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Research Letter

No Mutation in RAS-MAPK Pathway Genes in 30 Patients With Kabuki Syndrome

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To the Editor:

Kabuki syndrome (KS, OMIM 147920) also known as Niikawa–Kuroki syndrome, is a multiple congenital anomaly/mental retardation (MCA/MR) syndrome characterized by a distinctive facial appearance resembling the Kabuki actor's make-up, skeletal abnormalities, joint hypermobility, dermatoglyphic abnormalities, postnatal growth retardation, occasional visceral anomalies and immune abnormalities. The cause of KS remains unknown, even though a large number of patients from a variety of ethnic groups have been reported since 1981 [Wessels et al., 2002]. The prevalence was estimated to be 1/32,000 in Japan [Niikawa et al., 1988] and 1/86,000 in Australia and New Zealand [White et al., 2004]. Although most cases were sporadic, at least 14 familial cases have been reported. The equal male-to-female ratio of patients, and parent-child transmissions in some familial cases suggest an autosomal dominant of inheritance [Niikawa et al., 1988; Matsumoto and Niikawa, 2003]. At least six autosomal structural abnormalities have been reported in patients with KS or KS-like features [Matsumoto and Niikawa, 2003], but no concordant specific cytogenetic lesion have been found.

It is less likely that a large-scale genomic rearrangement is the common cause of KS, because array-based comparative genome hybridization (array-CGH) did not detect any abnormality in previously reported 8p22–p23.1, and in whole genome with 1.2/1.5 megabase resolution [Hoffman et al., 2005; Schoumans et al., 2005; Miyake et al., 2006]. Although it was reported that a patient with KS had a de novo 250 kilobase microdeletion of the exon 5 region in *C20orf133* gene, 19 additional patients with KS did not have any mutations or copy number changes of the gene [Maas et al., 2007].

Recently, germline mutations in some genes involving the RAS-mitogen-activated protein kinase (RAS-MAPK) signal transduction pathway have been shown to be causes of multiple congenital anomaly

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syndromes; i.e. Noonan syndrome due to *PTPN11* [Tartaglia et al., 2001], *KRAS* [Schubbert et al., 2006], *SOS1* [Roberts et al., 2007; Tartaglia et al., 2007] and *RAF1* [Pandit et al., 2007; Razzaque et al., 2007]; Costello syndrome due to *HRAS* [Aoki et al., 2005]; cardio-fadio-cutaneous (CFC) syndrome due to *KRAS* and *BRAF* [Niihori et al., 2006], and *BRAF*, *MEK1* and *MEK2* [Rodriguez-Viciana et al., 2006]. These achievements encouraged us that a disturbance of certain transcriptional factors or oncogenes related to the pathway may cause KS as an MCA syndrome due to their variety functions. To test the hypothesis, we screened in 30 patients with KS (14 females and 16 males) for mutations in 16 genes involving the RAS-MAPK pathway.

Experimental procedures were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University. Genomic DNA was extracted from their peripheral blood leukocytes or from EBV-transformed lymphoblastoid cells established after obtaining informed consent from all subjects and/or their parents. The selected genes for mutation analysis were following: *PTPN11*, *SOS1*, *GRB2*, *HRAS*, *KRAS*, *ERAS*, *NRAS*, *ARAF*, *BRAF*, *RAF1* (*CRAF*), *MEK1*, *MEK2*, *RASA1*, *RASA2*, *RASA3* and *RASA4*. These accession numbers are respectively NM_002834, NM_005633, NM_203506, NM_005343 and NM_176795 (HRAS1 and H-RAS), NM_033360 and NM_004985 (KRAS isoform a and b), NM_181532, NM_002524, NM_001654, NM_004333, NM_002880, NM_002755, NM_030662, NM_002890 and NM_022650 (RASA1 isoform 1 and 2), NM_006506, NM_007368, and NM_006989. Genomic sequences were retrieved from the UCSC genome browser (assembly: March 2006; http://genome.ucsc.edu/). The entire coding exons and splice junctions of the genes were directly sequenced using BigDye sequencing kit ver.3.1 (Applied Biosystems, Foster City, CA) and an automated sequencer Model 3100 (Applied Biosystems). PCR conditions and primer sequences are available in the online supplement (see the online supplementary file at http://www.interscience.wiley.com/jpages/1552-4825/suppmat/index.html).

