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II. 研究成果の刊行に関する一覧表

III. 研究成果の刊行物

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル	発表誌	出版年等
Nakayama M, Nozu K, Goto Y, Kamei K, Ito S, Sato H, Emi M, Nakanishi K, Tsuchiya S, Iijima K	HNF1B alterations associated with congenital anomalies of the kidney and urinary tract	Pediatric Nephrology	25(6):1073-9, 2010
Hayashi S, Imoto I, Aizu Y, Okamoto N, Mizuno S, Kurosawa K, Okamoto N, Honda S, Araki S, Mizutani S, Numabe H, Saitoh S, Kosho T, Fukushima Y, Mitsubuchi H, Endo F, Chinen Y, Kosaki R, Okuyama T, Ohki H, Yoshihashi H, Ono M, Takada F, Ono H, Yagi M, Matsumoto H, Makita Y, Hata A, Inazawa J	Clinical application of array-based comparative genomic hybridization by two-stage screening for 536 patients with mental retardation and multiple congenital anomalies	Journal of Human Genetics	56(2):110-24, 2011
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ORIGINAL ARTICLE

HNF1B alterations associated with congenital anomalies of the kidney and urinary tract

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Abstract Hepatocyte nuclear factor 1β (HNF1β) abnormalities have been recognized to cause congenital anomalies of the kidney and urinary tract (CAKUT), predominantly affecting bilateral renal malformations. To further understand the spectrum of HNF1β related phenotypes, we performed *HNF1B* gene mutation and deletion analyses in Japanese patients with renal hypodysplasia (n=31), unilateral multicystic dysplastic kidney (MCDK; n=14) and others (n=5). We identified *HNF1B* alterations in 5 out of 50 patients (10%). De novo heterozygous complete deletions of *HNF1B* were found in 3 patients with unilateral MCDK. Two of the patients showed contralateral hypodysplasia, whereas the other patient showed a radiologically normal contralateral kidney with normal renal function. Copy number variation analyses showed 1.4 Mb microdeletions involving the whole *HNF1B* gene with breakpoints in flanking segmental duplications. We also identified 1 novel truncated mutation (1007insC) and another missense mutation (226G>T) in patients with bilateral hypodysplasia. *HNF1B* alterations leading to haploinsufficiency affect a diverse spectrum of CAKUT. The existence of a patient with unilateral MCDK with normal renal function might provide genetic insight into the etiology of these substantial populations of only unilateral MCDK. The recurrent microdeletions encompassing *HNF1B* could have a significant impact on the mechanism of *HNF1B* deletions.

Keywords Hepatocyte nuclear factor 1β · Congenital anomalies of the kidney and urinary tract · Copy number variation · Heterozygous microdeletion · Unilateral multicystic dysplastic kidney

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Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT), developmental abnormalities of the kidney, occur with a frequency of 1 in 500 neonates and lead to major causes of chronic renal failure in infancy and childhood [1, 2]. To date, several gene mutations have been identified as a cause of human CAKUT, probably affecting the molecular pathogenesis of these disorders [3, 4].

Hepatocyte nuclear factor 1β (HNF1β) is a homeodomain-containing transcription factor that binds DNA and transactivates transcription [5]. HNF1β was initially described as liver-enriched transcription factors, but it was subsequently revealed that this protein is predominantly expressed in renal and pancreatic epithelia. HNF1β is the essential factor for embryogenesis in the kidney, pancreas, and liver, and is

expressed in the Wolffian duct and the Müllerian duct from very early developmental stage of the kidney [6]. In human metanephros, the transcript is strongly detected especially in the fetal medullary and cortical collecting ducts [7].

Alteration of the *HNF1B* gene, which is also known as *TCF2* and encodes HNF1β, originally known to be a gene responsible for the maturity-onset diabetes of the young type 5 (MODY5), has been recognized as a cause of renal structural abnormalities [8]. While a number of *HNF1B* mutations have been identified in individuals with CAKUT, whole-gene deletion of *HNF1B* is the most frequent molecular alteration observed in patients [9]. *HNF1B* gene abnormalities have been reported in a variety of individuals with renal malformations, such as renal hypodysplasia, multicystic dysplastic kidney (MCDK), cystic kidney disease, single kidney, and oligomeganephronia [9–12], suggesting the broad role this transcription factor plays throughout development.

Systematic mutational analyses of *HNF1B* in CAKUT have been carried out in Western countries. However, there have been no such analyses in Japan to date; thus, we have no information on the frequency and characteristics of *HNF1B* mutations in CAKUT in Japan. To address these questions, we analyzed the *HNF1B* gene in 50 children in a Japanese cohort who presented with CAKUT. We found that *HNF1B* alterations involve a diverse spectrum of CAKUT. We also identified *HNF1B* alteration in 1 out of 10 patients with unilateral MCDK and a radiologically normal contralateral kidney resulting in normal renal function, which may provide genetic insight into the etiology of unilateral MCDK. Moreover, using copy number variation (CNV) analyses, we confirmed that the recurrent microdeletions of 17q12 encompassing *HNF1B* could have a significant impact on the etiology of whole exonic deletions of *HNF1B*.

Materials and methods

Patient recruitment

We recruited 50 Japanese individuals with renal abnormalities based on ultrasound findings during the postnatal period or with onset of renal disease in early childhood. Patients selected for this study had at least one of the following renal phenotypes: uni- or bilateral renal hypo/dysplasia with or without cysts, unilateral multicystic dysplasia, single kidney, and uni- or bilateral cystic kidneys. Renal hypoplasia was defined as a kidney length of <2 standard deviations (SD) for age [13]. Renal dysplasia was considered when poor corticomedullary differentiation and/or diffuse hyperechogenicity were found. Patients were excluded if they had other known genetic anomalies, such as autosomal recessive polycystic kidney disease, autosomal dominant polycystic kidney disease, and syndromic forms of renal abnormalities related to mutations of paired-box 2 (*PAX2*), eye-absent homolog 1 (*EYA1*) and sine oculis homeobox homolog 1 (*SIX1*). Written informed consent was obtained from the patients or their parents. The Institutional Review Board of the National Center for Child Health and Development approved this study.

