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SHORT COMMUNICATION

Androgenetic/biparental mosaicism in a girl with Beckwith–Wiedemann syndrome-like and upd(14)pat-like phenotypes

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This report describes androgenetic/biparental mosaicism in a 4-year-old Japanese girl with Beckwith–Wiedemann syndrome (BWS)-like and paternal uniparental disomy 14 (upd(14)pat)-like phenotypes. We performed methylation analysis for 18 differentially methylated regions on various chromosomes, genome-wide microsatellite analysis for a total of 90 loci and expression analysis of *SNRPN* in leukocytes. Consequently, she was found to have an androgenetic 46,XX cell lineage and a normal 46,XX cell lineage, with the frequency of the androgenetic cells being roughly calculated as 91% in leukocytes, 70% in tongue tissues and 79% in tonsil tissues. It is likely that, after a normal fertilization between an ovum and a sperm, the paternally derived pronucleus alone, but not the maternally derived pronucleus, underwent a mitotic division, resulting both in the generation of the androgenetic cell lineage by endoreplication of one blastomere containing a paternally derived pronucleus and in the formation of the normal cell lineage by union of paternally and maternally derived pronuclei. It appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of BWS-like and upd(14)pat-like phenotypes, but not for the occurrence of other imprinting disorders or recessive Mendelian disorders.
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Keywords: androgenesis; Beckwith–Wiedemann syndrome; mosaicism; upd(14)pat

INTRODUCTION

A pure androgenetic human with paternal uniparental disomy for all chromosomes is incompatible with life because of genomic imprinting.[1,2] However, a human with an androgenetic cell lineage could be viable in the presence of a normal cell lineage. Indeed, an androgenetic cell lineage has been identified in six liveborn individuals with variable phenotypes.[3–7] All the androgenetic cell lineages have a 46,XX karyotype, and this is consistent with the lethality of an androgenetic 46,YY cell lineage.

Here, we report on a girl with androgenetic/biparental mosaicism, and discuss the underlying factors for the phenotypic development.

CASE REPORT

This patient was conceived naturally to non-consanguineous and healthy parents. At 24 weeks gestation, the mother was referred to us because of threatened premature delivery. Ultrasound studies showed Beckwith–Wiedemann syndrome (BWS)-like features,[8] such as macroglossia, organomegaly and umbilical hernia, together with polyhydramnios and placentomegaly. The mother repeatedly received amnioreduction and tocolysis.

She was delivered by an emergency cesarean section because of preterm rupture of membranes at 34 weeks of gestation. Her birth weight was 3730 g (+4.8 s.d. for gestational age), and her length 45.6 cm (+0.7 s.d.). The placenta weighed 1040 g (+7.3 s.d.).[9] She was admitted to a neonatal intensive care unit due to asphyxia. Physical examination confirmed a BWS-like phenotype. Notably, chest roentgenograms delineated mild bell-shaped thorax characteristic of paternal uniparental disomy 14 (upd(14)pat),[10] although coat hanger appearance of the ribs indicative of upd(14)pat was absent (Supplementary Figure 1). She was placed on mechanical ventilation for 2 months, and received tracheostomy, glossectomy and tonsillectomy in her infancy, due to upper airway obstruction. She also had several clinical features occasionally reported in BWS[8] (Supplementary Table 1). Her karyotype was 46,XX in all the 50 lymphocytes analyzed. On the last examination at 4 years of age, she showed postnatal growth failure and severe developmental retardation.

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MOLECULAR STUDIES

This study was approved by the Institutional Review Board Committee at the National Center for Child health and Development, and performed after obtaining informed consent.

Methylation analysis

We first performed bisulfite sequencing for the *H19*-DMR (differentially methylated region) and KvDMR1 as a screening of BWS[11,12] and that for the IG-DMR and the *MEG3*-DMR as a screening of upd(14)pat,[10] using leukocyte genomic DNA. Paternally derived clones were predominantly identified for the four DMRs examined (Figure 1a). We next performed combined bisulfite restriction analysis for multiple DMRs, as reported previously.[13] All the autosomal DMRs exhibited markedly skewed methylation patterns consistent with predominance of paternally inherited clones, whereas the *XIST*-DMR on the X chromosome showed a normal methylation pattern (Figure 1a).

Genome-wide microsatellite analysis

Microsatellite analysis was performed for 90 loci with high heterozygosities in the Japanese population.[14] Major peaks consistent with paternal uniparental isodisomy and minor peaks of maternal origin were identified for at least one locus on each chromosome, with the minor peaks of maternal origin being more obvious in tongue and tonsil tissues than in leukocytes (Figure 1b and Supplementary Table 2). There were no loci with three or four peaks indicative of chimerism. The frequency of the androgenetic cells was calculated as 91% in leukocytes, 70% in tongue cells and 79% in tonsil cells, although the estimation apparently was a rough one (for details, see Supplementary Methods).

Expression analysis

We examined *SNRPN* expression, because *SNRPN* showed strong expression in leukocytes (for details, see Supplementary Data). *SNRPN* expression was almost doubled in the leukocytes of this patient (Figure 1c).

DISCUSSION

These results suggest that this patient had an androgenetic 46,XX cell lineage and a normal 46,XX cell lineage. In this regard, both the androgenetic and the biparental cell lineages appear to have derived from a single sperm and a single ovum, because a single haploid genome of paternal origin and that of maternal origin were identified in this patient by genome-wide microsatellite analysis. Thus, it is likely that after a normal fertilization between an ovum and a sperm, the paternally derived pronucleus alone, but not the maternally derived pronucleus, underwent a mitotic division, resulting both in the generation of the androgenetic cell lineage by endoreplication of

