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Non-replication of an association between the *Odd-skipped related 1* c.654G>A (rs12329305) polymorphism and kidney volume in newborns

Brak replikacji związku polimorfizmu c.654G>A (rs12329305) genu *Odd-skipped related 1* z objętością nerek u noworodków

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ABSTRACT

Introduction: Odd-skipped related 1 (Osr1) protein is a marker of intermediate mesoderm from which all definitive kidney progenitor cells develop. The loss-of-function *OSR1* c.654G>A (rs12329305) polymorphism has been associated with a reduced kidney volume at birth and congenital renal anomalies.

The aim of this study was to re-investigate whether the *OSR1* c.654A allele is associated with a smaller kidney volume (a surrogate of the congenital nephron number) in newborns, either alone or in combination with *RET* c.1296G>A (*RET* x *OSR1* interaction).

Materials and methods: A total of 178 healthy, full-term Polish newborns were included in this study. Kidney size was measured by ultrasound on the third day of life and normalised for body surface area (TKV/BSA). The *OSR1* polymorphism was identified by mini-sequencing. The reduced model without interaction and the full model including interaction terms were compared using the F test.

Results: TKV/BSA did not differ across the genotype groups, $t(176) = -0.45$, $p = 0.653$ (106 ±24 and 108 ±25 mL/m^2 for GG and GA, respectively). Similarly, there was no effect of the c.654G>A genotype on TKV/BSA after controlling for the effect of covariates, $F(1,175) = 0.28$, $p = 0.598$. There was no association of the *RET* x *OSR1* interaction on the TKV/BSA.

Conclusion: We did not find an association between the *OSR1* c.645G>A polymorphism, alone and in combination with *RET* c.1296G>A and kidney size in a group of healthy full-term newborns.

Keywords: genetic association; kidney volume; nephron endowment; replication study.

ABSTRAKT

Wstęp: Białko 1 (Osr1) jest markerem mezodermy pośredniej, z której rozwijają się wszystkie komórki progenitorowe nerki ostatecznej. Polimorfizm c.654G>A (rs12329305) genu *OSR1*, prowadzący do utraty funkcji białka, był opisywany w związku ze zmniejszeniem objętości nerek u noworodków oraz wrodzonymi anomaliami nerek.

Celem pracy było określenie związku allelu c.654A ze zmniejszeniem objętości nerek (surogatu wrodzonej liczby nefronów) u noworodków, a także jego interakcji z polimorfizmem c.1296G>A genu RET (w populacji polskiej).

Materiały i metody: W badaniu wzięło udział 178 zdrowych, donoszonych polskich noworodków. Objętość nerek, normalizowaną względem powierzchni ciała (TKV/BSA), mierzono za pomocą badania ultrasonograficznego w 3. dniu życia. Polimorfizm genu *OSR1* identyfikowano przy użyciu minisekwencjonowania. Istotność interakcji badano przez porównanie modeli ze składnikiem i bez składnika interakcji za pomocą testu F.

Wyniki: Wartości TKV/BSA nie różniły się w zależności od genotypu, $t(176) = -0,45$, $p = 0,653$ (106 ±24 i 108 ±25 mL/m^2, odpowiednio dla GG i GA). Podobnie nie stwierdzono związku c.654G>A z TKV/BSA po uwzględnieniu zmiennych zaburzających, $F(1,175) = 0,28$, $p = 0,598$. Nie zaobserwowano związku interakcji *RET* x *OSR1* z TKV/BSA.

Wnioski: Nie wykazano związku polimorfizmu c.645G>A genu *OSR1*, analizowanego indywidualnie lub w połączeniu z polimorfizmem c.1296G>A *RET*, z wielkością nerki w grupie zdrowych noworodków urodzonych o czasie.

Słowa kluczowe: asocjacja genetyczna; objętość nerek; wrodzona liczba nefronów; badanie replikacyjne.