Laboratory assessment

We performed blood tests for characterizing general biochemical parameters, including liver enzymes and fasting blood glucose levels. Serum creatinine levels were measured with an enzymatic assay when patients were in a stable condition. Glomerular filtration rate (GFR) was estimated from the value of serum creatinine levels and height, according to the Schwartz formula. We used the Modified Diet in Renal Disease (MDRD) Study equation for Japanese adult patients. The lower limit of normal estimated GFR was defined as 80 ml/min/1.73 m^2.

Molecular analysis

Genomic DNA was extracted and purified from peripheral leukocytes in whole-blood samples using a QIAamp DNA blood kit (Qiagen, Tokyo, Japan). To detect *HNF1B* gene deletions, we performed semiquantitative polymerase chain reaction (PCR) amplification using capillary electrophoresis (Agilent 2100 Bioanalyzer with DNA 1000 Lab Chips; Agilent Technologies, Palo Alto, CA, USA), as previously described [14]. We applied this method to exons 2, 4, and 9 of the *HNF1B* gene. Probable identified deletions were confirmed by multiple ligation-dependent probe amplification (MLPA) assays [15] using an MLPA kit (SALSA MLPA P241-B1 MODY, Lot 0408; MCR-Holland, Amsterdam, The Netherlands), which contains all 9 exons of *HNF1B*. For patients with whole gene deletion of *HNF1B*, we subsequently performed genome-wide DNA screening for CNVs using deCODE-Illumina CNV chip (57K, i-select format; deCODE genetics, Reykjavik, Iceland) and array-based comparative genomic hybridization (array CGH) analysis (Early Access 400K CNV array; Agilent Technologies, Santa Clara, CA, USA), to identify the boundaries of the deleted region involving *HNF1B*. We identified CNVs by the deCODE-Illumina CNV chip by using DosageMiner software developed by deCODE genetics and loss-of-heterozygosity analysis [16]. For array CGH, we used Agilent Human Whole Genome CNV microarray, consisting of 487,008 probes, which include 392,824 CNV probes. Array CGH experiments were performed according to the manufacturer's instructions [17].

Patients without *HNF1B* deletions were screened for mutations by direct sequencing of all 9 exons and exon–intron boundaries, as previously described [18, 19]. We collected DNA samples from 100 healthy individuals as controls for mutation analysis.

When probands had *HNF1B* alterations, genetic studies were extended to family members whenever possible. For an affected relative whose blood sample was unavailable, we obtained a PCR-ready DNA sample from the autopsy liver tissue embedded in paraffin using a DNA extraction kit (DEXPAT; Takara Bio, Shiga, Japan).

Results

Patient characteristics

We studied 50 patients with renal structural abnormalities who were diagnosed with renal hypodysplasia (n=31), unilateral MCDK (n=14), single kidney (n=4), and glomerulocystic kidney disease (n=1). The mean age at genetic analysis was 10.4 years old (age range, 0.9–31 years) and the ratio of male to female patients was 37 to 13. Cortical cysts were observed in 20 out of 50 patients (40%); 3 patients had unilateral hypodysplasia, 14 patients had unilateral MCDK, and bilateral hypodysplasia, single kidney, and glomerulocystic kidney disease occurred in 1 patient each. Twenty patients (40%) had progressed to non-diabetic end-stage renal disease. Ten out of 14 patients with unilateral MCDK showed radiologically normal or compensatory hypertrophy of the contralateral kidney. Two probands had positive family histories of renal disease. All patients showed normal liver function, except for 1 patient. None of the patients had evidence of diabetes.

HNF1B molecular analysis

We identified *HNF1B* alterations in 5 out of 50 patients (10%); 2 out of 31 patients (7%) had hypodysplastic kidneys and 3 out of 14 patients (21%) had unilateral MCDK. No *HNF1B* alterations were detected in patients with single kidney and glomerulocystic kidney disease. Table 1 shows the clinical findings and *HNF1B* mutations of 5 patients and 2 family members (K7188f and K718s).

De novo heterozygous deletions of *HNF1B* were found in 3 patients with MCDK by semiquantitative PCR (Fig. 1). All deletions were confirmed and found to be complete deletion of *HNF1B* by repeated MLPA analyses in all 3 patients. Two out of 3 patients (S710, S746) showed contralateral renal dysplasia, whereas the other patient (S708) showed a radiologically normal length and appearance of the contralateral kidney with normal renal function. CNV analyses with a deCODE-Illumina CNV chip and array CGH showed 1.4 Mb deletions at 17q12 in all 3 patients with *HNF1B* deletions. Interestingly, the microdeletions found in the 3 patients were flanked by segmental duplications on both sides. The regions flanking the microdeletions in 2 unaffected individuals were polymorphic in copy number. The deleted regions involved *HNF1B* and 14 adjacent genes (Fig. 2).

One frameshift mutation and one missense mutation were identified in patients with bilateral renal hypodysplasia by direct sequencing (K718, S440). These mutations were not detected in 100 healthy controls or in the healthy mother of the affected patient.

