OPEN

Citation: Human Genome Variation (2015) 2, 15004; doi:10.1038/hgv.2015.4

www.nature.com/hgv

ARTICLE

DGCR6 at the proximal part of the DiGeorge critical region is involved in conotruncal heart defects

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Cardiac anomaly is one of the hallmarks of DiGeorge syndrome (DGS), observed in approximately 80% of patients. It often shows a characteristic morphology, termed as conotruncal heart defects. In many cases showing only the conotruncal heart defect, deletion of 22q11.2 region cannot be detected by fluorescence *in situ* hybridization (FISH), which is used to detect deletion in DGS. We investigated the presence of genomic aberrations in six patients with congenital conotruncal heart defects, who show no deletion at 22q11.2 in an initial screening by FISH. In these patients, no abnormalities were identified in the coding region of the *TBX1* gene, one of the key genes responsible for the phenotype of DGS. However, when copy number alteration was analyzed by high-resolution array analysis, a small deletion or duplication in the proximal end of DiGeorge critical region was detected in two patients. The affected region contains the *DGCR6* and *PRODH* genes. *DGCR6* has been reported to affect the expression of the *TBX1* gene. Our results suggest that altered dosage of gene(s) other than *TBX1*, possibly *DGCR6*, may also be responsible for the development of conotruncal heart defects observed in patients with DGS and, in particular, in those with stand-alone conotruncal heart defects.

Human Genome Variation (2015) **2,** 15004; doi:10.1038/hgv.2015.4; published online 12 February 2015

INTRODUCTION

Cardiac anomaly is one of the congenital abnormalities frequently observed in children, and some of them are known to be related to genetic abnormalities such as chromosome aberrations. The specific morphology of the cardiac anomaly often depends on the underlying genetic change. One of the syndromes that frequently includes a characteristic cardiac anomaly is DiGeorge syndrome (DGS), also known as 22q11.2 deletion syndrome or velo-cardio-facial syndrome.[1–3]

DGS is one of the most common microdeletion syndromes, with a frequency of around 1 in 4000 to 6000 live births.[4] Typical symptoms include multiple developmental anomalies such as cardiovascular malformations, palatal abnormalities, as well as characteristic facial appearance, immunodeficiency, endocrine dysfunction, hypocalcemia and intellectual disability. The types of cardiac defects seen in DGS include tetralogy of Fallot, pulmonary atresia, truncus arteriosus, interrupted aortic arch, type B, and ventricular septal defect.[2,3] Referred to as conotruncal heart defects, the morphology of these cardiac anomalies is often characteristic of DGS and frequently involves cardiac outflow tract abnormality. As patients with 22q11.2 deletion may not always have all the characteristic features of the syndrome, cases that are found by chance are often initially suspected due to the presence of the characteristic heart defect involving the outflow tract, and are then screened for 22q11.2 deletion by fluorescence *in situ* hybridization (FISH) or other methods for a diagnosis of the syndrome.

The chromosome 22q11.2 region contains several low copy repeat (LCR) sequences that mediate non-allelic homologous recombination and may cause copy number abnormalities.[5] There are eight sets of LCRs, termed LCR A to H, and because these LCRs share high homology with each other, the wrong LCRs can align inappropriately during recombination events, leading to a deletion on one resultant recombinant chromosome and a duplication on the other within the 22q11.2 region.[6–9] Approximately 90% of patients with DGS have a hemizygous 3-Mb deletion at the 22q11.2 region (LCR-A to LCR-D, also called the DiGeorge critical region) and 7% have a hemizygous 1.5-Mb deletion (LCR-A to LCR-B). The remaining 3% of patients show less common genomic alterations such as the deletion of a shorter region.[10] Although the distal breakpoint in the 22q11.2 deletion can be variable, the majority of patients (97–98%) have a proximal breakpoint within LCR-A.[11]

The DiGeorge critical region contains approximately 40 genes within a 3-Mb region and 30 genes within a proximal 1.5-Mb region.[12] Identification of the causative gene(s) within the deleted region has been intensively investigated to elucidate the etiology of the syndrome.