INTRODUCTION

A growing body of evidence suggests that nephron endowment may be a risk for cardiovascular and kidney diseases in later life [1, 2]. The number of nephrons has remarkable intra- and inter-population variation [3], and both genetic and environmental factors have been implicated in determining nephron endowment [4]. In addition to mutations associated with syndromic forms of renal hypoplasia [4], several common polymorphisms in genes active during kidney development, which

explain part of the inter-individual variation in nephron number, have been reported recently [5, 6, 7]. Owing to difficulties in direct counting of nephrons in vivo, several clinical surrogates have been proposed [3], including the kidney volume that has been validated to correlate well with nephron numbers [8].

Odd-skipped related 1 (Osr1), a zinc finger-containing transcription factor homologous to Drosophila odd-skipped family proteins [9], is an early marker of intermediate mesoderm (from embryonic day 8.5 in mice [10]) from which all metanephric progenitor cells develop, including collecting system cells, *Foxd1+* interstitial progenitors, and *Six2+* renal vesicle progenitors [11]. Disruption of the *Osr1* gene in mice leads to multiple organ defects such as agenesis of adrenal glands, kidneys, and gonads as well as structural heart abnormalities [10, 12]. Specifically, ureteric bud outgrowth, metanephric mesenchyme condensation, and the expression of critical regulatory factors for kidney development, such as *Six2*, *Eya1*, *Gdnf*, *Pax2*, and *Sall1*, are absent [12].

The *OSR1* gene encodes a 266 amino acid protein (NP_660303.1), which has three exons and is located on chromosome 2p24 [13]. Zhang et al. [6] described a synonymous variant in exon 2 of the human *OSR1* gene (c.654G>A, rs12329305), which affects mRNA expression in vitro and renders *OSR1* transcripts unstable. In healthy Canadian newborns, the rs12329305 T allele (equivalent to c.654A) has been associated with reductions in kidney size and function. The hypomorphic effect was additive to other variant altering ureteric bud branching, *RET* (NM_020975.4:c.1296G>A, rs1800860) [6]. More recently, Lozić et al. [14] found that the c.654A variant was overrepresented in cases of stillborn or neonatal death owing to congenital anomalies including the kidneys (congenital anomalies of the kidneys and urinary tract, CAKUT) compared with controls.

The aim of this study was to re-investigate whether the presumed loss-of-function *OSR1* c.654A allele, which is hypothesised to reduce the pool of renal progenitor cells [6], is associated with a smaller kidney volume in Polish newborns, either alone or in combination with *RET* c.1296G>A.

MATERIALS AND METHODS

The study was approved by the Pomeranian Medical University Ethics Committee. All parents provided written informed consent. A total of 178 healthy, full-term Polish newborns (77 female and 101 male individuals), born after the end of the 37th week of gestation, were included in this study. All newborns were breast fed and did not receive any medication. Twins and newborns of mothers with hypertension, preeclampsia, diabetes mellitus, a history of antenatal corticosteroid therapy, or illicit substance use were excluded. Other exclusion criteria included intra-uterine growth restriction, low birth mass (<2500 g), congenital infection, chromosomal anomalies, and congenital malformations.

Kidney size was measured by ultrasound on the third day of life using an EnVisor C system (Philips Canada, Markham, ON, Canada) with 5- and 10-MHz probes (Philips Canada). Kidney volume was approximated using the formula for an ellipsoid [5]. Left and right kidney volumes were summed (total kidney volume, TKV) and normalised for body surface area (BSA). BSA was calculated with the formula proposed by Mosteller [15].

The rs12329305 (NM_145260.2: c.654G>A, NC_000002.12: g.19353152C>T) polymorphism was identified by mini-sequencing. Genomic DNA extracted from cord blood (QIAamp Blood DNA Mini Kit, Qiagen, Hilden, Germany) was amplified for 38 cycles at 94°C for 20 s, 62°C for 40 s, and 72°C for 40 s. The primer pair was as follows: forward, 5′-CGACGAGCGGCCCTACACCT-3′; reverse, 5′-CCTGAACCCATGCTCCAAAACCTA-3′ (TIB Molbiol, Poznan, Poland). The PCR product was subject to a mini-sequencing reaction (ABI PRISM SNaPshot Multiplex Kit, Applied Biosystems, Foster City, CA, USA) with a SNaPshot extension primer 5′-gactgactgactgactgactgactgactTCCGGAGGCAAGACCACCT-3′ (Tib Molbiol). The *RET* c.1296G>A (rs1800860) polymorphism was genotyped as described previously [16].