A novel frameshift mutation (1007insC) found in a male patient (K718) resulted in a truncation at the transactivation domain. Absence of the vas deferens was discovered at the time of surgery for inguinal hernia. The frameshift mutation identified in the proband was observed in the patient's father (K718f) with a unilateral simple kidney cyst and normal contralateral kidney, and also in a sibling (K718s). The patient's father (K718f) was found to have a high urate level (urate 618 μmol/l, reference range: 220–416 μmol/l). The sibling was diagnosed with bilateral MCDK and the Potter sequence, and was aborted at 21 weeks' gestation. The autopsy specimen showed enlarged kidneys occupied by multiple cysts of various sizes, whereas no abnormalities were observed in the other organs including liver, pancreas, and genital organs.

A heterozygous missense mutation (226G>T) located between the dimerization domain and the DNA binding domain was detected in a male patient (S440). The resulting amino acid change (Gly76Cys) affects a residue highly conserved in the *HNF1B* sequence of different species. This *HNF1B* mutation has also been reported in patients with MCDK [9]. Our patient was diagnosed with bilateral hypodysplastic kidneys at 11 months old, developing end-stage renal disease at the age of 4 years. He received living-related renal transplantation at the age of 10 years. His healthy mother did not carry the same mutation.

Discussion

This study demonstrated, to the best of our knowledge for the first time, the frequency and characteristics of *HNF1B* mutations in CAKUT in Japan, and also in Asian countries. In this study, we identified *HNF1B* alterations comprising 3 whole deletions, 1 truncated mutation, and 1 missense mutation in patients with CAKUT. All of the cases who had whole *HNF1B* deletions presented with unilateral MCDK with/without contralateral hypodysplasia, whereas 1 familial case with a truncated mutation of *HNF1B* presented with various phenotypes between the proband and his family members. Our current study provides compelling evidence

Table 1 Clinical findings and mutation analyses

Patient number	Gender	Age at examination (years)	*HNF1B* gene abnormality	Renal phenotype	eGFR (ml/min/1.73m^2)
S708	Male	2.8	Complete deletion De novo	Right MCDK Left radiologically normal	96.4
S710	Female	2.1	Complete deletion De novo	Right MCDK Left dysplasia	83.5
S746	Female	5.6	Complete deletion De novo	Left MCDK Right dysplasia	94.3
K718	Male	4.0	1007insC	Bilateral hypodysplasia	70.3
K718f	Male	32	1007insC	Right simple renal cyst	45.7
K718s	Female	–	1007insC	Bilateral MCDK Potter syndrome	–
S440	Male	13	226G>T	Bilateral hypodysplasia	ESRD Post-transplantation

eGFR, estimated glomerular filtration rate; f, father; s, sibling; ins, insertion; MCDK, multicystic dysplastic kidney; ESRD, end-stage renal disease

that the clinical spectrum of *HNF1B* abnormalities consists of a wide range of phenotypes with various renal severities [20, 21].

We found that the frequency of *HNF1B* alterations was 10% (5 out of 50 patients), which is similar to that of previous studies (8.9–29%) of CAKUT [9, 12]. This finding indicates that *HNF1B* alterations are a major cause of CAKUT in Japan, as well as in Western countries. Interestingly, with the wide phenotypic variation found in recruited patients, *HNF1B* alterations were clustered in patients with renal cystic malformation including MCDK. Review of all of the individuals with *HNF1B* alterations showed that 5 out of 7 individuals shared a common feature of renal cystic malformation, suggesting that renal cysts seem to be the most frequent outcome when *HNF1B* haploinsufficiency occurs. These findings are in accordance with previous reports showing that *HNF1B* alterations are associated with bilateral renal cysts [9]. These results suggest that patients with renal cysts are good candidates for systematic *HNF1B* screening.

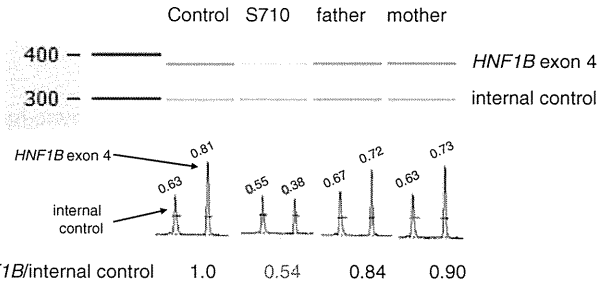

Fig. 1 Semiquantitative polymerase chain reaction (PCR) amplification of *HNF1B* exon 4. Representative result of semiquantitative PCR amplification shows heterozygous deletion of *HNF1B* exon 4 in patient S710. Peak concentration ratio of the patient's PCR product was compared with those of her parents and the normal control, indicating heterozygous deletion of the appropriate exon

In this study, we showed that *HNF1B* abnormalities encompass a wide clinical spectrum with various severities. Three out of 5 patients with *HNF1B* alterations presented with unilateral MCDK, with various phenotypes of contralateral kidney. Interestingly, we identified whole *HNF1B* deletion in 1 patient with unilateral MCDK and a radiologically normal contralateral kidney resulting in normal renal function. While previous studies have reported that *HNF1B* anomalies were only found to be associated with bilateral renal abnormalities [9, 12], various phenotypes in renal diseases also had distinct diagnoses, ranging from bilateral MCDK in autopsy samples [22, 23] to unilateral MCDK with normal renal length in single remaining kidney [24]. Recently, we examined *HNF1B* alterations in an additional 2 patients showing unilateral MCDK with a radiologically normal contralateral kidney and normal renal function. One of the patients showed a whole *HNF1B* deletion detected by MLPA analysis (personal communication (2010), Dr. Kaneko, Kansai Medical University, Japan and Drs. Nozu and Iijima, Kobe University Graduate School of Medicine, Japan), suggesting that *HNF1B* alterations are not rare in this common condition. Further studies are needed to confirm the contribution of *HNF1B* alterations in patients with unilateral MCDK and normal renal function.