So far, haploinsufficiency of two genes, *TBX1*[13] and *CRKL*,[14] in the DiGeorge critical region on chromosome 22q11.2 have been identified as causing the dysfunction of the neural crest cells and anterior heart field, two possible affected regions in the morphogenesis of the syndrome.[15,16] Although located distally to the DiGeorge critical region on 22q11.2, haploinsufficiency of *MAPK1* (*ERK2*)[17] and *HIC2*[18] also have been implicated as contributors to the phenotype of DGS. These genes, with the exception of *HIC2*, are considered to be essential for neural crest cell development owing to their important roles in FGF8 (Fibroblast growth factor 8) signaling. Although the *FGF8* gene is not located within the 22q11.2 region, FGF8 inactivation can also

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Received 5 November 2014; revised 12 December 2014; accepted 17 December 2014

produce the typical phenotype of DGS, including cardiac malformations of the outflow tract and great vessels.[19]

TBX1 belongs to an evolutionarily conserved T-box family of transcription factors whose expression is precisely regulated during embryogenesis, and it appears to regulate the proliferation and differentiation of various progenitor cells during organogenesis.[20] Importantly, an animal model indicated that haploinsufficiency of the *TBX1* gene could be responsible for congenital heart defects, including cardiac outflow tract anomalies similar to that observed in patients with DGS.[21] In addition, complete loss of *TBX1* in mice resulted in a wide range of developmental anomalies encompassing almost all of the common DGS/velo-cardio-facial syndrome features, indicating a dependence of these clinical phenotypes on *TBX1* gene dosage.[21]

Although these genes were reported to be responsible for the phenotype of DGS, several other genes were also identified in the DiGeorge critical region, named as *DGCR* genes, and some of them are also possible candidates for DGS phenotype. There are 14 *DGCR* genes located within this region and eight of them, such as *DGCR1*, *DGCR2*, *DGCR3*, *DGCR6*, *DGCR6L*, *DGCR8*, *DGCR14* and *DGCR15(FAM230A)*, are protein-coding genes.

Within these genes, some are known to be associated with the clinical phenotype of the DGS. *DGCR1*, also known as *HIRA* or *TUPLE1*, encodes a histone chaperone, and its haploinsufficiency was considered to contribute to at least some of the features of the DGS phenotype.[22] *DGCR6* encodes a nuclear phosphoprotein, and in a chicken model, suppression of *DGCR6* resulted in a high incidence of cardiovascular anomalies reminiscent of those found in DGS.[23] *DGCR8* encodes a subunit of the microprocessor complex, and its haploinsufficiency was considered to contribute to the DGS phenotype through dysregulating miRNA biogenesis.[24] Sequence variants in the *DGCR14* (*DGSI*) gene encoding a component of C complex spliceosomes has been identified in patients who had no detectable 22q11.2 deletion but had some of the clinical features of DGS.[25]

Conotruncal heart defects are often observed without the other characteristic symptoms of DGS as a sole abnormality, and in around 10% of these patients, typical deletions within the DiGeorge critical region could be detected,[26–30] suggesting the syndrome-causing roles of misregulated genes located within the critical region, such as *TBX1*, in the observed conotruncal heart defects. In other words, most of the patients with the typical cardiac phenotype of conotruncal heart defects alone showed no deletion of 22q11.2, as determined by FISH, leading to a hypothesis that other genes could possibly be involved in the cardiac malformation.

In this study, we investigated the presence of genomic aberrations at the 22q11.2 region in six non-related patients with conotruncal congenital heart defects, such as tetralogy of Fallot, pulmonary atresia and truncus arteriosus. In all cases, deletions of the 22q11.2 region could not be detected by FISH analysis using a *TUPLE1* probe nor was any mutation found in the *TBX1* gene, the key gene responsible for the phenotype of DGS. However, high-resolution array comparative genomic hybridization detected changes in copy number at the proximal end of the DiGeorge critical region in two patients, indicating that gene(s) other than *TBX1* might also be responsible for the conotruncal heart defect phenotype.

MATERIALS AND METHODS

Patients

Six unrelated patients with typical cardiovascular phenotype of DGS/velo-cardio-facial syndrome such as tetralogy of Fallot, pulmonary atresia, ventricular septal defect and truncus arteriosus in whom 22q11.2 deletion was not detected by FISH analysis using *TUPLE1* probe were included in this study. Major features associated with the DGS such as thymic hypoplasia, cleft palate and hypocalcemia were not observed in these cases. The detailed information of the patients is presented in Table 1. According to the Declaration of Helsinki, written informed consent was obtained from the parents of all patients participating in this study. The study was approved by the ethical committees of Ehime University and The University of Tokushima. The molecular and molecular cytogenetic analyses were performed using genomic DNA extracted from peripheral blood mononuclear cells.

Table 1. Clinical features of the patients analyzed

Case	*Age*	*Sex*	*Cardiac defect*	*Other symptoms*
1	6y	F	TOF, PA, RAA, MAPCA	—
2	2y	M	VSD, PA, RAA, Truncus	—
3	4y	F	TOF	Abnormal face
4	36y	F	TOF, PA	—
5	29y	M	TOF	—
6	4y	M	TOF, PA	—

Abbreviations: F, female; M, male; MAPCA, multiple aortopulmonary collateral arteries; PA, pulmonary atresia; PS, pulmonary stenosis; RAA, right aortic arch; TOF, tetralogy of Fallot; Truncus, truncus arteriosus; VSD, ventricular septal defect; y, year.