In 227 coding exons of the 16 genes analyzed among 30 patients with KS, we found 27 base substitutions (Table I). Nine base substitutions lead to nonsynonymous amino acid changes. Two missense mutations in *RASA1* gene in two patients with KS were detected, which were not found in 86 phenotypically normal Japanese controls, but each mutation was detected in only one patient. Unfortunately DNA samples from their parents were not available. TaqMan real-time quantitative PCR assay for the *RASA1* gene in 30 patients did not show any copy number changes (data not shown). Mutations in *RASA1*, most of them results in premature termination codon, are known as a cause

TABLE I. Nucleotide Changes Found in Genes Analyzed in the RAS-MAPK Pathway in 30 Patients With Kabuki Syndrome

		Change of				
	Gene	Nucleotide	Amino acid	Number of patient(s)	SNP ID	AF
Non-synonymous	*RASA1*	c.73G>A	A25T	1	NR	0.000
		c.473C>G	S158C	1	NR	0.000
	RASA4	c.379T>C	W127R	7	NR	0.800
		c.381G>C	W127R	9	NR	0.900
		c.401G>A	R134Q	12	NR	0.806
		c.674T>C	V225A	6	NR	0.051
		c.728G>A	R243Q	7	NR	0.063
		c.1054A>G	M352V	8	rs746316	
		c.1103T>C	L368P	4	rs886343	
Synonymous	*SOS1*	c.195A>C	R65R	1	NR	0.045
		c.1230G>A	Q410Q	1	NR	0.000
	BRAF	c.1689C>G	G563G	1	NR	0.000
	RASA4	c.330C>T	V110V	22	NR	0.847
		c.336C>T	P112P	25	NR	0.847
	KRAS	c.519T>C	D173D	10	rs1137282	
	HRAS	c.81T>C	H27H	9	rs2227994	
	BRAF	c.1929A>G	G643G	4	rs1042179	
	RAF1	c.1629A>G	T543T	1	rs5746244	
	MEK2	c.453C>T	D151D	5	rs17851657	
		c.660C>A	I220I	20	rs11539507	
	RASA1	c.3067T>C	L1023L	3	rs3747704	
	RASA2	c.2028T>C	N672N	16	rs295322	
		c.2172G>A	L720L	18	rs295323	
	RASA3	c.1326T>C	T442T	12	rs2274717	
	RASA4	c.339T>C	D113D	4	rs11547191	
		c.1512C>T	A504A	3	rs739735	
		c.2253C>T	G751G	7	rs3099742	

AF, allele frequency among 82–89 phenotypically normal Japanese controls; NR, not registered in NCBI database.

of capillary malformation-arteriovenous malformation (CM-AVM) [Boon et al., 2005], but the manifestations of CM-AVM are so different from that of KS that it is less likely responsible for KS. Seven base substitutions of the nonsynonymous amino acid changes were confirmed as single nucleotide polymorphisms (SNPs) listed in the database of SNP or found in 82–89 normal Japanese controls. Synonymous changes were found as 18 base substitutions including 13 SNPs registered, 3 base changes found in the controls, and 2 base changes not found in the controls. Consequently, no pathogenic mutations were detected in any of the genes analyzed in RAS-MAPK pathway and in any of the patients with KS examined. Although our results do not totally rule out the role of RAS-MAPK pathway in KS, it is less likely that the genes in this pathway are associated with KS.

Since there has been no clue to identify the putative gene causative for KS, candidate gene approaches would be valuable in a view of "inborn errors of development". In this connection, transforming growth factor β receptors (*TGFBR*) 1 and *TGFBR2*, relating *IRF6* gene which is causative for van der Woude syndrome (VWS), was added to candidate genes because of specific lower lip pits with VWS and with KS in common, but the two genes did not show any mutations and copy number changes among 14 patients with KS [Bottani et al., 2006]. We may need to perform an intensive PCR-based mutation screening in the genes involving the TGF-β intracellular signaling pathways.

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Increased level of cell-free placental mRNA in a subgroup of placenta previa that needs hysterectomy

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Objective The purpose of this study was to investigate whether cell-free placental mRNA levels have the potential to predict a placenta previa resulting in hysterectomy.