Renal function in our affected individuals ranged from normal to dialysis-dependent, which required a renal transplant. A similar variability in renal function has been reported in individuals with *HNF1B* abnormalities [20, 21]. Furthermore, renal function was considerably poorer in one affected family member (K718f), despite the renal morphology of a unilateral simple cyst on repeated ultrasound scans, which was predicted to be the mildest phenotype. Although examination of renal histology was not undertaken in this case, it is reasonable to consider that *HNF1B*

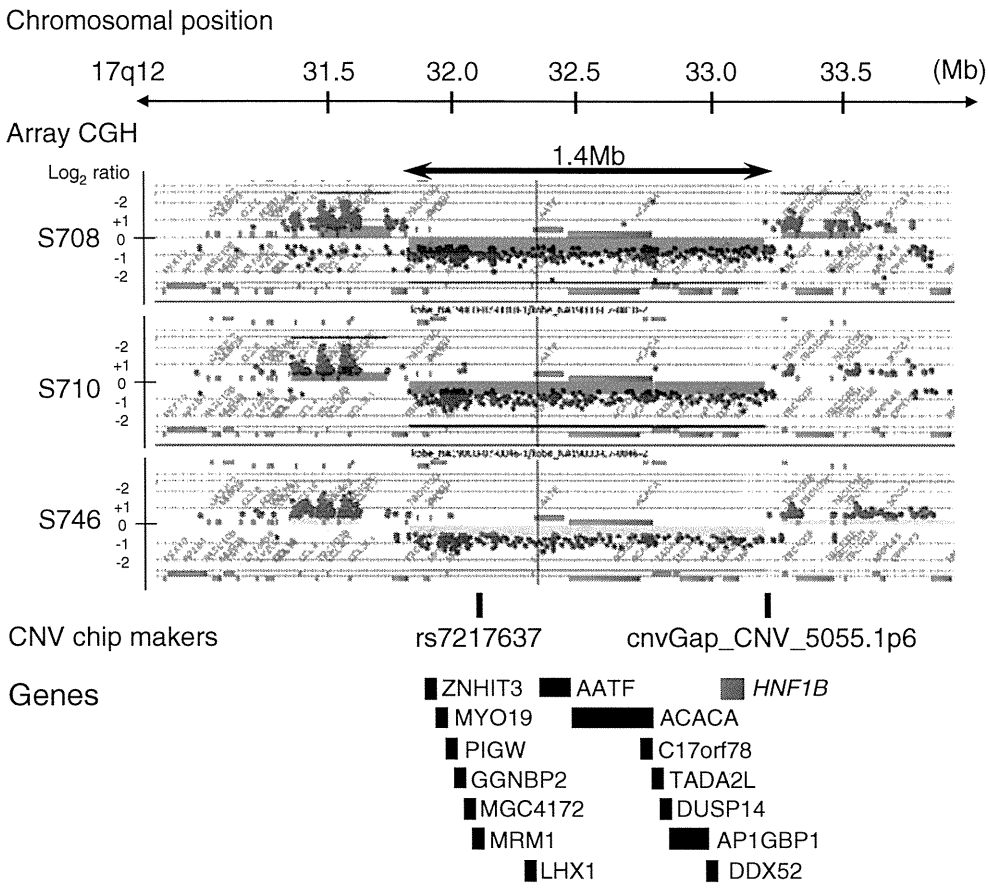

Fig. 2 Recurrent microdeletion at chromosome 17q12 involving the *HNF1B* gene. Agilent array comparative genomic hybridization (CGH) profile shows a heterozygous 1.4-Mb deletion in 3 patients with multicystic dysplastic kidney (MCDK). *Green plots* represent the deleted region and *red dots* indicate the flanking segmental duplication. This region includes *HNF1B* and 14 further genes

dysfunction pathologically affected renal function, which was not detected on renal ultrasound screenings. An important implication from this case is that screening for *HNF1B* alterations for those individuals may provide a better understanding for prognosis of renal function.

One male patient (K718) in our series with *HNF1B* mutation presented with an absence of vas deferens that was incidentally detected. The vas deferens is derived from the Wolffian duct during embryogenesis and is part of the excurrent duct system responsible for the transport, storage, and maturation of sperm. Congenital bilateral absence of the vas deferens is an important cause of male infertility in adulthood. Since the *HNF1B* gene is expressed in the Wolffian duct and Müllerian duct in the mouse embryo, it is possible that *HNF1B* alterations are associated with the genital tract malformation. To date, there have been 5 male patients with anomaly of the genital tract, including 1 case of bilateral agenesis of vas deferens [25, 26]. Although the frequency of male genital abnormalities is reported to be lower than that in females [21], there might be a certain number of potential male individuals carrying congenital genital malformation.

The frameshift mutation and the missense mutation that we found in our study are believed to be pathogenic. The frameshift mutation 1007insC is a novel mutation, which leads to truncation at the transactivation domain, probably affecting HNF1β function. In our study, the position of the missense mutation Gly76Cys was located between the dimerization domain and the DNA binding domain, and this amino acid change affects a residue highly conserved in the *HNF1B* sequence of different species. This *HNF1B* mutation has also been reported in patients with MCDK [9]. Finally, the absence of the same mutation in 200 chromosomes of unrelated Japanese control subjects or in the healthy mother of the affected patient would also support the pathogenetic role of this mutation.