Fluorescence *in situ* hybridization (FISH)

FISH analysis of peripheral blood samples was performed using a Vysis DiGeorge region probe (LSI TUPLE1 SpectrumOrange/LSI ARSA SpectrumGreen probe, Abbott Laboratories, Abbott Park, IL, USA) to detect the presence of 22q11.2 deletion, according to the manufacturer's instructions.

DNA extraction

Mononuclear cells of the peripheral blood were separated by Ficoll-Paque density centrifugation (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's instructions, and DNA was extracted from the mononuclear cells by using standard procedures.

PCR and sequence analysis

PCR was performed with AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and Tks Gflex DNA Polymerase (Takara, Otsu, Japan) at conditions recommended by the supplier. The obtained PCR product was analyzed by electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Direct sequencing of purified PCR products was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an automated ABI310 Genetic Analyzer (Applied Biosystems).

Multiplex ligation-dependent probe amplification

SALSA MLPA Congenital Heart Disease probemix (P311, MRC-Holland, Amsterdam, The Netherlands), which included three genes (*CDC45L*, *GP1BB* and *DGCR8*) located on chromosome 22q11 within the 3-Mb commonly deleted region, as well as other genes reported to be involved in cardiogenesis, was used for analysis, following the manufacturer's instructions. All runs included DNA from three normal controls to calibrate unknown samples. The reaction products were analyzed with an ABI310 Genetic Analyzer, and Coffalyser software (MRC-Holland) was used for copy number analysis.

TaqMan copy number assays for *TBX1*

The presence of any copy number alterations of the *TBX1* gene was evaluated by quantitative PCR with TaqMan copy number assays (Applied Biosystems) covering exon 2 (assay ID, Hs02267325_cn), exon 7 (Hs00856599_cn) and exon 10 (Hs01313390_cn) of the *TBX1* gene. TaqMan Copy Number Reference Assay for *RNase P* or *TERT* (Applied Biosystems) was used as an internal reference for copy number analysis.

Array-based copy number analysis

Copy number analysis was performed using a genome-wide high-resolution Affymetrix CytoScan HD array (Affymetrix, Santa Clara, CA, USA) containing >2.6 million copy number markers, including

approximately 750 000 single nucleotide polymorphisms, according to the manufacturer's protocols. After scanning hybridized arrays, CEL files were generated from Affymetrix GeneChip Command Console Software 4.1.2 (AGCC, Affymetrix) and imported into Chromosome Analysis Suite (ChAS) Software v.2.1 to generate and visualize copy number, loss of heterozygosity and genotyping data. For copy number analysis, five consecutive markers with the same polarity were required for the minimum number of markers per region, but no minimum size was set as the reporting threshold for each region in order to detect small aberrations. For the interpretation of the results, database of genomic variants (http://projects.tcag.ca/variation/), DECIPHER database (https://decipher.sanger.ac.uk/), Online Mendelian Inheritance in Man (http://www.omim.org), UCSC (http://genome.ucsc.edu/, hg19) and the International Standards for Cytogenomic Arrays (https://www.iscaconsortium.org/) were employed.

Quantitative PCR

Quantitative real-time PCR was performed using SYBR Premix ExTaq II (Takara) and Universal SYBR Select Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) with the PCR conditions recommended by the supplier. All measurements were performed in duplicate, and the difference in the duplicate threshold cycle was less than one cycle in all samples tested. All experiments were repeated at least three times. The primers used for real-time PCR are summarized in Table 2.

RESULTS

No abnormalities were detected in the *TBX1* gene in six patients with conotruncal heart anomaly

Among the genes within the commonly deleted DiGeorge critical region, *TBX1* is the most probable candidate for causing the phenotype observed in DGS. As mutations in the *TBX1* gene have been shown to be responsible for the phenotype in DGS/velo-cardio-facial syndrome patients without detectable 22q11.2 deletion, the presence of any mutation in this gene was examined by direct sequencing, using genomic DNA extracted from peripheral blood mononuclear cells of the patients. In all the six patients enrolled in this study, no mutation was detected in the coding regions of the *TBX1* gene (exons 1 to 8, 9A, 9B, 9C and 10) and in the surrounding exon–intron junction. In addition, copy number assessment of exons 2, 7 and 10 by quantitative real-time PCR analysis (TaqMan Copy number assay) showed neither deletion nor amplification of these exons in all the patients examined (data not shown). Moreover, there were no copy number alterations in the genes associated with cardiogenesis in all the patients analyzed using SALSA MLPA Congenital Heart Disease probemix.