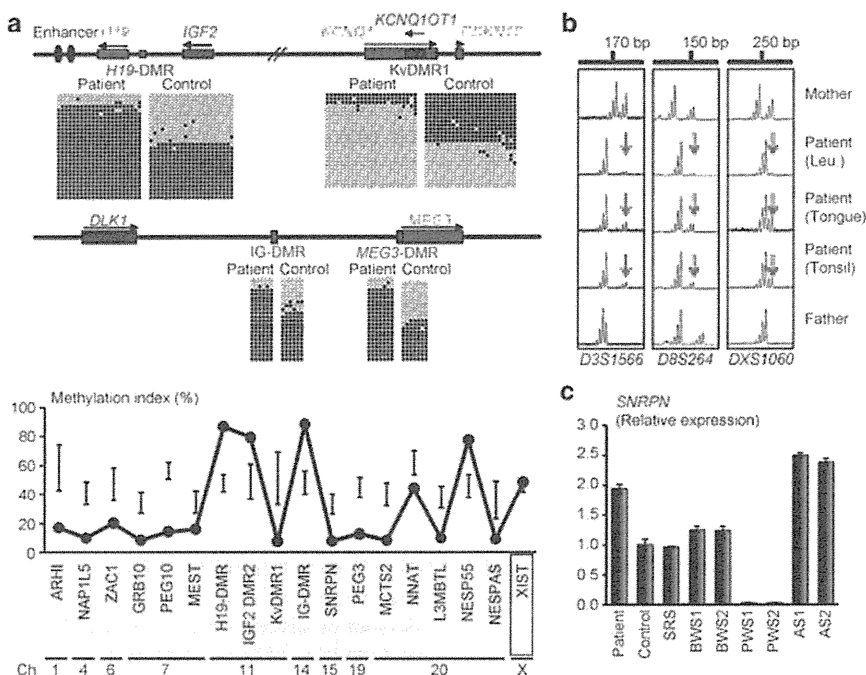

Figure 1 Representative molecular results. (**a**) Methylation analysis. Upper part: Bisulfite sequencing data for the *H19*-DMR and the KvDMR1 on 11p15.5, and those for the IG-DMR and the *MEG3*-DMR on 14q32.2. Each line indicates a single clone, and each circle denotes a CpG dinucleotide; filled and open circles represent methylated and unmethylated cytosines, respectively. Paternally expressed genes are shown in blue, maternally expressed gene in red, and the DMRs in green. The *H19*-DMR, the IG-DMR, and the *MEG3*-DMR are usually methylated after paternal transmission and unmethylated after maternal transmission, whereas the KvDMR1 is usually unmethylated after paternal transmission and methylated after maternal transmission.[10,11] Lower part: Methylation indices (the ratios of methylated clones) obtained from the COBRA analyses for the 18 DMRs. The DMRs highlighted in blue and pink are methylated after paternal and maternal transmissions, respectively. The black vertical bars indicate the reference data (maximum – minimum) in leukocyte genomic DNA of 20 normal control subjects (the *XIST*-DMR data are obtained from 16 control females). (**b**) Representative microsatellite analysis. Major peaks of paternal origin and minor peaks of maternal origin (red arrows) have been identified in this patient. The minor peaks of maternal origin are more obvious in tongue and tonsil tissues than in leukocytes (Leu.). (**c**) Relative expression level (mean ± s.d.) of *SNRPN*. The data are normalized against *TBP*. SRS: an SRS patient with an epimutation (hypomethylation) of the *H19*-DMR; BWS1: a BWS patient with an epimutation (hypermethylation) of the *H19*-DMR; BWS2: a BWS patient with upd(11)pat; PWS1: a Prader-Willi syndrome (PWS) patient with upd(15)mat; PWS2: a PWS patient with an epimutation (hypermethylation) of the *SNRPN*-DMR; AS1: an Angelman syndrome (AS) patient with upd(15)pat; and AS2: an AS patient with an epimutation (hypomethylation) of the *SNRPN*-DMR. The data were obtained using an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

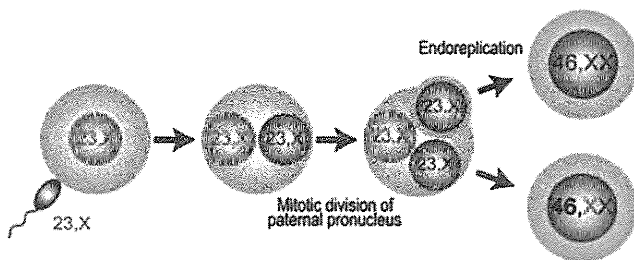

Figure 2 Schematic representation of the generation of the androgenetic/biparental mosaicism. Polar bodies are not shown.

one blastomere containing a paternally derived pronucleus and in the formation of the normal cell lineage by union of paternally and maternally derived pronuclei (Figure 2). This model has been proposed for androgenetic/biparental mosaicism generated after fertilization between a single ovum and a single sperm.[5,15,16] The normal methylation pattern of the *XIST*-DMR is explained by assuming that the two X chromosomes in the androgenetic cell lineage undergo random X-inactivation, as in the normal cell lineage. Furthermore, the results of microsatellite analysis imply that the androgenetic cells were more prevalent in leukocytes than in tongue and tonsil tissues.

A somatic androgenetic cell lineage has been identified in seven liveborn patients including this patient (Supplementary Table 1).[3–7] In this context, leukocytes are preferentially utilized for genetic analyses in human patients, and detailed examinations such as analyses of plural DMRs are necessary to detect an androgenetic cell lineage. Thus, the hitherto identified patients would be limited to those who had androgenetic cells as a predominant cell lineage in leukocytes probably because of a stochastic event and received detailed molecular studies. If so, an androgenetic cell lineage may not be so rare, and could be revealed by detailed analyses as well as examinations of additional tissues in patients with relatively complex phenotypes, as observed in the present patient.

Phenotypic features in androgenetic/biparental mosaicism would be determined by several factors. They include (1) the ratio of two cell lineages in various tissues/organs, (2) the number of imprinted domains relevant to specific features (for example, dysregulation of the imprinted domains on 11p15.5 and 14q32.2 is involved in placentomegaly[9,17]), (3) the degree of clinical effects of dysregulated imprinted domains (an (epi)dominant effect has been assumed for the 11p15.5 imprinted domains[18]), (4) expression levels of imprinted genes in androgenetic cells (although *SNRPN* expression of this patient was consistent with androgenetic cells being predominant in leukocytes, complicated expression patterns have been identified for several imprinted genes in both androgenetic and parthenogenetic fetal mice, probably because of perturbed *cis*- and *trans*-acting regulatory mechanisms[19]) and (5) unmasking of possible paternally inherited recessive mutation(s) in androgenetic cells. Thus, in this patient, it appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of BWS-like and upd(14)pat-like body and placental phenotypes, but remained below the threshold level for the occurrence of other imprinting disorders or recessive Mendelian disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL

GATA3 abnormalities in six patients with HDR syndrome

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Abstract. *GATA3* mutations cause HDR (*h*ypoparathyroidism, sensorineural *d*eafness, and *r*enal dysplasia) syndrome and, consistent with the presence of the second DiGeorge syndrome locus (*DGS2*) proximal to *GATA3*, distal 10p deletions often leads to HDR and DiGeorge syndromes. Here, we report on six Japanese patients with *GATA3* abnormalities. Cases 1–5 had a normal karyotype, and case 6 had a 46,XX,del(10)(p15) karyotype. Cases 1–6 had two or three of the HDR triad features. Case 6 had no DiGeorge syndrome phenotype except for hypoparathyroidism common to HDR and DiGeorge syndromes. Mutation analysis showed heterozygous *GATA3* mutations in cases 1–5, i.e., c.404–405insC (p.P135fsX303) in case 1, c.700T>C & c.708–709insC (p.F234L & p.S237fsX303) on the same allele in case 2, c.737-738insG (p.G246fsX303) in case 3, c.824G>T (p.W275L) in case 4, and IVS5+1G>C (splice error) in case 5. Deletion analysis of chromosome 10p revealed loss of *GATA3* and preservation of *D10S547* in case 6. The results are consistent with the previous finding that *GATA3* mutations are usually identified in patients with two or three of the HDR triad features, and provide supportive data for the mapping of *DGS2* in the region proximal to *D10S547*.