In our present study, screening of *HNF1B* deletions by semiquantitative PCR amplification and MLPA analysis revealed that all 3 cases with *HNF1B* deletions were found to show deletions of whole exons. This tendency toward complete exonic deletions as a major pattern for heterozygous *HNF1B* deletions is similar to that found in previous reports [9, 12, 20]. Furthermore, subsequent CNV analyses of these 3 cases showed that the microdeletions at 17q12 extended to the 1.4-Mb region, including the entire *HNF1B* gene. High resolution mapping of the deleted region by the array CGH showed microdeletions with breakpoints in flanking segmental duplications, indicating that the microdeletions were mediated by flanking segmental duplications. The same mechanism was proposed in patients with

congenital renal abnormalities with or without mental retardation or MODY5 [22, 27], suggesting that recurrent non-allelic homologous recombination occurs in region 17q12. Collectively, this recombination possibly explains the high rate of de novo *HNF1B* deletions detected in previous studies [9, 20], and thus evaluation of this microdeletion by conventional gene dosage analysis should be considered in individuals suspected of having *HNF1B* alterations.

The recurrent microdeletion in the 17q12 region identified in 3 patients in this study involved *HNF1B* and 14 adjacent genes, which is predicted to result in haploinsufficiency of these affected genes. One of the genes in this region is *LHX1*, a limb homeodomain gene important for renal development in mouse studies [28, 29]. It has been proposed that the microdeletion of *LMX1* is associated with an earlier onset of renal pathology, suggesting that haploinsufficiency of *LHX1* as well as *HNF1B* influence this onset variability [22]. In the current study, however, although all 3 patients with microdeletions showed the shared phenotype of unilateral MCDK, no apparent difference was observed in the renal phenotype, severity of renal function or onset of disease between patients with *HNF1B* deletion and those with mutations. Our results suggest that heterozygous deletions of the affected adjacent 14 genes do not seem to influence the core phenotype. It is possible that *HNF1B* is the predominant gene among deleted regions contributing to the renal phenotype. Further studies are needed to confirm our findings.

Copy number variations can be an important source of genetic variation among human populations of different ethnic groups as well as among individuals. It is likely that different location and frequency spectra of CNVs exist for different populations, especially different ethnic groups, such as occurs in cases of single nucleotide polymorphisms and insertion–deletion polymorphisms [30, 31]. It is possible that there are interpopulation differences in the copy number due to non-allelic homologous recombination mediated by flanking segmental duplications [32]. This study demonstrated, for the first time to our knowledge, the existence of the CNV resulting in the 1.4-Mb microdeletion encompassing the *HNF1B* gene in Japanese patients, which has already been shown in several reports performed in the USA and European countries [9, 20, 22, 27].

In conclusion, the current study provides further evidence that *HNF1B* alterations leading to haploinsufficiency affect a wide variety of renal disease spectrum. The existence of an affected patient with unilateral MCDK and a radiologically normal contralateral kidney resulting in normal renal function might provide genetic insight into the etiology of the substantial population of unilateral MCDK. Identifying *HNF1B* deletions and mutations in patients with heterogeneous phenotypes should provide a better understanding of renal function, as well as early detection of extrarenal manifestation related to this gene.

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Disclosure All of the authors declare no competing interests.

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ORIGINAL ARTICLE

Clinical application of array-based comparative genomic hybridization by two-stage screening for 536 patients with mental retardation and multiple congenital anomalies

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Recent advances in the analysis of patients with congenital abnormalities using array-based comparative genome hybridization (aCGH) have uncovered two types of genomic copy-number variants (CNVs); pathogenic CNVs (pCNVs) relevant to congenital disorders and benign CNVs observed also in healthy populations, complicating the screening of disease-associated alterations by aCGH. To apply the aCGH technique to the diagnosis as well as investigation of multiple congenital anomalies and mental retardation (MCA/MR), we constructed a consortium with 23 medical institutes and hospitals in Japan, and recruited 536 patients with clinically uncharacterized MCA/MR, whose karyotypes were normal according to conventional cytogenetics, for two-stage screening using two types of bacterial artificial chromosome-based microarray. The first screening using a targeted array detected pCNV in 54 of 536 cases (10.1%), whereas the second screening of the 349 cases negative in the first screening using a genome-wide high-density array at intervals of approximately 0.7 Mb detected pCNVs in 48 cases (13.8%), including pCNVs relevant to recently established microdeletion or microduplication syndromes, CNVs containing pathogenic genes and recurrent CNVs containing the same region among different patients. The results show the efficient application of aCGH in the clinical setting.
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Keywords: array-CGH; congenital anomaly; mental retardation; screening

INTRODUCTION

Mental retardation (MR) or developmental delay is estimated to affect 2–3% of the population.[1] However, in a significant proportion of cases, the etiology remains uncertain. Hunter[2] reviewed 411 clinical cases of MR and reported that a specific genetic/syndrome diagnosis was carried out in 19.9% of them. Patients with MR often have congenital anomalies, and more than three minor anomalies can be useful in the diagnosis of syndromic MR.[2,3] Although chromosomal aberrations are well-known causes of MR, their frequency determined by conventional karyotyping has been reported to range from 7.9 to 36% in patients with MR.[4–8] Although the diagnostic yield depends on the population of each study or clinical conditions, such studies

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suggest that at least three quarters of patients with MR are undiagnosed by clinical dysmorphic features and karyotyping.

In the past two decades, a number of rapidly developed cytogenetic and molecular approaches have been applied to the screening or diagnosis of various congenital disorders including MR, congenital anomalies, recurrent abortion and cancer pathogenesis. Among them, array-based comparative genome hybridization (aCGH) is used to detect copy-number changes rapidly in a genome-wide manner and with high resolution. The target and resolution of aCGH depend on the type and/or design of mounted probes, and many types of microarray have been used for the screening of patients with MR and other congenital disorders: bacterial artificial chromosome (BAC)-based arrays covering whole genomes,[9,10] BAC arrays covering chromosome X,[11,12] a BAC array covering all subtelomeric regions,[13] oligonucleotide arrays covering whole genomes,[14,15] an oligonucleotide array for clinical diagnosis[16] and a single nucleotide polymorphism array covering the whole genome.[17] Because genome-wide aCGH has led to an appreciation of widespread copy-number variants (CNVs) not only in affected patients but also in healthy populations,[18–20] clinical cytogenetists need to discriminate between CNVs likely to be pathogenic (pathogenic CNVs, pCNVs) and CNVs less likely to be relevant to a patient's clinical phenotypes (benign CNVs, bCNVs).[21] The detection of more CNVs along with higher-resolution microarrays needs more chances to assess detected CNVs, resulting in more confusion in a clinical setting.