Methods Twenty-eight singleton pregnant women with placenta previa were classified into the following four groups: 16 women with placenta located at a posterior part of the uterine wall (Group A); 4 each with placenta located at the anterior wall without (Group B) or with (Group C) previous cesarean section; and the other 4 with a history of previous cesarean section and who had the placenta located at an anterior part of uterine wall and underwent a cesarean hysterectomy (Group D). The maternal plasma concentrations of the cell-free placental lactogen (PL) mRNA measured by real-time reverse-transcription polymerase chain reaction (PCR) were converted into multiples of the median (MoM).

Results The MoM (range) values of cell-free PL mRNA in the control group and Groups A to D were 1.00 (0.39–2.35), 2.04 (0.91–3.93), 2.58 (1.90–3.90), 3.50 (1.20–4.30), and 6.28 (5.24–7.63), respectively. The concentration of cell-free PL mRNA was significantly higher in Group D than in Group A, B, or C (Mann–Whitney's U-test, $P < 0.05$).

Conclusion The cell-free PL mRNA concentration in maternal plasma has the potential to predict a subgroup of placenta accreta resulting in hysterectomy. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: cell-free placental mRNA; ultrasonography; placenta previa; placenta accreta; cesarean hysterectomy

INTRODUCTION

Placenta accreta is defined as an abnormal adherence of the placenta to the uterine wall with partial or complete absence of the deciduas basalis. The conditions in which the trophoblastic tissue invades within the myometrium, and through the serosal surface of the uterus, are called placenta increta and placenta percreta, respectively.

Placenta previa, when located at the anterior uterine wall and having history of a cesarean section, has a high risk of placenta accreta (Finberg and Williams, 1992). However, placenta accreta is usually found at the time of delivery and its final diagnosis is confirmed by pathological examination after the surgery. Therefore, the obstetrician's main concern is whether such a complication can be predicted prenatally, because prenatal diagnosis allows a planned approach under a more controlled condition with possible treatment. Ultrasonography and magnetic resonance imaging (MRI) play roles in predicting the invasiveness of placenta previa (Comstock, 2005), and an elevated level of α-fetoprotein in maternal serum has been suggested to be a biological marker of placenta accreta (Zelop *et al.*, 1992; Kupferminc *et al.*, 1993). Nevertheless, diagnostic accuracy of these predictive tests is still unsatisfactory.

Circulating cell-free placental mRNA can stably be isolated and quantified in maternal plasma (Ng *et al.*, 2003; Tjoa *et al.*, 2006; Lo and Chiu, 2007; Maron and Bianchi, 2007). As recent advances in molecular studies have shown that the main source of circulating fetal RNA was from the placenta (Ng *et al.*, 2003), potential clinical applications of several markers for prenatal monitoring have been proposed. The quantitative aberrations of selected placental mRNA species have been reported in various pregnancy-associated disorders. They included corticotropin releasing hormone (CRH), placenta specific-1 (PLAC1), selectin-P mRNA in preeclampsia (Ng *et al.*, 2003), β-hCG (CGB) mRNA in gestational trophoblastic disease (Masuzaki *et al.*, 2005a), and placental lactogen (PL) in twin–twin transfusion syndrome (Miura *et al.*, 2007). Our previous study has demonstrated that the levels of circulating cell-free placental mRNA for PL and CGB can monitor the effect of methotrexate (MTX) treatment in a woman with placenta percreta with a residual placental mass at the internal os, for which she was subsequently treated with MTX (Masuzaki *et al.*, 2005b). Thus, cell-free placental mRNA in the maternal plasma has become a molecular marker for noninvasive evaluations of placental invasion (Mazouni *et al.*, 2007). Here we report

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that the maternal plasma concentration of such placental mRNA has the potential as a predictive marker of placenta previa-associated placenta accreta, which will result in hysterectomy.