Microdeletion and duplication were detected at the proximal part of the DiGeorge critical region in two patients

Copy number variation (CNV) analysis on a genomic scale was performed using the Affymetrix CytoScan HD array in all patients. In the six patients, 1036 CNVs (211 gains and 825 losses) were observed, indicating 172.7 CNVs per individual analyzed (35.2 gains and 137.5 losses). In two out of the six patients, copy number alterations were detected in the proximal side of the DiGeorge critical region, although results obtained by array-based analysis left some uncertainty regarding the genomic breakpoint/borderline of copy number alteration on the 22q11.2 region owing to scarcity of probes located around the region (Figure 1). One patient (case 1) showed a monoallelic deletion, spanning approximately 108.0–375.6 kb, and the other (case 2) showed a duplication, spanning approximately 87.9–375.6 kb around this region (Figure 1). The other four patients showed no copy number alteration within the DiGeorge critical region. In addition, no other copy number alteration that is likely to be responsible for the phenotypes was detected in any of the six patients. Consents for

Table 2. Primer sequences used in the study

Region[a]	*Gene*	*Name*	*Sequence*
	PGK2	PGK2–392f	5′-CTGTTGCTGTTGAGCTCAAATCC-3′
		PGK2–445r	5′-CCACTTCTGCGCCTACACAGTC-3′
	B2M	b2MG-142f	5′-CAGGTTTACTCACGTCATCCAG-3′
		b2MG-194r	5′-GGATGAAACCCAGACACATAGC-3′
	HPRT1	HPRT1–305f	5′-TGAACGTCTTGCTCGAGATGTG-3′
		HPRT1–344r	5′-ACAGAGGGCTACAATGTGATGG-3′
1	*TUBA8*	TUBA8-g39100f	5′-GAACATGTGCCCTGTGAAGTGC-3′
		TUBA8-g39145r	5′-AAATGAGGCAGTCGGTGACCAC-3′
2		USP18g-11849f	5′-AGATGGTCCATCACTTACCGTG-3′
		USP18g-11898r	5′-GTCAAGTCCCAGTGAACCCATC-3′
3	*USP18*	USP18g-26519f	5′-ACTCGGAGCACCTGTTGACCC-3′
		USP18g-26569r	5′-TAATCTCCTGAGGGCCGCTAAC-3′
4		USP18g-41603f(m)	5′-GAATGCGTGGGCTTTATGTGAAGA-3′
		USP18g-41733r(m)	5′-GTGGGAACTCTGGAAGTCCTTC-3′
5		TMEM191B-5up 1F(m)	5′-CACCAAGGAACCTACTTCAAGGA-3′
		TMEM191B-5up 1R(m)	5′-CACCACCTAGGCTGGCCTGT-3′
6		DGCR6g-10000f	5′-CTGGGTGCAGCCTGATGTTCTG-3′
		DGCR6g-10094r	5′-AGAGAAACAGCTCTGCCGTGAGA-3′
7		DGCR6g-12568f	5′-GCCAGGTGTACAGGATGACTAGG-3′
		DGCR6g-12621r	5′-TCCCTGTGATGCTGTGCTTCAG-3′
8	*DGCR6* (intron 3)	DGCR6g-18001f	5′-GTGCTCCCCTGGAGTCCATTAG-3′
		DGCR6g-18077r	5′-GCCCCATCACCCCACCATTGC-3′
9	*PRODH* (exon 15)	PRODH-1837f	5′-GTGTACAAGTACGTGCCCTATGG-3′
		PRODH-1900r	5′-TCATGAGGCTGCTGTTCTCCAG-3′
10	*PRODH* (exon 1)	PRODH-90f	5′-TCCCCACCATGCAGCTGAAGTG-3′
		PRODH-136r	5′-TTCCTGACAAATGCCAGACAAGG-3′
11	*DGCR9*	DGCR9–29f	5′-CTCAAAGAGCCAAAGTCATCCTC-3′
		DGCR9–120r	5′-CCATGCTGTTTTGGTTACCATGC-3′
12	*DGCR2* (exon 10)	DGCR2–1645f	5′-ACGATGATGCTTTTGAGCCTGTG-3′
		DGCR2–1700r	5′-CCGGAGTAATGCACCTTCACTC-3′

[a]Region numbers correspond to the numbers of Figure 1b indicating the approximate sites of the primer pairs.

