing detected an association of the *OSR1* rs12329305 polymorphism with stillborn/neonatal death due to malformations/genetic disorders (χ^2=11.454, p=7×10^{-4}), while 2 other investigated polymorphisms – rs9936833 near *FOXF1* and

HOXA1 rs10951154 – did not show such association (χ^2=1.462, p=0.2266 and χ^2=1.267, p=0.2603, respectively) (Table 2). The *OSR1* rs12329305 polymorphism also showed genotyping association with stillborn/neonatal death due to malformations/genetic disorders (χ^2=13.25, p=0.0013). In the group of cases, TT genotype was found more frequently than in controls (Figure 1). Two other analyzed SNPs did not show genotypic association (*HOXA1* rs10951154 χ^2=2.96, p=0.227; near *FOXF1* rs9936833 χ^2=5.15, p=0.076 (Figure 1).

Second-stage association analysis of the 2 subgroups of cases (with isolated and multiple anomalies) and controls showed allelic association of the *OSR1* rs12329305 polymorphism with

isolated anomalies (χ^2=19.092, p=1.25×10^{-5}) (Table 3). Detailed analysis showed allelic association of the *OSR1* rs12329305 polymorphism with kidney and heart anomalies (χ^2=21.809, p=4.18×10^{-5}; χ^2=29.671, p=5.12×10^{-8}, respectively) (Table 3).

Our study had 80% statistical power to detect (α=0.05) an effect with odds ratio (OR) = 2.1 for rs10951154 and OR=1.9 for rs9936833, assuming an additive model. The OR for rs12329305 is not presented because MAF for the general population is 2.2% and recessive homozygotes are not found in HapMap CEU population since it is a low-frequency functional variant (according NCBI dbSNP).

Discussion

This is the first study to investigate the role of the *OSR1* rs12329305, *HOXA1* rs10951154, and rs9936833 near *FOXF1* SNPs in cases of stillborn/neonatal deaths due to malformations of various organs.

The *OSR1* rs12329305 polymorphism showed allelic and genotype association with stillborn/neonatal death due to malformations/genetic disorders. We observed that the *OSR1* rs12329305 T allele is over-represented in some isolated congenital malformations. Due to the significant presence of *OSR1* rs12329305 T allele, mainly in isolated malformations of the heart and kidneys, we concluded that the polymorphic variant of this gene can contribute to susceptibility of congenital kidney and heart developmental defects in humans. Polymorphism rs9936833 near *FOXF1* and *HOX1* rs10951154 polymorphism did not show allele or genotype association with stillborn/neonatal deaths due to malformations of different organs in our study.

To date, only 1 study has observed frequency of the rare T allele in 6% of white neonates, which is in accordance with our finding of 5.6% in the control group [14]. The NCBI dbSNP database reported a 2.2% MAF for rs12329305 in the HapMap CEU population with no recessive homozygous, and we found no recessive homozygous in our control group. Accordingly, rs12329305 is a low-frequency polymorphism, which showed large effect size to stillborn/neonatal death due to malformations, especially to the isolated malformations of the heart and kidneys. Some studies defined variants with MAFs between 0.1% and 3% as rare variants and concluded that rare variants associated with complex phenotypes sometimes have larger effect sizes to the trait than common variants [24]. Associations of rare variants with complex phenotypes, metabolic pathways, or polygenetic disease are under-investigated [25]. However, a recent study has shown that *Osr1* has an essential role in the early stages of kidney and heart development in an animal model [16]. It seems that OSR1 protein is equally important