Key words: HDR syndrome, *GATA3*, DiGeorge syndrome, *DGS2*, Phenotypic spectrum

HDR (*h*ypoparathyroidism, sensorineural *d*eafness, and *r*enal dysplasia) syndrome is an autosomal dominant disorder first reported by Bilous *et al.* [1]. This condition is primarily caused by haploinsufficiency of *GATA3* on chromosome 10p15, although *GATA3* mutations have not been identified in a small portion of patients with clinical features compatible with HDR syndrome [2, 3]. *GATA3* consists of six exons, and encodes a transcription factor with two transactivating domains and two zinc finger domains on exons 2–6 [2]. *GATA3* is expressed in the developing parathyroid glands, inner ears, and kidneys, together with thymus and central nervous system (CNS) [4, 5].

Distal 10p deletions involving *GATA3* often lead to DiGeorge syndrome associated with hypoplastic thymus, T-cell immunodeficiency, hypoparathyroidism, congenital cardiac defects, and facial dysmorphism, in addition to HDR syndrome [6, 7]. Thus, deletion mappings have been performed, localizing the second DiGeorge syndrome locus (*DGS2*) to a ~1 cM region proximal to *D10S547* (the locus order: 10pter–*GATA3*–*D10S547*–*DGS2*–10cen) [6, 7].

Here, we report clinical and molecular findings in five patients with intragenic *GATA3* mutations and one patient with distal 10p deletion involving *GATA3*, and discuss the clinical features in *GATA3* mutation posi-

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Table 1 Summary of six patients with *GATA3* mutation or deletion

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Present age	40 years	39 years	4 years	31 years	17 years	4 years
Sex	Female	Female	Male	Female	Male	Female
Karyotype	46,XX	46,XX	46,XY	46,XX	46,XY	46,XX,del(10)(p15)
Hypoparathyroidism	Yes	Yes	Yes	Yes	Yes	Yes
Symptom	Convulsion	Tetany	No[b]	Convulsion	Convulsion	Convulsion
Ca (mg/dL)	3.4	3.4	2.7	4.3	3.0	4.7
P (mg/dL)	8.0	7.9	8.1	7.9	8.7	8.6
Intact PTH (pg/mL)	Undetected	Undetected	14	Undetected	Undetected	15
Age at diagnosis	10 years	13 years	17 months	3 years	17 months	2 weeks
Sensorineural deafness	Yes	Yes	No	Yes	Yes	Yes
Hearing level (dB)[a]	50 (B)	>70 (B)	Normal	60 (B)	50 (B)	90 (B)
Age at diagnosis	13 years	6 years		11 years	12 months	6 months
Renal lesion	Yes	Yes	Yes	Equivocal[c]	Yes	Yes
Malformation	RH (L)	PCD (B)	PD (R)	Absent	RH (L)	VUR (B)
Age at diagnosis	9 years	27 years	17 months		17 months	2 months

Abbreviations: PTH, parathyroid hormone; dB, decibel; B, bilateral; L, left; R, right; RH, renal hypoplasia; PCD, pelvicalyceal deformity; PD, pelvic duplication; and VUR, vesicoureteral reflux.
[a] Degree of hearing loss: normal, <25 dB; mild 26–40 dB; moderate 41–55 dB; moderately severe, 56–70 dB; and profound, >90 dB.
[b] Hypocalcemia was revealed by routine biochemical studies, when this boy was admitted because of bronchopneumonia.
[c] Renal malformation was absent, but renal dysfunction with increased serum creatinine was noticed during pregnancy.
Normal reference data: Ca: 8.84–10.44 mg/dL; P: 4.5–6.5 mg/dL; and intact PTH: 10–65 pg/mL.

tive patients and the chromosomal location of *DGS2*.

Patients and Methods

Patients

We studied six hitherto unreported Japanese patients (cases 1–6) with two or three HDR triad features. Cases 1–5 had a normal karyotype, and case 6 had a 46,XX,del(10)(p15) karyotype. Cases 1–4 and 6 were apparently sporadic cases, whereas case 5 was a possible familial case: the father received renal dialysis due to chronic renal failure from his twenties, and the paternal grandmother had unilateral renal hypoplasia, although they lacked clinical features suggestive of hypoparathyroidism and hearing difficulty.

Clinical phenotypes of the HDR triad features are summarized in Table 1. Hypoparathyroidism was noticed by convulsion in cases 1 and 4–6 and by tetany in case 2; in case 3, it was incidentally found by biochemical examinations at the time of admission due to bronchopneumonia. After confirming parathyroid hormone deficiency, 1α(OH) vitamin D therapy was started, successfully normalizing serum calcium and phosphate values in cases 1–6. Sensorineural deafness was demonstrated in cases 1, 2, and 4–6 by auditory brainstem response or audiometry, and they required hearing aids in their daily life. Case 3 had no hearing difficulty with normal auditory brainstem response. Renal lesion was radiologically confirmed in cases 1–3, 5, and 6. Although case 4 had no discernible renal malformation, she manifested renal dysfunction during pregnancy. In addition, case 6 exhibited developmental delay but lacked hypoplastic thymus, T-cell immunodeficiency, congenital cardiac defects, and facial dysmorphism characteristic of DiGeorge syndrome.

Mutation analysis of GATA3

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. After obtaining informed consent, leukocyte genomic DNA samples of cases 1–6 were amplified by PCR for the coding regions on exons 2–6 and their flanking splice sites, and the PCR products were subjected to direct sequencing from both directions on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). The primer sequences and the PCR conditions were as described previously [2, 3]. To confirm a heterozygous mutation, the corresponding PCR products were subcloned with a TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA), and normal and mutant alleles were sequenced separately.