The Hardy–Weinberg equilibrium was checked by the χ^2 test. Allele frequencies were calculated from genotype counts. Continuous variables were compared between groups using the Student's *t*-test or analysis of covariance for covariate adjustment (Statistica version 10, www.statsoft.com). A power analysis was conducted using PWR package in R (https://cran.r-project.org). The power to detect a small, medium, or large effect was 17%, 69%, and 98%, respectively, considering the sample sizes (149 vs 29) and alpha level (5%). By convention, small-, medium-, and large-effect sizes denote standardised mean differences of 0.2, 0.5, and 0.8, respectively [17]. The *OSR1* x *RET* interaction (n = 174) was analysed using SNPassoc package for R under a general, dominant and recessive genetic model for the *RET* polymorphism (there were only two *OSR1* genotypes). Two generalized linear models, the reduced without interaction and the full model including interaction terms were compared using the F test.

A p-value of less than 0.05 was considered significant.

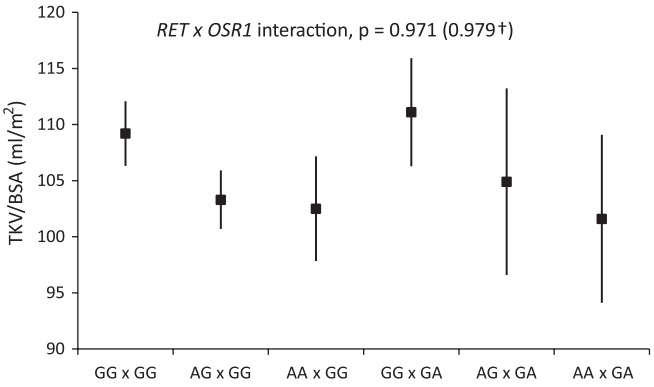
RESULTS

The c.654G>A genotype frequencies (83.7% and 16.3% for GG and GA, respectively) were in accordance with the Hardy–Weinberg expectation ($c^2 = 1.4$, $p = 0.237$). The minor allele (c.645A) frequency was 8.1% compared with 2.8% in Montreal newborns [6], 5.6% in Croatian children [14], and 2.5% in Utah residents with ancestry from northern and western Europe (CEU, 1000 Genomes Project, phase 3). Birth characteristics and kidney measurements with respect to sex and genotype are shown in Tables 1 and 2, respectively.

When newborns were stratified according to the c.654G>A genotype (Tab. 2), significant differences were observed in the gestational age, $t(176) = 2.06$, $p = 0.041$, birth mass (BM), $t(176) = 2.35$, $p = 0.020$, birth length (BL), $t(176) = 3.04$, $p = 0.003$, and BSA, $t(176) = 2.58$, $p = 0.011$. TKV/BSA did not differ across the genotype groups, $t(176) = -0.45$, $p = 0.653$. Similarly, there was no effect of the

c.654G>A genotype on TKV/BSA after controlling for the effect of gestational age, F(1,175) = 0.28, p = 0.598. BM, BL, and BSA were not entered as covariates into the analysis of covariance, because a dependent variable was normalised to BSA that was in turn approximated from BM and BL.

There was no association of the *RET* x *OSR1* interaction on the TKV/BSA under a general model, F(2,168) = 0.030, p = 0.971 (Fig. 1). A similar *RE*T-dependent genotype-phenotype pattern was observed among GG and GA *OSR1* genotypes.