We have applied aCGH to the diagnosis and investigation of patients with multiple congenital anomalies and MR (MCA/MR) of unknown etiology. We constructed a consortium with 23 medical institutes and hospitals in Japan, and recruited 536 clinically uncharacterized patients with a normal karyotype in conventional cytogenetic tests. Two-stage screening of copy-number changes was performed using two types of BAC-based microarray. The first screening was performed by a targeted array and the second screening was performed by an array covering the whole genome. In this study, we diagnosed well-known genomic disorders effectively in the first screening, assessed the pathogenicity of detected CNVs to investigate an etiology in the second screening and discussed the clinical significance of aCGH in the screening of congenital disorders.

MATERIALS AND METHODS

Subjects

We constructed a consortium of 23 medical institutes and hospitals in Japan, and recruited 536 Japanese patients with MCA/MR of unknown etiology from July 2005 to January 2010. All the patients were physically examined by an expert in medical genetics or a dysmorphologist. All showed a normal karyotype by conventional approximately 400–550 bands-level G-banding karyotyping. Genomic DNA and metaphase chromosomes were prepared from peripheral blood lymphocytes using standard methods. Genomic DNA from a lymphoblastoid cell line of one healthy man and one healthy woman were used as a normal control for male and female cases, respectively. All samples were obtained with prior written informed consent from the parents and approval by the local ethics committee and all the institutions involved in this project. For subjects in whom CNV was detected in the first or second screening, we tried to analyze their parents as many as possible using aCGH or fluorescence *in situ* hybridization (FISH).

Array-CGH analysis

Among our recently constructed in-house BAC-based arrays,[22] we used two arrays for this two-stage survey. In the first screening we applied a targeting array, 'MCG Genome Disorder Array' (GDA). Initially GDA version 2, which contains 550 BACs corresponding to subtelomeric regions of all chromosomes except 13p, 14p, 15p, 21p and 22p and causative regions of about 30 diseases already reported, was applied for 396 cases and then GDA version 3, which contains 660 BACs corresponding to those of GDA version 2 and pericentromeric regions of all chromosomes, was applied for 140 cases. This means that a CNV detected by GDA is certainly relevant to the patient's phenotypes. Subsequently in the second screening we applied 'MCG Whole Genome Array-4500' (WGA-4500) that covers all 24 human chromosomes with 4523 BACs at intervals of approximately 0.7 Mb to analyze subjects in whom no CNV was detected in the first screening. WGA-4500 contains no BACs spotted on GDA. If necessary, we also used 'MCG X-tiling array' (X-array) containing 1001 BAC/PACs throughout X chromosome other than pseudoautosomal regions.[12] The array-CGH analysis was performed as previously described.[12,23]

For several subjects we applied an oligonucleotide array (Agilent Human Genome CGH Microarray 244K; Agilent Technologies, Santa Clara, CA, USA) to confirm the boundaries of CNV identified by our in-house BAC arrays. DNA labeling, hybridization and washing of the array were performed according to the directions provided by the manufacturer. The hybridized arrays were scanned using an Agilent scanner (G2565BA), and the CGH Analytics program version 3.4.40 (Agilent Technologies) was used to analyze copy-number alterations after data extraction, filtering and normalization by Feature Extraction software (Agilent Technologies).

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed as described elsewhere[23] using BACs located around the region of interest as probes.

RESULTS

CNVs detected in the first screening

In the first screening, of 536 cases subjected to our GDA analysis, 54 (10.1%) were determined to have CNV (Figure 1; Tables 1 and 2).

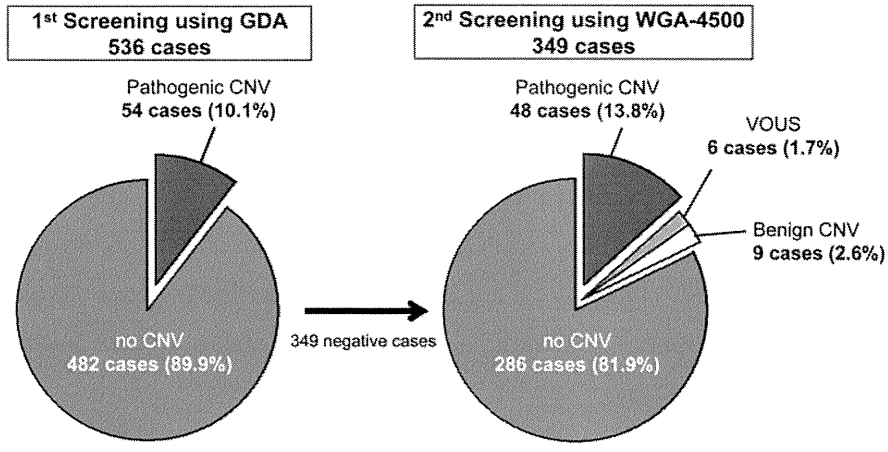

Figure 1 Percentages of each screening in the current study.