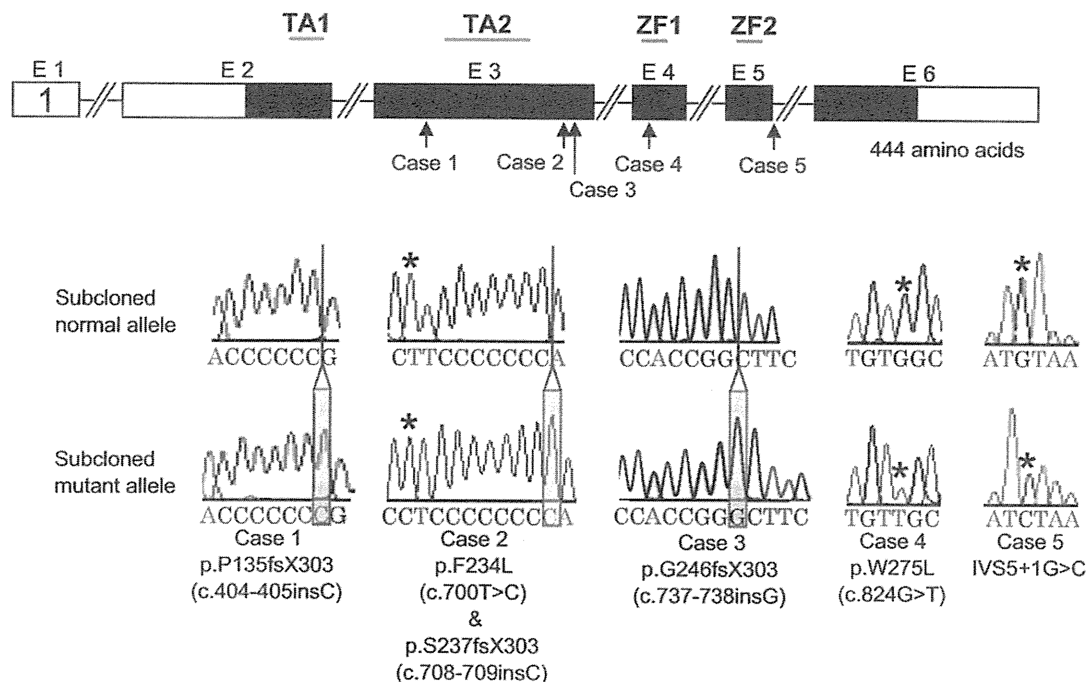

Fig. 1 Mutation analysis of *GATA3*.
Upper part: The structure of *GATA3* and the position of the mutations identified in cases 1–5. *GATA3* consists of exons 1–6 (E1–E6) and encodes two transactivating domains (TA1 and TA2) and two zinc finger domains (ZF1 and ZF2). The black and white boxes denote the coding regions and the untranslated regions, respectively.
Lower part: Electrochromatograms showing the subcloned normal and mutant sequences in cases 1–5.

Deletion analysis of 10p

To indicate an extent of the 10p deletion in case 6, oligoarray comparative genomic hybridization (CGH) was carried out with 1x244K Human Genome Array (catalog No. G4411B) (Agilent Technologies, Palo Alto, CA), according to the manufacturer's protocol. Furthermore, fluorescence *in situ* hybridization (FISH) was performed with an RP11-554F11 BAC probe containing the whole *GATA3* gene [3] and an RP11-17E09 BAC probe containing *D10S547* (BACPAC Resources Center, Oakland, CA), together with a CEP 10 probe for *D10Z1* (Abbott, Chicago, IL) utilized as an internal control. The two BAC probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the control probe was detected according to the manufacturer's protocol.

Results

Mutation analysis of GATA3

Direct sequencing identified heterozygous *GATA3* mutations in cases 1–5, i.e., a frameshift mutation (c.404–405insC, p.P135fsX303) in case 1, a missense mutation (c.700T>C, p.F234L) and a frameshift mutation (c.708–709insC, p.S237fsX303) on the same allele in case 2, a frameshift mutation (c.737-738insG, p.G246fsX303) in case 3, a missense mutation (c.824G>T, p.W275L) in case 4, and a splice donor site mutation (IVS5+1G>C) in case 5 (Fig. 1). Unfortunately, the renal phenotype positive father and paternal grandmother of case 5 were not examined. These mutations were absent from 200 control subjects. No intragenic mutation was identified in case 6 with distal 10p deletion.

Deletion analysis of 10p

CGH revealed a ~10 Mb terminal deletion from chromosome 10p of case 6 (Fig. 2). FISH analysis showed that the 10p deletion chromosome was missing *GATA3* and retained *D10S547*.

Discussion

Cases 1–6 had two or three of the HDR triad features and heterozygous *GATA3* abnormalities. This is consistent with the previous notion that *GATA3* mutations

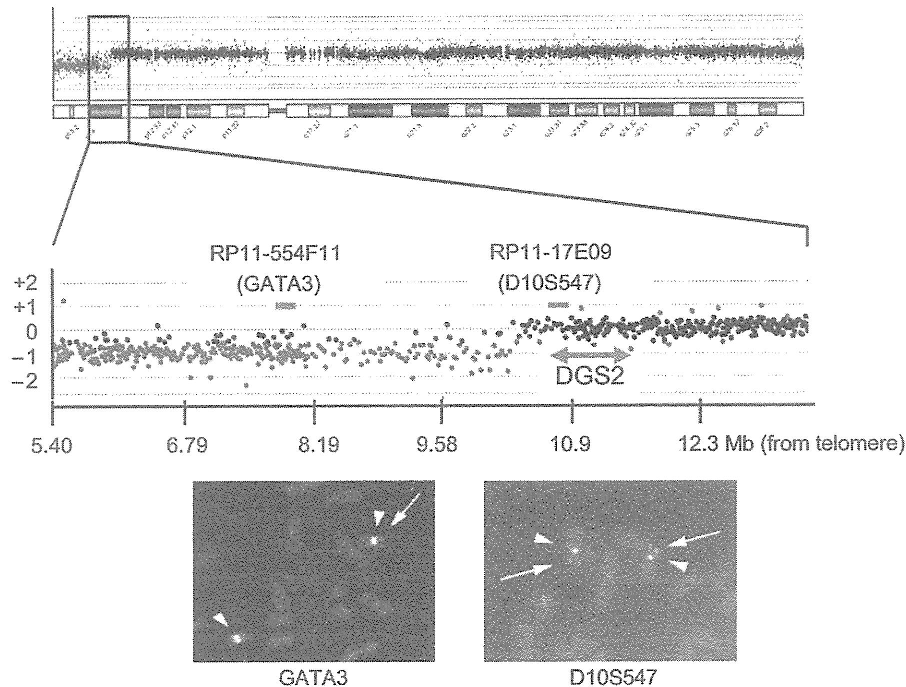

Fig. 2 Deletion analysis of 10p. The green and black signals in CGH indicate the deleted and preserved regions on the 10p deleted chromosome, respectively. The critical region for *DGS2* is indicated. The RP11-554F11 probe containing *GATA3* detects only a single signal (an arrow), whereas the RP11-17E09 probe containing *D10S547* identifies two signals (arrows). The arrowheads indicate *D10Z1* detected by a control CEP 10 probe.

are usually identified in patients with two or three of the HDR triad features [8, 9]. However, this would more or less be due to an ascertainment bias that *GATA3* are usually examined in patients diagnosed as having HDR syndrome. Indeed, familial studies of probands with typical HDR syndrome have identified *GATA3* mutations in subjects with apparently deafness only phenotype [3, 10], although there has been no report documenting apparently normal phenotype in individuals with *GATA3* mutations. It is possible, therefore, that *GATA3* mutations are associated with a relatively wide penetrance and expressivity of the HDR triad features. In this context, it is notable that the father and the paternal grandmother of case 5 had renal abnormalities as the sole discernible clinical phenotype. This suggests that *GATA3* mutations may cause renal abnormalities alone in exceptional patients, although mutations analysis could not be performed for the father and the grandmother.

Case 6 lacked T-cell immunodeficiency, congenital cardiac defects, and abnormal facial appearance characteristic of DiGeorge syndrome. While case 6 had hypoparathyroidism, this is explained by loss of *GATA3*. In addition, developmental delay is ascribed to chromosome aberration. Thus, genotype-phenotype correlation in case 6 is consistent with the previous mapping of *DGS2* to a region proximal to *D10S547* [6, 7].