MATERIALS AND METHODS

Between April 2002 and October 2007, we had 61 singleton pregnant women who had low-lying placentae at 20 weeks as study participants who visited Nagasaki University Hospital for management of their placenta previa. An inclusion criterion in this study was pregnant women who had a placenta previa at 29–32 weeks of gestation, without any other complications, that may influence the levels of cell-free placental mRNA in maternal plasma, e.g. preeclampsia, preterm labor, or intrauterine growth restriction. Patients who had a tocolytic agent or uterine bleeding before or at blood sampling were also excluded from this study, because theses factors might also affect the concentration of cell-free mRNA in the maternal plasma. Two out of 33 cases that met the above exclusion criteria underwent cesarean hysterectomy. One case had a tocolytic agent to suppress uterine contraction and uterine bleeding during blood sampling, and the other case had preeclampsia and a tocolytic agent to suppress uterine contraction. Both cases, in which the placenta was located at the anterior of uterine wall with a previous cesarean section, were diagnosed as placenta previa-increta by pathological examination of placenta/uterus. Twenty-eight out of 61 pregnant women with placenta previa were included finally in this study. All participants gave written informed consent, and the study protocol was approved by IRB for Ethical, Legal, and Social Issues of Nagasaki University. They were classified into four groups (Groups A–D), according to their ultrasonographic findings, history of delivery, and the presence or absence of hysterectomy (Table 1); 16 women whose placenta was located at the posterior part of the uterine wall had no history of previous cesarean section, and their uterus were conserved because a separation of their placenta and control of uterine bleeding were possible (Group A); 4 women each had the placenta located at an anterior part of the wall without (Group B) and with (Group C) previous cesarean sections, and their uterus were conserved because a separation of their placenta and control of uterine bleeding were possible; and the last 4 had a history of previous cesarean section, and had the placenta located at an anterior part of uterine wall and received a cesarean hysterectomy because of placenta previa-increta or -percreta and uncontrolled uterine bleeding (Group D). The prenatal prediction of placenta accreta in Groups B, C, and D was estimated by ultrasonographic findings, which were suggestive of placenta accreta, including (1) irregularly shaped placental lacunae within the placenta, (2) thinning of the myometrium overlying the placenta, (3) loss of retroplacental clear space, and (4) increased vascularity of the uterine serosa/bladder interface. As ultrasonographic assessment for the placenta located at the posterior wall of the uterus was not adequate (Comstock, 2005), the cases in Group A were excluded from the ultrasonographic examination. The same surgeon decided to perform a cesarean hysterectomy when bladder invasion of placenta was visible or separation of placenta was impossible and massive uterine bleeding was not controlled during the cesarean section. Pathological examination of placenta/uterus was performed in cases of Group D, but not in cases of Groups A–C.

To get the samples from the cases of placenta previa that did not move away from the cervix by the placental migration, blood samples (8 mL) were collected with EDTA from the women when they visited our hospital to undergo maternal/fetal health check up at 29–32 weeks of gestation. The plasma samples were frozen at -80 °C for an average of 3 months (ranging from 1 to 12 months). For performing quantitative analysis, 1.6 mL of each plasma sample was centrifuged at 11 500 *g* for 10 min to remove any residual cells, and cell-free mRNAs were extracted from 1.2 mL of the supernatant of their plasma as described previously (Ng *et al.*, 2003). None of them had any complications such as preterm labor and uterine bleeding at the time of blood sampling. Sixty healthy singleton pregnant women without any obstetric complications were included as the control group, and EDTA blood sampling and cell-free mRNA extraction were performed at 29–32 weeks of gestation. Among the control group, and Groups A, B, C and D, there were no significant differences in maternal age, nulliparous number, and gestational age at the time of sampling (data not shown).

Table 1—The classification of Groups A–D

Group (cases)	Diagnosis	Location of placenta	Previous cesarean section	Separation of placenta	Bleeding during a cesarean section	Cesarean hysterectomy
Group A ($n = 16$)	Placenta previa*	Posterior of uterine wall	−	Possible	Controlled	−
Group B ($n = 4$)	Placenta previa*	Anterior of uterine wall	−	Possible	Controlled	−
Group C ($n = 4$)	Placenta previa*	Anterior of uterine wall	+	Possible	Controlled	−
Group D ($n = 4$)	Placenta previa--increata or -percreta	Anterior of uterine wall	+	Impossible	Uncontrolled	+

The minus symbol indicates a subgroup with the background, while the plus symbol a subgroup without the background.
The asterisk indicates that although a pathological examination of placenta/uterus was impossible in the cases that did not have a cesarean hysterectomy, some cases in Groups A–C might have a possibility of placenta previa-accreta.