Table 1 A total of 40 cases with CNV at subtelomeric region(s) among 54 positive cases in the first screening

	Position where CNV detected				
Gender	*Loss*	*Gain*	*Corresponding disorder*[a]	*OMIM or citation*	*Parental analysis*[b]
M	1p36.33		Chromosome 1p36 deletion syndrome	#607872	
M	1p36.33p36.32		Chromosome 1p36 deletion syndrome	#607872	
M	1p36.33p36.32		Chromosome 1p36 deletion syndrome	#607872	
M	1p36.33p36.32		Chromosome 1p36 deletion syndrome	#607872	
M	1q44		Chromosome 1q43-q44 deletion syndrome	#612337	
F	2q37.3		2q37 monosomy[c]	Shrimpton *et al.*[24]	
F	2q37.3		2q37 monosomy[c]	Shrimpton *et al.*[24]	
M	3q29		Chromosome 3q29 deletion syndrome	#609425	
F	5p15.33p15.32		Cri-du-chat syndrome	#123450	
M	5q35.2q35.3		Chromosome 5q subtelomeric deletion syndrome	Rauch *et al.*[25]	
F	6p25.3		Chromosome 6pter-p24 deletion syndrome	#612582	
M	7q36.3		7q36 deletion syndrome[d]	Horn *et al.*[26]	
F	7q36.3		7q36 deletion syndrome[d]	Horn *et al.*[26]	
M	9p24.3p24.2		Chromosome 9p deletion syndrome	#158170	
F	9q34.3		Kleefstra syndrome	#610253	
F	10q26.3		Chromosome 10q26 deletion syndrome	#609625	
F	16p13.3		Chromosome 16p13.3 deletion syndrome	#610543	
F	22q13.31		Chromosome 22q13 deletion syndrome	#606232	
M	22q13.31q13.33		Chromosome 22q13 deletion syndrome	#606232	
M		15q26.3	15q overgrowth syndrome[c]	Tatton-Brown *et al.*[27]	
F		15q26.3	15q overgrowth syndrome[c]	Tatton-Brown *et al.*[27]	
M		21q22.13q22.3	Down's syndrome (partial trisomy 21)	#190685	
M		Xp22.33	A few cases have been reported; e.g. V5-130 in Lu *et al.*[28]		
M		Xq28	Chromosome Xq28 duplication syndrome	#300815	
F	1q44		Chromosome 1q43-q44 deletion syndrome	#612337	
		8p23.2p23.3			
M	3p26.3		3p deletion syndrome[d]	Fernandez *et al.*[29]	
		12p13.33p11.22			
F	3p26.3		3p deletion syndrome[d]	Fernandez *et al.*[29]	
		16p13.3	Chromosome 16p13.3 duplication syndrome	#613458	
F	4q35.2		4q− syndrome[d]	Jones *et al.*[30]	
		7q36.3			
M	5p15.33		Cri-du-chat syndrome	#123450	
		20p13			
M	5p15.33p15.32		Cri-du-chat syndrome	#123450	
		2p25.3			
F	6q27		6q terminal deletion syndrome[d]	Striano *et al.*[31]	
		11q25			
F	6q27		6q terminal deletion syndrome[d]	Striano *et al.*[31]	
		8q24.3			
M	7q36.3		7q36 deletion syndrome[d]	Horn *et al.*[26]	*dn*
		1q44			
M	9p24.3p24.2		Chromosome 9p deletion syndrome	#158170	
		7q36.3			
F	10p15.3p15.2		Chromosome 10p terminal deletion[d]	Lindstrand *et al.*[32]	*pat*
		7p22.3p22.2			
M	10p15.3		Chromosome 10p terminal deletion[d]	Lindstrand *et al.*[32]	
		2p25.3			
M	10q26.3		Chromosome 10q26 deletion syndrome	#609625	
		2q37.3	Distal trisomy 2q[d]	Elbracht *et al.*[33]	
M	18q23		Chromosome 18q deletion syndrome	#601808	
		7q36.3			
F	22q13.31q13.33		Chromosome 22q13.3 deletion syndrome	#606232	*pat*
		17q25.3	One case was reported	Lukusa *et al.*[34]	
M	Xp22.33/Yp11.32		Contiguous gene–deletion syndrome on Xp22.3[d]	Fukami *et al.*[35]	
		Xq27.3q28	Chromosome Xq28 duplication syndrome	#300815	

Abbreviations: F, female; CNV, copy-number variant; M, male; OMIM, Online Mendelian Inheritance in Man; *dn, de novo* CNV observed in neither of the parents.
[a]The name of disorder is based on entry names of OMIM, expect for entry names in DECIPHER and description in each cited article.
[b]*pat*, father had a balanced translocation involved in corresponding subtelomeric regions.
[c]Entry names in DECIPHER.
[d]Description in each cited article.

All the CNVs detected in the first screening were confirmed by FISH. Among the positive cases, in 24 cases one CNV was detected. All the CNVs corresponded to well-established syndromes or already described disorders (Table 1). In 16 cases two CNVs, one deletion and one duplication, were detected at two subtelomeric regions, indicating that one of parents might be a carrier with reciprocal translocation involved in corresponding subtelomeric regions, and at least either of the two CNVs corresponded to the disorders. We also performed parental analysis by FISH for three cases whose parental samples were available, and confirmed that in two cases the subtelomeric aberrations were inherited from paternal balanced translocation and in one case the subtelomeric aberrations were *de novo* (Table 1). In the other 14 cases, CNVs (25.9%) were detected in regions corresponding to known disorders (Table 2).