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ORIGINAL ARTICLE

Maternal age effect on the development of Prader–Willi syndrome resulting from upd(15)mat through meiosis 1 errors

Keiko Matsubara[1,2,3], Nobuyuki Murakami[2,3], Toshiro Nagai[2] and Tsutomu Ogata[1]

Prader–Willi syndrome (PWS) is primarily caused by deletions involving the paternally derived imprinted region at chromosome 15q11.2–q13 and maternal uniparental disomy 15 (upd(15)mat). The underlying mechanisms for upd(15)mat include trisomy rescue (TR), gamete complementation (GC), monosomy rescue and post-fertilization mitotic error, and TR/GC is mediated by non-disjunction at maternal meiosis 1 (M1) or meiosis 2 (M2). Of these factors involved in the development of upd(15)mat, M1 non-disjunction is a maternal age-dependent phenomenon. We studied 117 Japanese patients with PWS and identified deletions in 84 patients (Deletion group) and TR/GC type upd(15)mat through M1 non-disjunction in 15 patients (TR/GC (M1) group), together with other types of abnormalities. Maternal age was significantly higher in TR/GC (M1) group than in Deletion group (median (range), 37 (35–45) versus 30 (19–42); $P=1.0\times10^{-7}$). Furthermore, delayed childbearing age became obvious since the year 2003 in Japan, and relative frequency of TR/GC (M1) group was significantly larger in patients born since the year 2003 than in those born until the year 2002. The results imply that the advanced maternal age at childbirth is a predisposing factor for the development of upd(15)mat because of increased M1 errors.
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Keywords: maternal age effect; meiosis 1; non-disjunction; Prader-Willi syndrome; upd(15)mat

INTRODUCTION

Prader–Willi syndrome (PWS) is a developmental disorder associated with various dysmorphic, neurologic, cognitive, endocrine and behavioral/psychiatric features.[1] It is caused by absent expression of paternally derived genes on the imprinted region at chromosome 15q11.2–q13, and previous studies have indicated that deletions of the paternally derived imprinted region and maternal uniparental disomy 15 (upd(15)mat) account for ~70 and ~25% of PWS patients, respectively.[1] The remaining PWS patients have rare abnormalities such as epimutations (hypermethylation) of the PWS imprinting center (IC), at the differentially methylated region encompassing exon 1 of *SNRPN* and microdeletions involving the PWS-IC or HBII-85 small nucleolar RNAs distal to the PWS-IC.[2–4]

Upd(15)mat are primarily caused by four mechanisms; that is, trisomy rescue (TR), gamete complementation (GC), monosomy rescue (MR) and post-fertilization mitotic error (PE).[5] TR refers to a condition in which chromosome 15 of paternal origin is lost from a zygote with trisomy 15, formed by fertilization between a disomic oocyte and a normal sperm. GC results from fertilization of a disomic oocyte with a nullisomic sperm. MR refers to a condition in which chromosome 15 of maternal origin is replicated in a zygote with monosomy 15, formed by fertilization between a normal oocyte and a nullisomic sperm. PE is an event after formation of a normal zygote. In this regard, a disomic oocyte specific to TR and GC is produced by non-disjunction at meiosis 1 (M1) or meiosis 2 (M2), and non-disjunction at M1 is known to increase with maternal age, probably because of a long-term (10–50 years) meiotic arrest at prophase 1.[6]

It is predicted, therefore, that the relative frequency of TR/GC-type upd(15)mat through M1 non-disjunction is high in PWS patients born to aged mothers and is increasing in countries where childbearing age is rising. In this context, previous studies have revealed a significantly higher maternal age in PWS patients with upd(15)mat than in those with deletions,[7,8] a significantly higher relative frequency of upd(15)mat in patients born to mothers aged $\geqslant$35 years than in those born to mothers aged $<$35 years[9] and a significantly increased relative frequency of upd(15)mat in PWS patients $<$5 years of age in United Kingdom where childbearing age is increasing.[10] In these

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studies, however, as underlying mechanisms for upd(15)mat have not been examined, it remains to be clarified whether such maternal age effect on the occurrence of upd(15)mat is primarily mediated by M1 non-disjunction. Furthermore, after studying underlying mechanisms for upd(15)mat by microsatellite analysis, Robinson *et al.*[11] have mentioned that maternal age effect is similar between M1 and M2 errors. Thus, it remains to be clarified whether advanced maternal age is relevant to the occurrence of TR/GC type upd(15)mat through M1 errors.

Here, we report that the advanced maternal age at childbirth constitutes a risk factor for TR/GC type upd(15)mat through M1 non-disjunction.

MATERIALS AND METHODS

This study was approved by the Institute Review Board Committees at the National Center for Child Health and Development and Dokkyo University Koshigaya Hospital, and performed after obtaining informed consent.

PWS patients

This study consisted of 117 Japanese PWS patients (72 male patients and 45 female patients) who satisfied the following selection criteria: (1) normal karyotype in all the 50 lymphocytes examined, (2) hypermethylated PWS-IC that was confirmed by methylation analysis for bisulfite-treated leukocyte genomic DNA, using methylated and unmethylated allele-specific PCR primers (Supplementary Figure 1),[12] and (3) positive data on the maternal age at childbirth (parental age was not found in two aged patients who had left our follow-up and whose hospital records had been discarded and in one patient who was born after artificial insemination by donor).

Molecular studies

We performed fluorescence *in situ* hybridization analysis, microsatellite analysis and multiplex ligation-dependent probe amplification (MLPA) analysis. For fluorescence *in situ* hybridization analysis, an ~125-kb probe identifying a region encompassing *SNRPN* was hybridized to lymphocyte metaphase spreads, together with a CEP 15 probe for *D15Z1* and a probe for *PML* on 15q22 utilized as internal controls. The probe for the *SNRPN* region was labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the control probes were detected according to the manufacturer's protocol (Abbott, Chicago, IL, USA). For microsatellite genotyping, PCR amplification was performed for 13 microsatellite loci on chromosome 15, using fluorescently labeled forward primers and unlabeled reverse primers. Subsequently, the PCR products were determined for size on a CEQ8000 autosequencer (Beckman Coulter, Fullerton, CA, USA). For MLPA analysis, we utilized a commercially available MLPA probe mix (ME028-B1) for multiple segments on the chromosome 15 imprinted region, including the PWS-IC and three portions within the HBII-85 small nucleolar RNAs (MRC-Holland, Amsterdam, The Netherlands). The procedure was as described in the manufacturer's instructions. The primers utilized in this study are summarized in Supplementary Table 1.