The dots and bars depict a mean ±standard error, † p value adjusted by sex

FIGURE 1. *RET* x *OSR1* interaction on the total kidney volume normalised to body surface area (TKV/BSA)

No evidence for epistasis was found under a dominant, F(1,170) = 0.017, p = 0.897 (sex adjusted p = 0.936) or recessive model, F(1,170) = 0.064, p = 0.800 (sex adjusted p = 0.825).

DISCUSSION

A reduced nephron number has been associated with a number of clinical outcomes such as blood pressure, chronic kidney disease, impaired kidney graft function, and survival [18, 19]. Knowledge about factors that underlie the variation in nephron number would therefore assist in identifying children at risk of cardiovascular disease, and have potentially significant implications for future health. In the present study, we did not confirm an association between the *OSR1* c.645G>A polymorphism and kidney size, a surrogate of nephron number, in a group of healthy full-term Polish newborns.

A low reproducibility rate in genetic association studies is a well-recognised issue [20]. However, high expectations for successful replication of initially significant findings are continuously held. In the case of non-replication, the risk of a false-positive result in the initial report should first be addressed [21]. Several factors such as population stratification, confounding, inadequate sample size, misgenotyping, and inappropriateness of statistical methods have been recognised to generate false-positive association signals [22]. A spurious relationship may

TABLE 1. Demographic and sonographic characteristics of newborns

Characteristic	All (n = 178)	Female (n = 77)	Male (n = 101)	p*
Gestational age (weeks)	39.4 ±1.4	39.3 ±1.5	39.5 ±1.3	0.364
Birth mass, BM (grams)	3458 ±453	3349 ±370	3540 ±493	0.005
Birth length, BL (cm)	55.6 ±2.9	55.4 ±2.6	55.8 ±3.1	0.372
Body surface area, BSA (m^2)	0.230 ±0.020	0.226 ±0.017	0.234 ±0.021	0.008
Right kidney volume, RKV (mL)	11.9 ±3.1	11.4 ±2.9	12.2 ±3.3	0.103
Left kidney volume, LKV (mL)	12.7 ±3.2	12.2 ±3.0	13.0 ±3.4	0.115
Total kidney volume, TKV (mL)	24.5 ±5.8	23.6 ±5.6	25.2 ±5.9	0.076
TKV/BSA (mL/m^2)	107 ±24	105 ±25	108 ±23	0.455

* two-tailed *t*-test probability (Females vs Males), TKV/BSA – total kidney volume normalised for body surface area, mean ±standard deviation

TABLE 2. Demographic and sonographic characteristics of newborns with respect to c.654G>A genotype

Characteristic	GG (n = 149)	GA (n = 29)	p*
Gender (% female)	57.1	55.2%	0.852
Gestational age (weeks)	39.5 ±1.4	38.9 ±1.4	0.041
Birth mass, BM (grams)	3492 ±462	3279 ±357	0.020
Birth length, BL (cm)	55.9 ±2.7	54.2 ±3.3	0.003
Body surface area, BSA (m^2)	0.232 ±0.020	0.222 ±0.017	0.011
Right kidney volume, RKV (mL)	11.9 ±3.3	11.4 ±2.4	0.377
Left kidney volume, LKV (mL)	12.7 ±3.2	12.5 ±3.3	0.813
Total kidney volume, TKV (mL)	24.6 ±5.9	23.9 ±5.2	0.540
TKV/BSA (mL/m^2)	106 ±24	108 ±25	0.653†

* two-tailed *t*–test probability (GG vs GA); † p = 0.598 (analysis of covariance), adjusted for the gestational age, TKV/BSA – total kidney volume normalised for body surface area, mean ±standard deviation

occur if a study population has been admixed with other ethnic groups that differ in allele frequencies and the quantitative trait mean [23], and the appropriate counteracting measures are not undertaken (e.g. stratified analysis). The level of population homogeneity was assessed in the initial study, and there was no evidence of significant stratification [6]. The geographic area from which the subjects had originated in the current study coincides with the West Pomerania province in Poland. Its population genetic structure has been recently investigated in detail and shown to be remarkably homogenous with a genome-wide linkage disequilibrium pattern resembling the Kuusamo Finish isolate population [24].