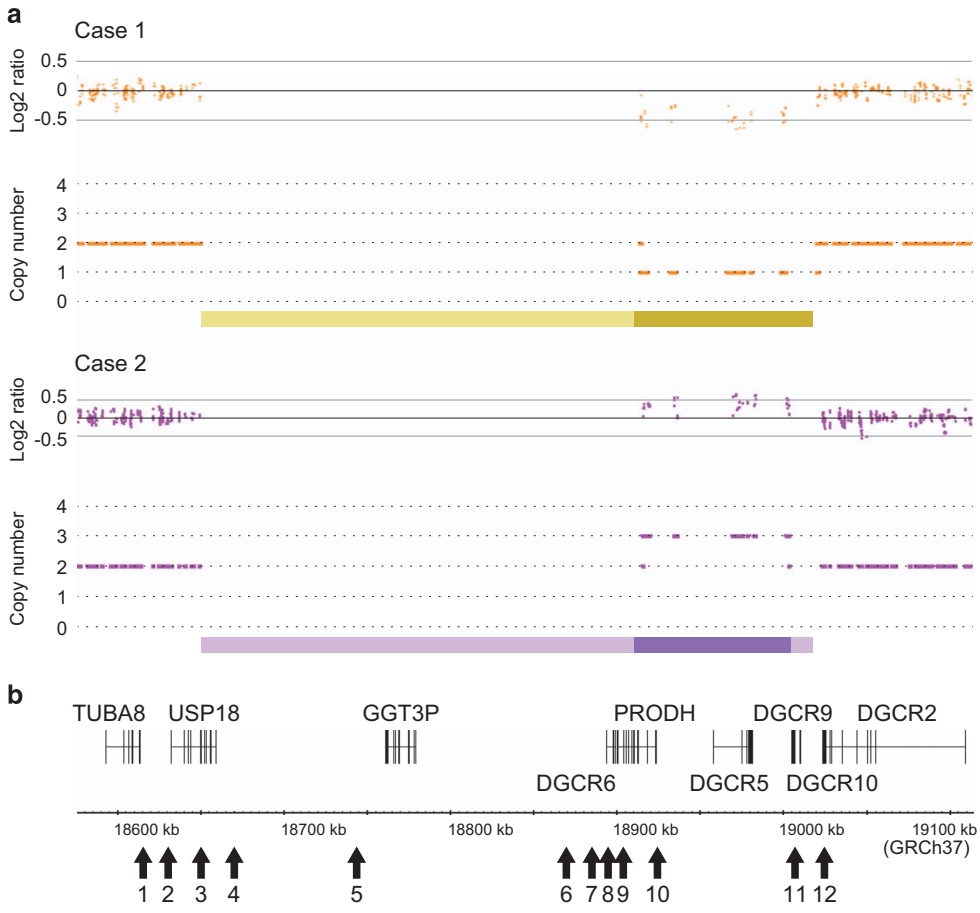

Figure 1. Microdeletion and duplication at the proximal part of the DiGeorge syndrome critical region detected in two patients by high-resolution array and quantitative PCR analysis. (**a**) Result of high-resolution array analysis in two cases. A small deletion, including at least *DGCR9*, *DGCR5* and *PRODH*, was observed in case 1, whereas almost the same region was duplicated in case 2. The area without any dots is where the array probe does not exist. The light and the dark brown bars show the estimated maximum and minimum deleted area in case 1, respectively. The light and dark purple bars show the estimated maximum and minimum duplicated area in case 2, respectively. (**b**) Genes located in this area are presented. The arrows indicate the locations of primers used in quantitative PCR analysis shown in Figure 2.

blood sampling from the parents to determine the origin of these copy number alterations could not be obtained.

The affected region contains the *DGCR6* and *PRODH* genes

Within the proximal side of the DiGeorge critical region, showing copy number alterations in two patients, there are several genes, including *USP18* at the proximal end and *DGCR6* and *PRODH* at the distal end. Copy number alterations of these genes were analyzed by quantitative real-time PCR analysis. In case 1, the genomic area, including the *PRODH*, *DGCR6*, *DGCR5* and *DGCR9* genes was deleted, resulting in only one remaining allele of these genes, whereas both alleles of *DGCR2* seemed to be retained. In case 2, the same genomic area that was lost in case 1 was duplicated instead, including *PRODH* and *DGCR6* to *DGCR9* genes (Figure 2). In addition, small isolated copy number alteration at the 3′ downstream region of the *USP18* gene was detected in case 2 by quantitative PCR analysis, which was not observed in any other patient or control samples. Regardless of this observation, coding regions of the *DGCR2* and *USP18* genes at the distal and proximal end of the altered region remained intact in both cases. As array comparative genomic hybridization probes are usually not located in the region containing *DGCR6* gene, and because public database data concerning the copy number changes of the region in healthy individuals may be restricted, we analyzed the gene dosage of *DGCR6* in 50 control samples by quantitative PCR. There was no CNV of the gene in all individuals analyzed (data not shown). This indicated that copy number alteration of the *DGCR6* gene is not a common event in healthy individuals, and that copy number changes of the gene observed in two out of the six patients with conotruncal heart defects could be one of the disease-causing variations.