Classification of PWS patients

The PWS patients were classified into several groups, according to the underlying (epi) genetic causes (Figure 1). In particular, upd(15)mat was divided into three groups by the previously reported methods[13] (Supplementary Figure 2): (1) heterodisomy for at least one of the three adjacent pericentromeric (<4 Mb from the centromere) microsatellite loci (*D15S541*, *D15S542* and *D15S1035*) was regarded as indicative of TR/GC type upd(15)mat through M1 non-disjunction (TR/GC (M1) group), (2) the combination of isodisomy for the pericentromeric microsatellite loci and heterodisomy for at least one middle to distal microsatellite loci was interpreted as indicative of TR/GC type upd(15)mat through M2 non-disjunction (TR/GC (M2) group) and (3) isodisomy for all the informative microsatellite loci was regarded as indicative of MR/PE type upd(15)mat (MR/PE group). However, it is usually impossible to distinguish between TR and GC, and between MR and PE on the basis of microsatellite data, although identification of segmental isodisomy or mosaicism with a normal cell lineage is unique to PE.[14,15]

Analysis of parental ages

We compared parental ages between different groups and between two different time periods (until the year 2002 and since the year 2003), and relative frequency of each group between the two time periods. The setting of the two time periods was based on the Annual Vital Statistics Data from the Japanese Ministry of Health, Labor and Welfare (http://www.mhlw.go.jp/toukei/list/81-1.html). The maternal age producing the largest number of live births changed from 25–29 years to 30–34 years, and that producing the third largest number of live births changed from 20–24 years to 35–39 years, between the two time periods (Supplementary Figure 3).

Statistical significance of the median age was examined by the Mann–Whitneys *U*-test, that of the correlation between parental ages by Spearman's rank correlation test, and that of relative frequency by the Fisher's exact probability test. $P<0.05$ was considered significant.

RESULTS

Classification of PWS patients

The results are shown in Figure 1. Fluorescence *in situ* hybridization analysis revealed heterozygous deletions in 84 of the 117 patients (Supplementary Figure 4;Deletion group). Then, microsatellite genotyping was carried out in 27 of the 33 patients without deletions, classifying 15 patients as TR/GC (M1) group, seven patients as TR/GC (M2) group and three patients as MR/PE group (Figure 2;in the remaining six patients, further studies were refused by the parents). There was no finding indicative of segmental isodisomy or mosaicism. Finally, MLPA was performed in the remaining two non-upd(15)mat patients, identifying no microdeletion affecting the PWS-IC. Thus, the two patients were classified as Epimutation group.

Analysis of parental ages

Distribution of parental ages in each group is shown in Figure 3a, and parental age data are summarized in Table 1. Maternal ages were invariably ⩾35 in TR/GC (M1) group. Furthermore, comparison of maternal ages in Deletion, TR/GC (M1) and TR/GC (M2) groups with >5 patients revealed significant difference between Deletion and TR/GC (M1) groups ($P=1.0\times10^{-7}$), but not between Deletion and TR/GC (M2) groups ($P=0.19$), and between TR/GC (M1) and TR/GC (M2) groups ($P=0.085$). Paternal ages showed similar tendency, with

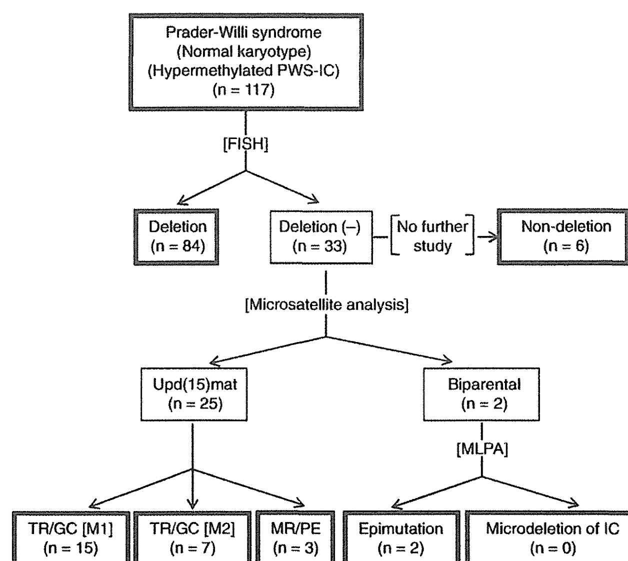
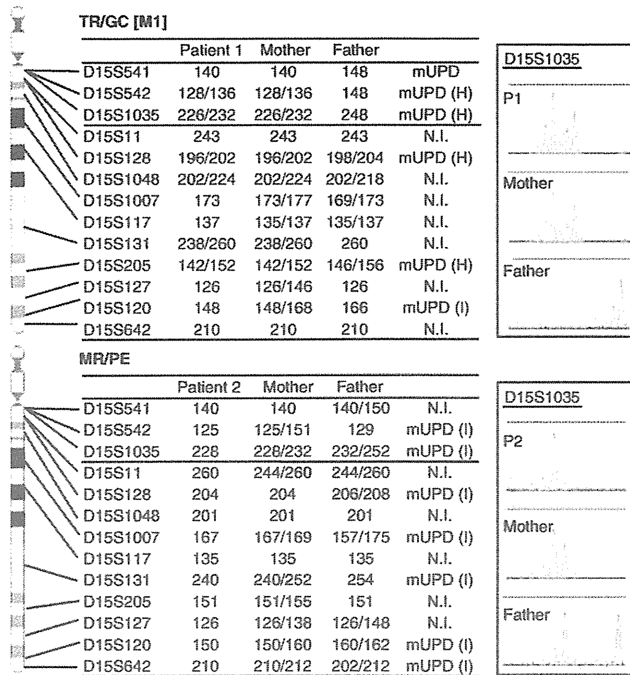


Figure 1 Classification of 117 Japanese patients with Prader–Willi syndrome phenotype.


TR/GC [M1]

	Patient 1	Mother	Father	
D15S541	140	140	148	mUPD
D15S542	128/136	128/136	148	mUPD (H)
D15S1035	226/232	226/232	248	mUPD (H)
D15S11	243	243	243	N.I.
D15S128	196/202	196/202	198/204	mUPD (H)
D15S1048	202/224	202/224	202/218	N.I.
D15S1007	173	173/177	169/173	N.I.
D15S117	137	135/137	135/137	N.I.
D15S131	238/260	238/260	260	N.I.
D15S205	142/152	142/152	146/156	mUPD (H)
D15S127	126	126/146	126	N.I.
D15S120	148	148/168	166	mUPD (I)
D15S642	210	210	210	N.I.

