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Original Research Article

A pilot study conducted in the Czech Republic relating to the molecular analysis of the *RET* and *GDNF* genes in patients with cakut, predominantly unilateral renal agenesis

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Abstract

Renal anomalies are quite a common medical condition thought to be genetically influenced. The subject of this work was to conduct molecular genetic analysis of two human renal agenesis causing candidate genes encoding the *RET receptor thyrosin kinase* and *GDNF neutrophic factor*. The mutational analysis of twenty *RET* exons and three *GDNF* exons in twenty patients diagnosed with unilateral renal agenesis was carried out. Furthermore, copy number changes in both genes were investigated. The aim of this work was to identify potential mutations of *RET/GDNF* genes and thus to prove their association with renal agenesis. In this group of patients, no known pathogenic mutations were discovered, only three known single nucleotide polymorphisms of the *RET* gene were detected. Polymorphism rs1800860 (GCG-GCA, Ala432) was detected in 11/20 patients, two of them in a homozygous state. Polymorphism rs1800861 (CTT-CTG, Leu769) was identified in 6/20 patients, where one patient was a homozygote for the minor allele G. Polymorphism rs1800863 (TCC-TCG, Ser904) was detected in 5/20 patients, always in heterozygous combinations. All these polymorphisms are common ones with the minor allele frequency in population higher than 10%. Results of this study did not confirm an increased incidence of *RET* and *GDNF* mutations in patients diagnosed with renal agenesis and thus the association of these genes with renal agenesis cannot be proved. Nevertheless, with regard to a limited size of the patient group, the association between RET/GDNF signal complex and renal agenesis cannot be excluded. Submitted paper is a pilot study of patients with CAKUT in the Czech Republic, we intend to continue collecting samples and examine more genes relating to these anomalies.

Keywords: CAKUT, GDNF, Pheochromocytoma, Polymorphism, Renal agenesis, RET

INTRODUCTION

Kidney anomalies can be classified as common birth defects which significantly influence the chance of survival and the quality of life on the whole. Undoubtedly, renal agenesis is one of the most serious conditions from

this group of developmental defects. Despite the fact that, in some cases, unilateral renal agenesis may be unidentified, the majority of affected individuals suffer from various medical complications which are the primary consequence of compensatory hypertrophy of the contralateral kidney and can lead to, for example, arterial hypertension, proteinuria or decreased glomerular filtration. Bilateral renal agenesis is responsible for the significant percentage of abortions and early deaths of newborn children. Congenital renal agenesis is a relatively common developmental defect, according to the birth defect registers its average annual incidence ranges from 0.15 to 0.2 in 1,000 births (Schedl, 2007; Schulman et al., 1993), or 0.6 in 1,000 newborns (Pohl et al., 2002).

Bilateral renal agenesis (BRA) is incompatible with life. It occurs in about one in 30,000 births (Schedl, 2007). Unilateral renal agenesis (URA) is more common than BRA. The most quoted URA frequency is one in 4,000-5,000 births (Schedl, 2007; Reiterová and Merta, 2008). The remarkable fact is that renal agenesis as well as other anomalies belonging to CAKUT (Congenital anomalies of the kidney and urinary tract) indicate the male predisposition, ratio of affected men to women is 2.5:1 (Carter et al., 1979; Harewood et al., 2010; Sanna-Cherchi et al., 2009). Apart from the male sex of a fetus, mother's diabetic disease is another risk factor (Davis et al., 2010; Nielsen et al., 2005).

Family occurrence of CAKUT (congenital anomalies of the kidney and urinary tract) gives evidence of genetic factors being present at the creation of kidney diseases, however, the heredity pattern indicates variable expressivity with the occurrence of different phenotypes throughout their genealogy trees and incomplete penetrance enabling the influence of environmental factors. This situation is also complicated by an asymptomatic nature of URA and a high degree of genetic heterogeneity (Sanna-Cherchi et al., 2009; Cain et al., 1974; Fitch, 1977; Renkema et al., 2011; Squiers et al., 1987).

The risk of renal defects is higher in fetuses whose first degree relatives suffer from any kidney anomaly, in such cases the empirical risk for CAKUT is about 10% (Renkema et al., 2011), for BRAHD (bilateral renal agenesis/ hypoplasia/ dysplasia) about 5% (Harewood et al., 2010; Fitch, 1977). Occurrence of various kidney anomalies, including renal agenesis, as one of the symptoms accompanying some multiorgan syndromes with a specific type of heredity, such as autosomal dominant diseases (Townes-Brocks Syndrome), Multiple endocrine neoplasia (MEN) 2A; pheochromocytoma; recessive syndromes (Fraser Syndrome) or X chromosome linked disorders (Kallman Syndrome) indicates the importance of genetic factors in renal agenesis and other CAKUT (Pope et al., 1999). And last but not least, renal agenesis is the part of some chromosome aberrations, such as Down, Patau, Edwards or Turner Syndrome (Nicolaides et al., 1992).