DISCUSSION

A characteristic cardiac anomaly is one of the hallmarks of DGS, observed in approximately 80% of the patients,[1–3] and its rather specific morphology often leads to the diagnosis of DGS. Although the deletion within the 22q11.2 region is observed in about 10% of patients with stand-alone conotruncal heart defects lacking any other symptoms of DGS, most of the patients with these stand-alone conotruncal heart defects show no abnormality when evaluated by the standard FISH screening for 22q11.2 deletion.[26–30]

Two out of six cases presented in this report have atypical small deletion/duplication at the proximal end of the commonly deleted region in DGS. Both copy number analysis by high-resolution array and mutation analysis failed to detect any abnormalities within the *TBX1* gene in these patients. In both cases, copy number alterations resided in the so-called LCR-A region, which is located at the proximal end of the commonly deleted region in DGS. Detailed copy number analysis by quantitative real-time PCR suggested that this small deletion/duplication region involves four

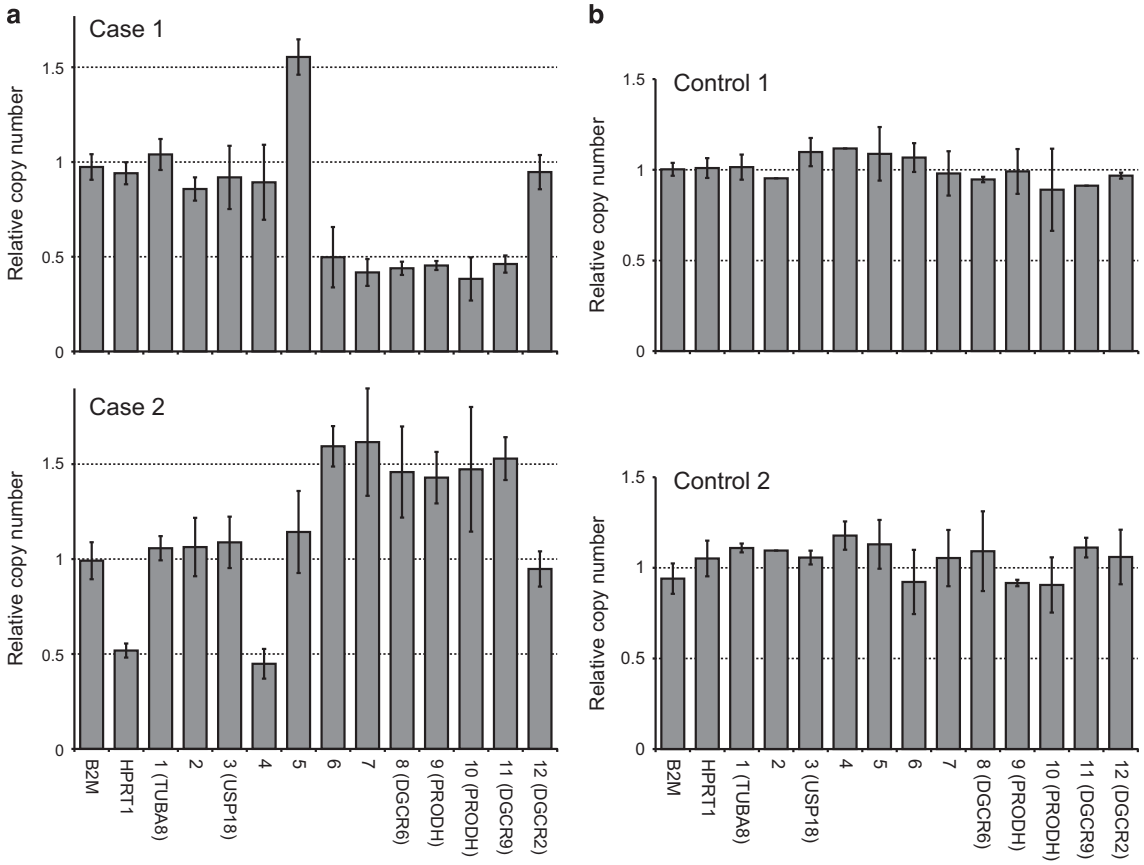

Figure 2. Identification of genes within microdeletion and duplication areas at the proximal part of the DiGeorge critical region, as confirmed by quantitative PCR analysis. (**a**) The affected regions in cases 1 and 2 contain *DGCR6*, *PRODH* and *DGCR9* genes, as confirmed by quantitative PCR. *PGK2* was used as an internal reference, and the average value of six control DNA samples was set as one for each primer set. Case 2 is a male showing a half dose of the *HPRT1* gene located on the X chromosome. (**b**) Results of quantitative PCR in representative female control samples.

genes at the proximal end of the DiGeorge critical region: *DGCR6*, *PRODH*, *DGCR5* and *DGCR9*. The products of *DGCR5* and *DGCR9* are non-coding RNA with unknown significance, whereas the other two genes, *DGCR6* and *PRODH*, encode proteins with several functions.[23,31,32] These two genes reside close together on 22q11.2 region, with a distance of about 0.7 kb between their respective tail positions.