in the early stage of embryonic human development as well as in murine development [15]. To our knowledge, mutations in *OSR1* have not been described in humans, and no associations with congenital malformations have been found until now. Our study showed association of rs12329305 *OSR1* minor allele with kidney and heart anomalies. A Montreal study showed an association of rs12329305 *OSR1* minor allele with reduced kidney volume in healthy white neonates [14]. The same study showed that *OSR1* rs12329305 polymorphism in humans altered the secondary *OSR1* transcript to be relatively unstable, and this apparent effect may change renal progenitor pools, leading to a decreasing size of the kidneys and alteration of their function in healthy neonates [14]. Accordingly, a possible explanation for our finding is that the presence of the T allele in cases alters the secondary *OSR1* transcript, leading to further impaired production or reduced bioavailability of transcriptional regulatory OSR1 protein. Osr1 protein and its homolog Osr2 protein, which exists in the following 2 isoforms: Osr2B containing 3 and Osr2A containing 5 zinc finger motifs, have proven to be functionally equivalent in mouse development [26]. Osr1 protein is the structural homolog of Osr2B, and they share 65% identity in amino acid sequence and 98% identity in amino acid sequence of the conserved region of their zinc finger domains in mice [27]. Osr2A represses transcriptional activity, while Osr2B activates transcription [28]. We assume that their structural homology, as well as partially overlapping expression patterns, is probably a very important biological mechanism in human embryonic development and organogenesis, as well as in mouse development [27]. In a mouse model it was shown that Osr1 protein is a component in the signaling pathway in the regulation of embryonic cardiovascular development. It phosphorylates and regulates the function of many angiogenesis factors and their receptors. On the other hand, some cases with impaired production or reduced bioavailability of transcriptional regulatory Osr1 protein rescue the cardiovascular defect because of residual kinase activity of other factors involved in the *Osr1* signaling cascade [29]. There is a possibility that *Osr1* is also involved in complex pathological developmental processes of cardiovascular system diseases. Recent studies that investigated different polymorphisms within genes potentially associated with cardiovascular system diseases emphasize the benefit of their detection in early childhood [30, 31].

We also speculate that the *OSR1* rs12329305 polymorphism may be involved in the regulation of developmental pathways in cases in which complex genetic architecture is present (e.g., 3 copies of chromosome 21 or chromosome 18) and can enhance the phenotype severity in these cases.

It was shown that FOX proteins are transcription factors essential for gastrointestinal and lung organogenesis [11, 17, 32]. A recent study showed that heterozygous mutations in

FOXF1, mostly located within the putative DNA-binding domain, were found in patients with a lethal developmental disorder of the lung (ACD/MPV), always accompanied with other multiple malformations of heart, gastrointestinal, and urinary tract, similar to those seen in VACTERL association [32]. Agochukwu et al. (2011) did not find an association of *FOXF1* mutations with VACTERL association in a small cohort of patients [33]. Recently, a genome-wide association study (GWAS) identified the rs9936833 polymorphism located near *FOXF1*, surrounded by additional binding sites for transcription factors like *FOXP2*, which controls *FOXF1* expression [34]. The minor allele C of rs9936833 near *FOXF1* was associated with risk of Barrett's esophagus, indicating its involvement in esophageal development and structure [34]. Our study did not showed allelic or genotype association of the rs9936833 polymorphism near *FOXF1* with stillborn/neonatal death due to malformations.

A recent study indicated that *HoxA1* may have a role in cardiovascular system development in mice [35]. Homozygous mutations in *HoxA1* in mice often cause neonatal death [36]. Ingram et al. [37] identified the rs10951154 polymorphism within a highly conserved domain of *HOXA1* – an A-to-G substitution at codon 218 that changes the codon for 1 histidine in a series of histidine repeats to an arginine at the position of the 73rd amino acid. This allelic variant has been linked to both cerebellar and cranial development, and susceptibility to autism spectrum disorders has been suggested [37]. However, the role of

this particular series of histidine repeats has not been established and we found no evidence to link the rs10951154 polymorphism with multiple organ malformations. Consequently, we conclude that it does not have an effect on *HOXA1* pathways during early embryogenesis.

Conclusions

This study, for the first time, provides evidence implicating the *OSR1* rs12329305 polymorphism in the development of 1 or more organ system malformations in cases of stillbirth/neonatal death, particularly in those with congenital kidney and heart human developmental defects. Future analysis of the *OSR1* rs12329305 polymorphism using a large sample set will provide additional evidence of its important role in the development of heart and kidney or, potentially, other organs. This evidence would be of great benefit for the health of the population because of the high incidence of the broad clinical spectrum of CAKUTs and CHD.

Studies using a large sample set are also required to elucidate the role of the *OSR1* rs12329305 polymorphism as a potential genetic factor that increases the risk of specific malformations in the presence of the extra chromosome in the genome. We also emphasize the importance of autopsy findings of archived samples in the investigation of very rare genetic malformations.

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