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One-step quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) assay was performed using an ABI 7900T Sequence Detector (Perkin-Elmer, Foster City, CA) as described previously (Ng *et al.*, 2003a). PL was selected as placental cell-free mRNA in maternal plasma, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was taken as the total cell-free mRNA in the maternal plasma. Primer sets and TaqMan probes for PL and GAPDH, and single-strand and synthetic oligonucleotides for calibration curve were used as described previously (Ng *et al.*, 2003b). Calibration curves for PL mRNA ranged from 1×10^7 to 1×10^1 copies/mL, and the curve for GAPDH mRNA from 1×10^{10} to 1×10^4 copies/mL. Each sample was analyzed in triplicate with thermal cycling. The interassay coefficient of variations (the ratio between the standard deviation and the mean of repeated measurements) for PL mRNA and GAPDH mRNA concentrations in copies/mL, which were calculated from 18 consecutive runs over the study period, were 12.1% and 4.9% for low titer samples (1×10^2 copies/mL of PL mRNA and 1×10^5 copies/mL of GAPDH mRNA) and 4.3% and 1.8% for high titer samples (1×10^6 copies/mL of PL mRNA and 1×10^9 copies/mL of GAPDH mRNA), respectively. As the cell-free mRNA levels in maternal circulation could be affected by the gestational age at the time of blood drawing, plasma concentrations of cell-free PL mRNA in placental mRNA and of cell-free GAPDH mRNA in total mRNA were measured, and data were converted into multiples of the median (MoM) of 60 control pregnant women adjusted for gestational age as described previously (Purwosunu *et al.*, 2007). Differences among the four groups were evaluated with Mann–Whitney's *U*-test. Significant difference was defined as a *P*-value of 0.05 or less. As the results of quantitative real-time RT-PCR assay were not given to the surgeon, the decision to perform hysterectomy for each of the patients in Group D was never based on the plasma concentrations of cell-free PL mRNA and of cell-free GAPDH mRNA.

RESULTS

Each ultrasonographic finding we investigated was suggestive of placenta accreta, which was detected in Groups B, C, and D (Table 1). One case in Group B, two cases in Group C, and all four cases in Group D had all four ultrasonographic findings examined. Pathological examination confirmed that all four cases in Group D were diagnosed as placenta accreta including three cases of placenta increta and one case of placenta percreta (Figure 1). There was no finding of placental infarction in the cases of cesarean hysterectomy.

The median (minimum to maximum range) MoM values of cell-free PL mRNA in the control group and Groups A to D were 1.00 (0.39–2.35), 2.04 (0.91–3.93), 2.58 (1.90–3.90), 3.50 (1.20–4.30), and 6.28 (5.24–7.63), respectively (Table 2). The concentration of cell-free PL mRNA was significantly higher in Group A–D than in the control group (Mann–Whitney's *U*-test, $P = 0.0001$, 0.0019, 0.0051, and 0.0009 respectively) (Figure 2(a)). The concentration of cell-free PL mRNA was significantly higher in Group D than in Group A, B, or C (Mann–Whitney's *U*-test, $P = 0.0025$, 0.0209, and 0.0209, respectively), while there was no significant difference between Group A, B, or C ($P > 0.05$) (Figure 2(a)). The MoM (range) values of cell-free GAPDH mRNA in the control group and Groups A to D were 1.00 (0.58–1.96), 1.10 (0.59–1.47), 1.09 (0.97–1.54), 1.16 (0.96–1.22), and 1.20 (1.11–1.35), respectively (Figure 2(b)). There was no significant difference of cell-free GAPDH mRNA levels in the maternal plasma among the five groups ($P > 0.05$).

DISCUSSION

The concentration of the cell-free PL mRNA (placental RNA) in maternal plasma was significantly higher in women with placenta previa who had hysterectomy (Group D) than those with placenta previa of other groups. However, the plasma concentration of cell-free GAPDH mRNA (total RNA including maternal and fetal molecules) showed no significant difference

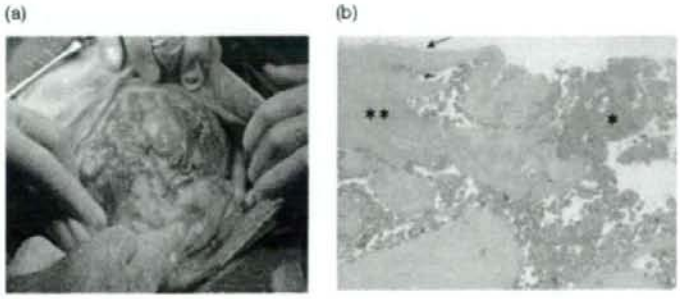

Figure 1—Placenta percreta in a woman of Group D. (a) The bladder wall was involved and a cesarean supravaginal hysterectomy was performed. (b) Pathological finding (hematoxylin–eosin stain, ×40 magnification). Placenta (*) penetrated through the myometrium (**) to the serosa (black arrow)