Although many studies have concluded that the phenotype does not correlate to the length of the deletion in the 22q11.2 region, a few studies indicated that some phenotype–genotype correlation could be observed by detailed analysis.[11] In patients with aberrant cardiac morphology, although the length and breakpoints of the deleted regions on 22q11.2 varied, the presence of conotruncal heart defects was almost always accompanied by a large deletion measuring 3 or 1.5 Mb in length, involving the *TBX1* gene region.[11] Patients with deletions distal to these commonly deleted regions showed rather atypical heart defects.[11]

Although only a few reports describing the actual mapping of the proximal breakpoints in DGS are available,[33,34] proximal breakpoints were usually located around the region that contains the *DGCR6* and *PRODH* genes, which exactly corresponds to the affected regions in the two cases reported here. The more proximally located genes *USP18* and *TUBA8* were both often retained in patients with typical 3 Mb or nested 1.5 Mb proximal 22q11.2 deletions, as observed in the two patients analyzed in this study.

Figure 3 presents the location of copy number alterations reported in cases that have a small deletion or duplication proximal to the DiGeorge critical region. In all the six cases evaluated, *DGCR6* and *PRODH* are included or suspected to be included in the altered fragment, and they all show a heart defect that is observed in DGS. These reports also support our notion that *DGCR6* may also be responsible for the heart anomaly observed in DGS.

DGCR6 is located most centromeric within the DGS critical region.[35] The DGCR6 protein is a nuclear phosphoprotein highly expressed in the heart, liver and skeletal muscle.[31] During murine embryogenesis, *Dgcr6* shows widespread distribution, with the highest expression being observed in the pharyngeal arches, the central and peripheral nervous systems, and cardiac ganglion cells.[36] In a chicken model, *DGCR6* suppression in neural crest cells by retrovirus-mediated antisense transduction resulted in a high incidence of severe cardiovascular anomalies reminiscent of those found in DGS, possibly through the modulation of the expression level of some of the genes located within the DiGeorge critical region, including *TBX1*.[23]

PRODH encodes for proline dehydrogenase, a mitochondrial rate-limiting enzyme in the proline degradation process. Homozygous mutations in the *PRODH* gene lead to hyperprolinemia type I, a rare neurologic disorder with variable manifestations such as seizures, mental retardation, and psychiatric and behavioral disorders. Although multiple studies in human and mouse models indicate that abnormalities of *PRODH* are associated with the risk of psychiatric disorders,[37–39] no positive relationship between congenital heart defects and aberrant *PRODH* gene copy number was reported prior to this study. Collectively, these observations, in conjunction with the roles of *DGCR6* and *PRODH* described in