MR/PE

	Patient 2	Mother	Father	
D15S541	140	140	140/150	N.I.
D15S542	125	125/151	129	mUPD (I)
D15S1035	228	228/232	232/252	mUPD (I)
D15S11	260	244/260	244/260	N.I.
D15S128	204	204	206/208	mUPD (I)
D15S1048	201	201	201	N.I.
D15S1007	167	167/169	157/175	mUPD (I)
D15S117	135	135	135	N.I.
D15S131	240	240/252	254	mUPD (I)
D15S205	151	151/155	151	N.I.
D15S127	126	126/138	126/148	N.I.
D15S120	150	150/160	160/162	mUPD (I)
D15S642	210	210/212	202/212	mUPD (I)

Figure 2 Chromosomal locations of the examined microsatellite loci and representative results. MUPD, maternal uniparental disomy (unknown for heterodisomy or isodisomy); mUPD (H), maternal uniparental heterodisomy; mUPD (I), maternal uniparental isodisomy; N.I., not informative. Pericentromeric loci are present in a heterodisomic status in patient 1, and this is consistent with trisomy rescue/gamete complementation (meiosis 1) (TR/GC (M1)) type maternal uniparental disomy 15 (upd(15)mat). For D15S1035, for example, both of the heterozygous maternal alleles are inherited by patient 1, whereas the homozygous paternal alleles are not transmitted to patient 1; this demonstrates mUPD (H) for this locus. In patient 2, all informative loci are present in an isodisomic condition, and this is compatible with monosomy rescue/post-fertilization mitotic error (MR/PE) type upd(15)mat. For D15S1035, for example, one of the two heterozygous maternal alleles is transmitted to patient 2, whereas both of the heterozygous paternal alleles are not inherited by patient 2; this demonstrates mUPD (I) for this locus.

significant difference between Deletion and TR/GC (M1) groups (P=8.8$\times 10^{-5}$), but not between Deletion and TR/GC (M2) groups (P=0.39), and between TR/GC (M1) and TR/GC (M2) groups (P=0.39). However, whereas a significant correlation was observed between maternal and paternal ages in Deletion and TR/GC (M2) groups, there was no significant correlation between maternal and paternal ages in TR/GC (M1) group because of relatively advanced maternal ages in this group (Figure 3b). In addition, whereas maternal ages at childbirth were grossly similar between Deletion and TR/GC (M2) groups and the Japanese general population (the mean parental ages at childbirth in Japan were based on the data registered in the Ministry of Health, Labor and Welfare; http://www.mhlw.go.jp/toukei/list/81-1.html), they were obviously higher in TR/GC (M1) group than in the Japanese general population. Paternal ages at childbirth were grossly similar between Deletion group and the Japanese general population and tended to be higher in TR/GC (M1) and TR/GC (M2) groups than in the Japanese general population.

Relative frequency of each group markedly differed between 75 patients born until 2002 and 42 patients born since 2003 (Figure 3c). Here, TR/GC (M1) was indicated in three of the 75 patients born until the year 2002, and six non-deletion type patients were invariably born until the year 2002. Thus, TR/GC (M1) group accounted for at least three and up to nine of the 75 patients born until the year 2002, and 12 of the 42 patients born since the year 2003. Thus, the relative frequency of TR/GC (M1) was assessed to be significantly different, with the P-values being 1.8×10^{-7} for 3/75 versus 12/42, and 0.025 for 9/75 versus 12/42. In addition, there was no significant change in the parental ages of each group between the two time periods, although the maternal ages at birth of all the patients significantly differed between the two time periods.

DISCUSSION

The present study revealed deletions in 84 patients, upd(15)mat in 25 patients and epimutations in 2 patients. In addition, whereas microsatellite and MLPA analyses were not performed in six patients with non-deletion, the present and the previous studies argue that most of them have upd(15)mat, especially TR/GC (M1) type upd(15)mat.[1,13] Thus, the relative frequency of deletions, upd(15)mat and other rare causes appears to be similar between Japanese patient and previously reported Caucasian patients.[1]

Notably, the present study implies that advanced maternal age at childbirth constitutes a risk factor for the development of TR/GC (M1) type upd(15)mat. Indeed, maternal ages were significantly higher in TR/GC (M1) group than in Deletion group, which is free from maternal age effect. Although a significant difference was not found between maternal age-dependent TR/GC (M1) group and maternal age-independent TR/GC (M2) group, this would primarily be due to the small number of TR/GC (M2) group. Furthermore, the relative frequency of TR/GC (M1) group significantly increased since the year 2003 when delayed childbearing age became obvious, and the advanced maternal ages at birth since the year 2003 were primarily associated with the high frequency of TR/GC (M1) group rather than the advanced maternal ages in each group. Although it was impossible to distinguish between TR and GC, and between MR and PE,[16] this would not pose a major problem. The patients with M1 non-disjunction are included only in TR/GC (M1) group.

Paternal and environmental factors should also be considered for the present results. For a paternal factor, the frequencies of micro-deletions and nullisomic sperms might increase with age.[17] However, paternal ages at childbirth in each group were similar between the two time periods, and the relative frequency of Deletion group actually decreased since the year 2003. Furthermore, whereas nullisomic sperms can be a background of the development of GC, concomitant occurrence of a nullisomic sperm and a disomic oocyte must be extremely rare. Rather, nullisomic sperms would primarily constitute an underlying factor for the development of maternal age-independent MR. For an environmental factor, it is predicted that chemical materials are increasing with time and that aged parents are exposed to such materials for a long time. In this regard, it has been reported that exposure to environmental chemicals may exaggerate the occurrence of aneuploidies in females.[18] Thus, the environmental factor might be relevant to the recent increase of TR/GC (M1) group, although it is unlikely that this factor constitutes the major cause of the increased TR/GC (M1) type upd(15)mat. In males, whereas it has been reported that exposure to chemical materials might facilitate the occurrence of PWS, the relative frequency of genetic causes remained unchanged in PWS patients born to such males.[19–21] Collectively, the effects of such non-maternal age factors would remain small, if any, although further careful examinations are required for the precise evaluation of the maternal age effect on the occurrence of TR/GC (M1).