However, the majority of genes potentially connected with physiological nephrogenesis and thus their developmental disorders in humans were described on the basis of animal (especially mice) studies. In recent years the studies of targeted gene knockout mice and experiments with tissue and organ cultures have been used for identification of specific controlling proteins. So far, more than 40 genes that participate in mouse organogenesis have been identified (Bates, 2000). *RET* (Schuchardt et al., 1994), *GDNF* (Sanchez et al., 1996), *GFRA1* (Cacalano et al., 1998), *PAX 2* (Torres et al., 1995) and *WT1* (Kreidberg et al., 1993) are among the most important ones. Furthermore, all these genes are considered to be the candidate genes for renal agenesis in humans.

Two embryonic structures are essential and sufficient as well for the formation of fully working kidneys – ureteric bud (UB) and metanephric mesenchyme (MM), between which reciprocal inductive interactions occur.

This work is focused on detection of potential mutations in two candidate genes for renal agenesis, namely *RET* and *GDNF*, which are expressed by these two early nephrogenesis tissues. Both genes are confirmed to cause renal agenesis in mice (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996). Increased occurrence of *RET /GDNF* mutations was also detected in patients and aborted fetuses with both unilateral and bilateral renal agenesis (Skinner et al., 2008).

MATERIALS AND METHODS

Genomic DNA isolated from peripheral blood of 20 patients with unilateral renal agenesis was used as genetic material. Clinical characteristics of study objects are described in Table 1.

Isolation of genomic DNA, from peripheral blood lymphocytes, was done on the QIAcube (Qiagen) using the QIAamp DNA Mini Kit. Before participating in this research, all patients gave the written informed consent with molecular genetic investigation. Our study was approved by ethical committee. Mutation analysis was carried out by using polymerase chain reaction (PCR) and direct sequencing. The twenty exons of the *RET* gene and three exons of *GDNF* gene were amplified in 25 µl reaction volume with 0,5 µM of each primer, 1x PCR buffer, 1,5 mM $MgCl_2$, 100µM of each dNTP, between 50-300 ng of genomic DNA as template and 1 unit of Taq DNA polymerase (MBI Fermentas). PCR was performed for 35 cycles (30 s; 94 °C, 45 s; 57-62 °C, 40 s; 72 °C) with a final extension of 10 min at 72°C. PCR samples were analyzed on an automatic fluorescent ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer´s instructions. Primers have been designed based on GenBank sequences using the Primer 3 software (available on

Table 1. Clinical characteristics of study objects

Age	Gender	
35	F	unilateralagenesis, R
36	M	unilateralagenesis, L
28	M	unilateralagenesis L, R- withhydronephrosis
6	F	unilateralagenesis, L
34	F	unilateralrenal, ovary, oviductagenesis
37	M	unilateralagenesis L
16	M	unilateralagenesis
38	F	unilateralagenesis, L
35	M	unilateralagenesis,
26	M	unilateralagenesis L
18	F	unilateralagenesis
41	M	small, dystopic L kidney
20	M	unilateralagenesis, R glioma
24	F	unilateralagenesis, L
28	M	unilateralagenesis, R
17	M	unilateralagenesis
34	M	unilateralagenesis, pheochromocytoma
27	F	unilateralagenesis, L
41	F	unilateralagenesis, L
27	F	unilateralagenesis, R

F female; M male; R right; L left

http://www.hgmp.mrc.ac.uk/GenomeWeb/nuc-primer.html) including intron-exon boundaries.

Mutation detection using MLPA:

Genomic DNA was extracted from peripheral blood of the patient using kit method (Qiagen). MLPA was carried out using the Hirschsprung SALSA MLPA kits P169-C1 (RET 10q11.2, ZFHX1B 2q22.3, EDN3 20q13.3 and GDNF 5q13.2) (MRC Holland, Netherlands, http://www.mlpa.com) according to the manufacturer's instructions. Briefly, target DNA (200ng) was denatured for 5 min at 98 C, probe mix was added, after which the mixture was heated for 1 min at 95 C and incubated at 60 C overnight (16 h); after addition of ligase the mixture was incubated at 54 C for 15 min. Ligase was subsequently inactivated at 98 C for 5 min. Next, PCR mix was added to ligation product. The PCR reaction was carried out for 35 cycles (30 s at 95 C, 30 s at 60 C, and 60 s at 72 C). The fragments were analyzed on an ABI model 3130 capillary sequencer with a 50-cm capillary array and POP-4™ polymer (Applied Biosystems, UK) by mixing with 0.2 µl of the GeneScan™-500 ROX™ size standard (Applied Biosystems, UK) and 10 µl of HiDi Formamide (Applied Biosystems, UK). MLPA data were analyzed by Coffalyser software (MRC Holland, Netherlands, http://www.mlpa.com)

RESULTS

The main objective of this work was to identify potential mutations in both genes and on the basis of this to define the potential role of these genes in connection with renal agenesis. After mutational analysis of two candidate genes (*RET* and *GDNF*), which can cause renal agenesis in humans, in 20 patients with unilateral renal agenesis, three already known single nucleotide polymorphisms (SNP) were detected. It was namely in *RET* exon 7 (rs1800860), in *RET* exon 13 (rs1800861) and in *RET* exon 15 (rs1800863). According to the dbSNP NCBI database, all these polymorphisms are common ones with minor allele frequency in population exceeding 10%. No known pathogenic mutation was identified. MLPA analysis detected no deviation from control samples.