In favour of a true positive association is the discovered effect of the (apparently synonymous) c.645G>A polymorphism on *OSR1* expression, which was observed in vitro. The sequencing of reversed-transcribed mRNA isolated from a Wilm's tumour cell line (WitS) heterozygous for c.654G>A revealed a c.654A-specific exon 2-skipping event [6]. However, it is unclear whether this allele-dependent effect on *OSR1* exon 2 expression is unique in WitS cells or whether it can be observed in other cell lines, including in vivo conditions. Additionally, Lozić et al. [14] found significant over-representation of the presumed loss-of-function c.654A allele in foetal or neonatal death cases owing to CAKUT and congenital heart defects compared with the control group (25% vs 5.6% and 35% vs 5.6%, respectively). Interestingly, in contrast to *Osr1*$^{-/-}$ null mutant mice, heterozygous mice with one *Osr1* allele display apparently normal nephrogenesis and exhibit no urogenital or heart defects [10, 11].

Our study was sufficiently powered (82%) to detect the true effect size comparable with the effect observed in the initial report (a standardised mean difference of −0.59). However, the true effect tends to be smaller than the observed effect size of the initial study ("winner's curse") [25]. In such cases, the type 2 error would exceed the acceptable level. Recently, much effort has been made to establish a statistical framework to predict the probability of a successful replication. Several methods have been developed [26, 27, 28]. Not only did they fail to gain wide acceptance, but they also received considerable criticism [29, 30, 31]. According to one study [32], accurate estimates of replication probability are rarely feasible in practice. Clearly, such knowledge would help to identify a so-called pseudo-failure [26] which is a low *a priori* probability of a successful replication (replication power or replicability defined as "the estimated probability that an exact replication of an initial null hypothesis rejection will similarly reject the null hypothesis" [33]).

The studies of model organisms suggest that gene-gene interaction (or epistasis) is a common feature of the genetic architecture of complex traits [34]. Unfortunately, it is rarely addressed in complex trait studies (being still statistically and computationally challenging [34]), although it can be a factor contributing to low replication rate in human association studies [35]. We analysed an interaction between *OSR1* c.654G>A and *RET* hypomorphic variant c.1296G>A (the effect of the c.1296A allele on the kidney size in Polish newborns has recently been confirmed [16]). There was no evidence of non-linear interaction between the two loci. It should be noted, however, that any statistical evidence of epistasis does not necessarily indicate the interaction in a biological sense.

Of note, between-study heterogeneity is also one of the major impediments for successful replication [36]. It should be noted that the original report [6] and this study were similar or identical in many respects including the age of subjects, genetic marker and model, phenotype and its measurement, recruitment strategy, sample size, and ethnicity (European descent), although both populations have been influenced by founder effects [24, 37]. Thus, as mentioned above, although internally homogenous, the two populations may differ substantially from one another with respect to their genetic composition. Regarding between-study heterogeneity, the mean TKV/BSA in Polish newborns (107 mL/m^2) was about 80% of that in newborns of the Montreal cohort (132 mL/m^2). However, raw kidney volumes in the Polish cohort were almost identical to those reported in a large European cohort of newborns [38], whereas greater (by 9–10%) normalised volumes (TKV/BSA) could have been related to the Du Bois formula used by Schmidt et al. [38], which is prone to underestimating the surface area of newborns [39]. Other sources of between-study heterogeneity include gene–gene and gene–environment interactions [36]. Interestingly, both types of interactions may also jeopardise the ability to replicate an initial report, contributing at least in part to an inconsistency between association studies [20].

In conclusion, we could not confirm an association between the synonymous c.645G>A polymorphism in the *OSR1* gene and kidney size in a group of Polish newborns. It should be noted that a replication process needs to face statistical limitations, and this fact tends to be overlooked by the scientific community (the "replication fallacy") [26]. Even if high replicability is expected, other population-specific factors, such as epistasis or gene–environmental interactions, may preclude successful replication.

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