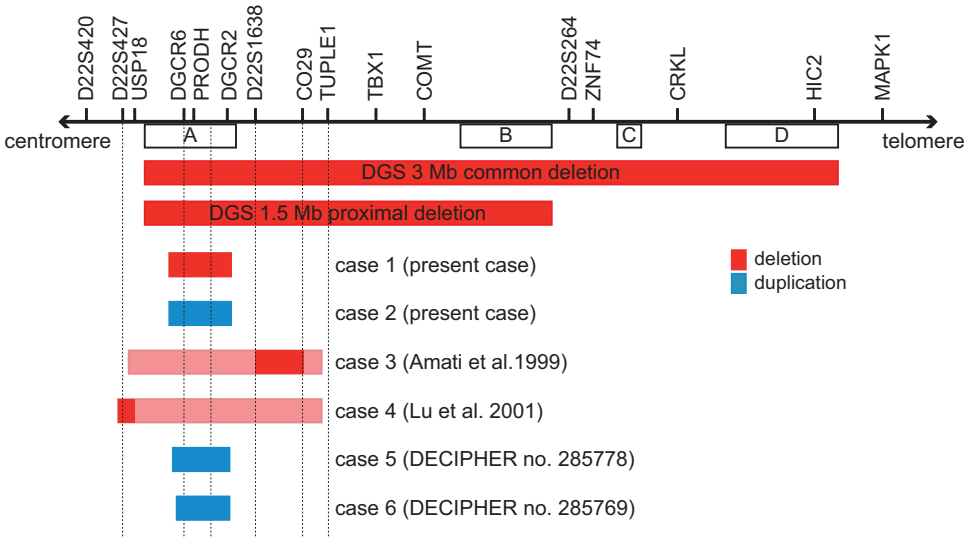

Figure 3. Reported cases with copy number alteration in the proximal part of the DiGeorge critical region. The bars with case numbers show deleted or duplicated regions in cases with heart defects. Cases 1 and 2 are present cases, whereas cases 3 to 6 are previously reported cases. Red bars indicate deleted regions, and blue bars indicate duplicated regions. Light and dark red bars show the estimated maximum and minimum deleted area respectively in case 3 and 4. The 3-Mb and 1.5-Mb commonly deleted regions in DGS are shown as the two red bars at the top. LCR A to LCR D in 22q11.2 region are shown as open boxes, and STS markers (D22S427 and D22S1638), FISH probes (TUPLE1), and genes located around the area are also indicated. The heart defects observed in cases 3 to 6 are as follows: case3; interrupted aortic arch type B, pulmonary stenosis,[47] case 4; tetralogy of Fallot,[48] case 5; pulmonary stenosis (DECIPHER no. 285778), case 6; abnormal tricuspid valve (DECIPHER no. 285769). DGS, DiGeorge syndrome; FISH, fluorescence *in situ* hybridization; LCR, low copy repeat; STS, sequence-tagged site.

the literature, strongly indicate that *DGCR6*, not *PRODH*, might be involved in the pathogenesis of conotruncal heart defects by some yet unknown mechanisms, although the roles of non-coding RNAs such as *DGCR5* and *DGCR9* that also reside in the affected region cannot be completely excluded.

Though interstitial deletions are the most common genomic variation associated with DGS by far,[40,41] other genomic instabilities that disrupt chromosome 22q11.2 have been reported in patients with a phenotype similar to that of DGS. In particular, individuals carrying microduplications mediated by the LCRs have also been reported.[42] The phenotype of patients with 22q11.2 microduplications is extremely variable, ranging from multiple defects to mild learning difficulties, and shares some features of DGS such as velopharyngeal insufficiency, urogenital abnormalities and conotruncal heart defects.[43] In addition, a gain-of-function mutation of *TBX1* was also identified in some patients with the phenotype of DGS.[44] In a BAC transgenic mouse model, heterozygous deletion of the *TBX1* gene region and duplication of the region containing *TBX1* showed development of conotruncal heart defects as observed in DGS.[45,46] These observations indicate that whether deleted or duplicated, a change in the gene dosage in any of the causative genes may contribute to the phenotype of 22q11.2 syndrome such as conotruncal heart defects. Although a detailed mechanism is still unknown, Liao *et al.*[46] suggested a model for *TBX1* dosage effects by hypothesizing that TBX1 acts as heterodimer for transcriptional regulation of its target genes. Both reduction and increase of the amount of TBX1 protein hinders appropriate heterodimer formation with its partner and target DNA binding, resulting in a loss/reduction of transcriptional regulation. The same explanation may apply to DGCR6, which is also a transcriptional regulator related to TBX1.

CNVs of *DGCR6* were only identified in two patients out of the six analyzed in our study. There may be two explanations for this. One possibility is that some genetic changes that have significant impact on expression or function of the *DGCR6* gene are present within the region but could not be detected by array comparative genomic hybridization (for example, very small copy number change(s) or some genetic alteration in promoter or enhancer of *DGCR6*). The other possibility is that there is a genetic alteration in some other gene(s) that is responsible for the phenotype. In this regard, we have undergone mutational analysis of possible candidate genes, such as *FGF8, CRKL*, *MAPK1* and *HIC2* genes.[14,17–19] We could not detect any genetic variation in the coding region of these genes in all six cases analyzed in this study. Further analysis is necessary to identify the responsible genetic abnormalities in these four cases.

In summary, the six patients analyzed in this study have typical conotruncal heart defects that are frequently observed in DGS. However, we have failed to identify any abnormalities in the *TBX1* gene in any of the six patients. Instead, small genomic copy number alterations that involve the *DGCR6* and *PRODH* genes were identified in two patients. Although one presented deletion, while the other showed duplication, these two genes may be responsible in the pathogenesis of conotruncal heart defects in these patients by some common mechanism. As the altered expression of *DGCR6* was previously reported to be a cause of the cardiac malformation observed in a chicken model of DGS,[23] the findings of our study further support the pathological role of DGCR6 in conotruncal heart defects. Further analysis of more cases and a possible functional relationship between DGCR6 and TBX1 during cardiogenesis is necessary to clarify the role of DGCR6 in conotruncal heart defects.

ACKNOWLEDGEMENTS

The authors thank Ms Tokiko Mizushiro, Yuko Kajita and Chihiro Tanaka (Department of Pediatrics, Ehime University Graduate School of Medicine) for technical assistance, as well as Dr Hideaki Horikawa (the Support Center for Advanced Medical Sciences, Institute of Health Biosciences, the University of Tokushima Graduate School) for assistance provided during array-based copy number analysis. This work was supported by Grants-in-Aid for Scientific Research (JSPS KAKENHI) Grant Number 22591181.

COMPETING INTERESTS

The authors declare no conflict of interest.

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