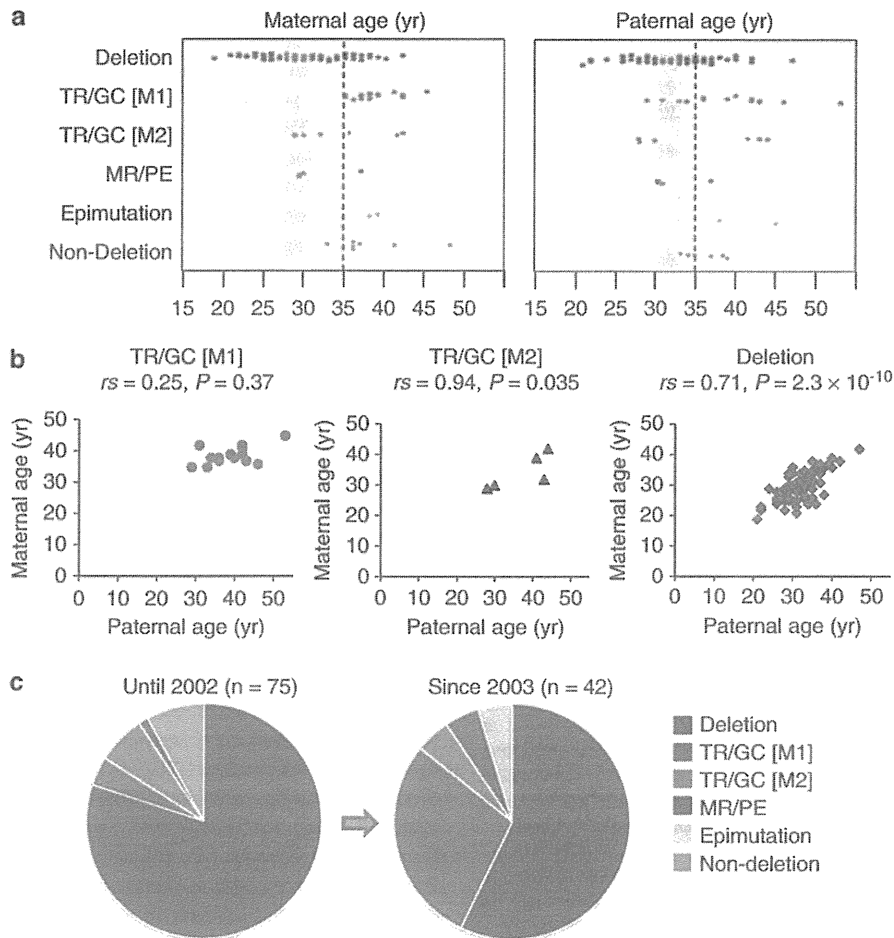


Figure 3 Analysis of parental ages at childbirth. (**a**) The distribution of parental ages in each group. The light pink and blue vertical bars represent the mean maternal and paternal ages at childbirth from the year 1970 to the year 2008. (**b**) Correlation between maternal and paternal ages at childbirth. Significant correlation is observed in trisomy rescue/gamete complementation (meiosis 2) (TR/GC (M2)) and Deletion groups, but not in trisomy rescue/gamete complementation (meiosis 1) (TR/GC (M1)) group because of relatively advanced maternal age. (**c**) Relative frequency of each group in 75 patients born until the year 2002 (n=60, 3, 5, 1, 0 and 6 for Deletion, TR/GC (M1), TR/GC (M2), monosomy rescue/post-fertilization mitotic error (MR/PE), epimutation and non-deletions groups, respectively) and in 42 patients born since the year 2003 (n=24, 12, 2, 2, 2 and 0 for Deletion, TR/GC (M1), TR/GC (M2), MR/PE, Epimutation and Non-deletions groups, respectively).

Several points should be made with regard to the present study. First, we classified upd(15)mat primarily on the basis of the results of three pericentromeric microsatellite loci, with the assumption of no recombination between the centromere and the three loci, as have been employed in the previous study.[13] The methods would be basically acceptable, because the three loci reside within a 4 Mb region from the centromere and a recombination is relatively rare in the centromeric regions.[22] However, it remains possible that a cryptic recombination(s) might have occurred in the pericentromeric region.

Second, upd(15)mat may also be caused by maternal age-dependent meiotic sister chromatid pre-division that can lead to aneuploid oocytes, including disomic oocytes specific to TR/GC.[23] In this regard, as such disomic oocytes can have various patterns of isodisomic and heterodisomic regions, it is impossible to discriminate between upd(15)mat through sister chromatid pre-division and that through conventional meiotic non-disjunction by microsatellite analysis. Thus, the patients classified as TR/GC (M1) group may have upd(15)mat due to maternal age-dependent conventional non-disjunction at M1 and maternal age-dependent sister chromatid pre-division, whereas those classified as TR/GC (M2) group may have upd(15)mat due to maternal age-independent conventional non-disjunction at M2 and maternal age-dependent sister chromatid pre-division. However, even if not all the patients classified as TR/GC (M1) group have upd(15)mat due to conventional non-disjunction at M1, it can be concluded that maternal age-dependent factors still have a critical role in the occurrence of upd(15)mat in patients classified as TR/GC (M1) group. In addition, possible mixture of maternal age-dependent and -independent factors in patients classified as TR/GC (M2) group may be relevant to the lack of significant difference in the maternal age between TR/GC (M2) and Deletion groups, and between TR/GC (M2) and TR/GC (M1) groups.

Lastly, whereas fluorescence *in situ* hybridization analysis has been routinely performed at commercial laboratories since the year 1993 in Japan, detailed molecular studies including microsatellite analysis are usually available only in institutional laboratories. Thus, a substantial fraction of patients without deletions may have remained undiagnosed or misdiagnosed, without receiving further studies including micro-

Table 1 Parental ages (year) at childbirth

	Deletion	*TR/GC (M1)*	*TR/GC (M2)*	*MR/PE*	*Epimutation*	*Non-deletion*	*All patients*	*General population*
Maternal age								
Total								
Median	30	37	31	30	38.5	36	32	27.5–30.9
Range	19–42	35–45	29–42	29–37	38–39	30–48	19–48	
Number	84	15	7	3	2	6	117	
Until 2002								
Median	29	37	32	29	—	36	30[a]	
Range	19–42	35–37	29–42	—	—	30–48	19–48	
Number	60	3	5	1	0	6	75	
Since 2003								
Median	32.5	38.5	35.5	33.5	38.5	—	35[a]	
Range	23–39	35–45	30–41	30–37	38–39	—	23–45	
Number	24	15	2	2	2	0	42	
Paternal age								
Total								
Median	32.5	40	35.5	31	41.5	36	33	30.6–33.0
Range	21–47	29–53	28–44	28–37	38–45	33–39	21–53	
Number	82[b]	15	6[c]	3	2	6	114[b,c]	
Until 2002								
Median	32.5	43	35.5	28	—	36	33	
Range	21–47	33–43	28–44	—	—	33–39	21–47	
Number	58[b]	3	4[c]	1	0	6	72[b,c]	
Since 2003								
Median	32.5	39.5	35.5	34	41.5	—	34.5	
Range	22–40	29–53	30–41	31–37	38–45	—	22–53	
Number	24	12	2	2	2	0	42	

Abbreviations: GC, gamete complementation; M1, meiosis 1; M2, meiosis 2; MR,monosomy rescue; PE, post-fertilization mitotic error; TR, trisomy rescue.
The data of the general population indicate the range of the mean parental ages at childbirth from the year 1970 to 2008.
[a]*P*-value=0.00017.
[b]Paternal age was not found in two old patients who had left our follow-up and whose hospital records had been discarded.
[c]Paternal age was not identified in one patient who was born after artificial insemination by donor.

satellite analysis at appropriate institutions. In this regard, considering the opportunity to receive detailed molecular studies, it is possible that upd(15)mat is overlooked more frequently in aged patients than in young patients. If so, this may be relevant to the significant difference in the relative frequency of TR/GC (M1) group between the two time periods ('since the year 2003' versus 'until the year 2002').

In summary, the results imply that the advanced maternal age at childbirth is a predisposing factor for the development of upd(15)mat because of increased M1 errors. This notion is applicable to maternal upd in general, as well as to trisomies. However, there are several caveats as discussed in the above, and the number of patients, especially those classified as TR/GC (M2) group, is small. Thus, further careful studies using a large number of patients are necessary in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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