DISCUSSION AND CONCLUSION

Polymorphism rs1800860 G/A, identified in 11/20 patients, out of which two cases were in a homozygous state, had been associated with the reduction of nephrons in children with a minor A allele (Zhang et al., 2008). These children, whereas with one or two A alleles, showed kidney capacity reduced by 9.7% and, simultaneously, renal function decreased by 9.2% in comparison with homozygotes for a G allele. It was proved that the G-->A substitution, located in the 1476 position of *RET* proto oncogene exon 7, can influence the splicing in this gene area, since it is to be found in the sequence called exonic splicing enhancer. The result is that the aberrant RET lacks the place for interaction with

its GDNF ligand and GFRA1 co-receptor in the extracellular protein domain. These findings indicate that common polymorphism variants occurring in genes included into physiological nephrogenesis can influence number of nephrons, which fluctuates five times in normal population (Clark and Bertram, 1999), and can increase the risk of hypertension or renal insufficiency in later life. Combination of effects of particular polymorphisms in the *RET* gene, but also in other renal organogenesis genes, could account for the significant number of kidney hypoplasias, however, they cannot be associated with renal agenesis. None of our patients have elevated blood pressure.

No connection of two other polymorphisms identified in this study, namely rs1800861 and rs1800863, to nephrogenesis has been published.

Not surprisingly, on the basis of these arguments, efforts to study *RET/GDNF* of human patients with renal agenesis increased. Only a limited number of studies dealing with these two genes mutations in groups of patients with unilateral or bilateral renal agenesis have been published. Nevertheless, the authors of these studies assess the role of *RET/GDNF* genes for CAKUT formation oppositely. Whereas Skinner et al. and Chatterjee et al. confirm the role of these two genes for disease formation, (Jeanpierre et al., 2011) negates the importance of both genes. The discrepancy among these studies may be caused by several factors.

First of all, these four works differ in size of patient groups. Whereas our work covers the group of 20 living patients and Skinner's study works with 29 patients, Jeanpierre's study) is much wider dealing with 105 cases and even more extensive Chatterjee´s work examines 122 living patients. Naturally, results of this extensive research convey much more information since distortion of results is not so significant as in the case of small groups.

Skinner's study (Skinner et al., 2008; Jeanpierre et al., 2011) deal with the groups of aborted fetuses affected by severe bilateral kidney defects, URA or BRA, in combination with other malformations of a contralateral kidney which were, at the same time, evaluated as the cause of premature deaths of these fetuses. On the contrary, Chatterjee´s study deals with living patients with various CAKUT phenotypes. This work is focused on patients with predominantly unilateral renal agenesis. Of course, this form of disease is not so severe, some patients were asymptomatic and diagnosed accidentally with the help of medical imaging techniques. It is obvious that in case of bilateral kidney malformations, which are incompatible with life, the possibility of any genetic dysbalance is higher than in case of unilateral agenesis. Moreover, Jeanpierre's study includes 21 cases of families where renal abnormality has already occurred, which indicates genetic basis of this disease. Nevertheless, increased mutation incidence of investigated candidate genes was not detected in Jeanpierre et al., only six potential *RET* mutations were identified - in three cases the same variant was present in a healthy father. Our work includes only two patients with family incidence of a renal disease.

Certain influence on discrepancies between these four studies, related to *RET* mutation frequency, could be caused by diverse ethnicity of investigated patients. This work is the first one mapping the role of RET/GDNF genes in renal agenesis in Czech patients.

Concerning the influence of GDNF on the formation of renal agenesis, the results of this work correspond to the results of both foreign studies (Skinner et al., 2008; Jeanpierre et al., 2011). Our study, as well as Jeanpierre et al., did not identify any *GDNF* mutations, Skinner et al. detected only one *GDNF* mutation and the mutation was found in a patient who had two other *RET* mutations. Chatterjee et al. found *GDNF* mutation in two patients who harboured additional *RET* mutations

This study did not confirm the increased incidence assumption of *RET* and *GDNF* mutations in patients with renal agenesis. It is apparent that genetic basis and subsequent cellular signaling, participating in renal agenesis inception, are more complex than originally thought. The situation is complicated especially by high genetic heterogeneity of renal agenesis and the significant influence of modifying factors being exercised.

To obtain a more accurate analysis of these problems within the Czech population it will be necessary to extend the sample of patients focusing on this disease affected families where the genetic etiology probability is higher.

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