Table 2 Other cases among 54 positive cases in the first screening

	Position where CNV detected			
Gender	*Gain*	*Loss*	*Corresponding disorder*	*OMIM*
F		4p16.3	Ring chromosome	
		4q35.2		
M		3q22.323	BPES	#110100
M		2q22.3	ZFHX1B region	*605802
M		4q22.1	Synuclein (SNCA) region	*163890
F		7p21.1	Craniosynostosis, type 1	#123100
F		7q11.23	Williams syndrome	#194050
F		8q23.3q24.11	Langer–Giedion syndrome	#150230
M	15q11.2q13.1		Prader–Willi/Angelman	#176270/ #105830
F		17p11.2	Smith–Magenis syndrome	#182290
M		17q11.2	Neurofibromatosis, type I	+162200
M	22q11.21		DiGeorge syndrome	#188400
F		22q11.21	DiGeorge syndrome	#188400
F	Xp22.31		Kallmann syndrome 1	+308700
F	Whole X		Mosaicism	

Abbreviations: CNV, copy-number variant; F, female; M, male; OMIM, Online Mendelian Inheritance in Man.

CNVs detected in the second screening and assessment of the CNVs

Cases were subject to the second screening in the order of subjects detected no CNV in the first screening, and until now we have analyzed 349 of 482 negative cases in the first screening. In advance, we excluded highly frequent CNVs observed in healthy individuals and/or in multiple patients showing disparate phenotypes from the present results based on an internal database, which contained all results of aCGH analysis we have performed using WGA-4500, or other available online databases; for example, Database of Genomic Variant (http://projects.tcag.ca/variation/). As a result, we detected 66 CNVs in 63 cases (Figure 1; Table 3). Among them, three patients (cases 36, 42 and 44) showed two CNVs. All the CNVs detected in the second screening were confirmed by other cytogenetic methods including FISH and/or X-array. For 60 cases, we performed FISH for confirmation and to determine the size of each CNV. For five cases, cases 13, 36, 48, 57 and 63, with CNVs on the X chromosome, we used the X-array instead of FISH. For cases 4, 6, 16–19 and 34, we also used Agilent Human Genome CGH Microarray 244K to determine the refined sizes of CNVs. The maximum and minimum sizes of each CNV determined by these analyses are described in Table 3.

Well-documented pCNVs emerged in the second screening

CNVs identified for recently established syndromes. We assessed the pathogenicity of the detected CNVs in several aspects (Figure 2).[21,37,38] First, in nine cases, we identified well-documented pCNVs, which are responsible for syndromes recently established. A heterozygous deletion at 1q41–q42.11 in case 2 was identical to patients in the first report of 1q41q42 microdeletion syndrome.[39] Likewise a CNV in case 3 was identical to chromosome 1q43–q44 deletion syndrome (OMIM: #612337),[40] a CNV in case 4 was identical to 2q23.1 microdeletion syndrome,[41] a CNV in case 5 was identical to 14q12 microdeletion syndrome[42] and a CNV in case 6 was identical to chromosome 15q26-qter deletion syndrome (Drayer's syndrome) (OMIM: #612626).[43] Cases 7, 8 and 9 involved CNVs of different sizes at 16p12.1-p11.2, the region responsible for 16p11.2-p12.2 microdeletion syndrome.[44,45] Although an interstitial deletion at 1p36.23-p36.22 observed in case 1 partially overlapped with a causative region of chromosome 1p36 deletion syndrome (OMIM: #607872), the region deleted was identical to a proximal interstitial 1p36 deletion that was recently reported.[46] Because patients with the proximal 1p36 deletion including case 1 demonstrated different clinical characteristics from cases of typical chromosome 1p36 deletion syndrome, in the near term their clinical features should be redefined as an independent syndrome.[46]

CNVs containing pathogenic gene(s). In four cases we identified pCNVs that contained a gene(s) probably responsible for phenotypes. In case 10, the CNV had a deletion harboring *GLI3* (OMIM: *165240) accounting for Greig cephalopolysyndactyly syndrome (GCS; OMIM: 175700).[47] Although phenotypes of the patient, for example, pre-axial polydactyly of the hands and feet, were consistent with GCS, his severe and atypical features of GCS, for example, MR or microcephaly, might be affected by other contiguous genes contained in the deletion.[48] Heterozygous deletions of *BMP4* (OMIM: *112262) in case 11 and *CASK* (OMIM: *300172) in case 13 have been reported previously.[49,50] In case 12, the CNV contained *YWHAE* (OMIM: *605066) whose haploinsufficiency would be involved in MR and mild CNS dysmorphology of the patient because a previous report demonstrated that haploinsufficiency of *ywhae* caused a defect of neuronal migration in mice[51] and a recent report also described a microdeletion of *YWHAE* in a patient with brain malformation.[52]

Recurrent CNVs in the same regions. We also considered recurrent CNVs in the same region as pathogenic; three pairs of patients had overlapping CNVs, which have never been reported previously. Case 16 had a 3.3-Mb heterozygous deletion at 10q24.31–q25.1 and case 17 had a 2.0-Mb deletion at 10q24.32–q25.1. The clinical and genetic information will be reported elsewhere. Likewise, cases 14 and 15 also had an overlapping CNV at 6q12–q14.1 and 6q14.1, and cases 18 and 19 had an overlapping CNV at 10p12.1–p11.23. Hereafter, more additional cases with the recurrent CNV would assist in defining new syndromes.

CNVs reported as pathogenic in previous studies. Five cases were applicable to these criteria. A deletion at 3p21.2 in case 20 overlapped with that in one case recently reported.[53] The following four cases had CNVs reported as pathogenic in recent studies: a CNV at 7p22.1 in case 21 overlapped with that of patient 6545 in a study by Friedman *et al.*,[14] a CNV at 14q11.2 in case 22 overlapped with those of patients 8326 and 5566 in Friedman *et al.*,[14] a CNV at 17q24.1–q24.2 in case 23 overlapped with that in patient 99 in Buysse *et al.*[54] and a CNV at 19p13.2 in case 24 overlapped with case P11 in Fan *et al.*[55]

Large or gene-rich CNVs, or CNVs containing morbid OMIM genes. In cases inapplicable to the above criteria, we assessed CNVs