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Table 2—Ultrasonographic features, histological findings, and cell-free mRNA levels in Groups B, C, and D

Group	Ultrasonographic features				Histological findings of placenta/uterus	Cell-free mRNA levels	
	Irregularly shaped placental lacunae	Thinning of the myometrium overlying the placenta	Loss of the retroplacental clear space	Increased vascularity of the uterine serosa/bladder interface		Cell-free PL mRNA (MoM)	Cell-free GAPDH mRNA (MoM)
B-1	O	O	O	X	Not examined*	2.89	1.06
B-2	X	X	O	X	Not examined*	2.27	1.54
B-3	X	O	X	X	Not examined*	1.90	0.97
B-4	O	O	O	O	Not examined*	3.93	1.12
C-1	O	O	O	X	Not examined*	3.29	1.22
C-2	O	O	X	O	Not examined*	4.30	0.96
C-3	O	O	O	O	Not examined*	1.20	1.19
C-4	O	O	O	O	Not examined*	3.70	1.13
D-1	O	O	O	O	Placenta previa/increta	5.49	1.11
D-2	O	O	O	O	Placenta previa-percreta	7.63	1.35
D-3	O	O	O	O	Placenta previa/increta	7.06	1.28
D-4	O	O	O	O	Placenta previa/increta	5.24	1.12

'O' indicates a case with the feature, while 'X' is a case without the feature.
The asterisk indicates that histological examination of placenta/uterus was not performed in the subgroups without a cesarean hysterectomy.

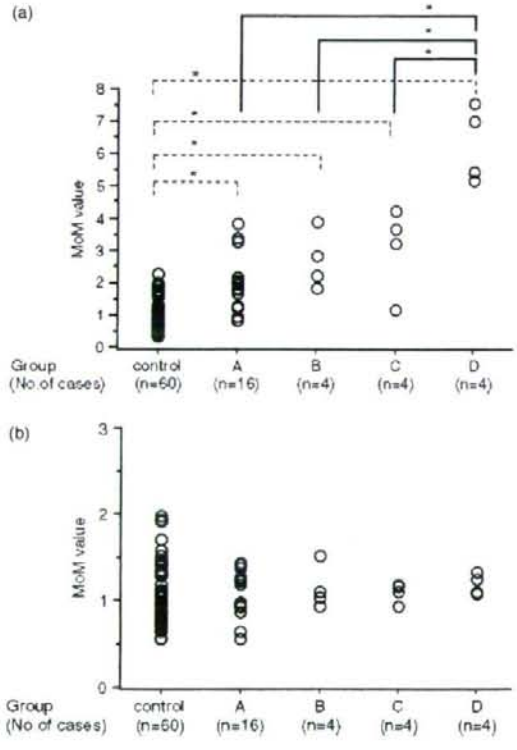

Figure 2—Scatter plots of the cell-free mRNA concentrations in maternal plasma in 28 women with placenta previa and 60 healthy control pregnant women. (a) Scatter plots of the cell-free PL mRNA concentrations in women of control group and Groups A, B, C, and D (open circle). A single asterisk shows significant differences ($P < 0.01$). (b) Scatter plots of the cell-free GAPDH mRNA concentrations in women of the control group and Groups A, B, C, and D (open circle). There was no significant difference among the five groups ($P > 0.005$)

between the groups. Both fetal hematopoietic cells and the placenta can contribute to the pool of cell-free fetal/placental mRNA detected in the maternal circulation (Maron and Bianchi, 2007). Although the usual explanation of the source of circulating DNA is apoptosis, necrosis and apoptosis that were caused by laser ablation of placenta in twin-to-twin transfusion syndrome did not affect circulating mRNA levels post procedure (Tjoa *et al.*, 2006). There was no finding of placenta infarction in the placentas of Group D, which also suggests that necrosis and apoptosis did not affect cell-free placental mRNA levels in maternal plasma. The higher incidence in women in Group D, having either placenta increta or placenta percreta, might be explained by a possible direct connection between the placenta and maternal circulation, because invasion of trophoblasts into the uterine muscle in cases of Group D led to increased concentrations of cell-free placental mRNA in maternal plasma. The concentration of circulating cell-free PL mRNA in the maternal plasma of Groups A–C was about 2–3 times the control level of placenta mRNA, suggesting that the presence of thin and dysfunctional decidua at the lower segment of the uterus might be related to this increase (Sekizawa *et al.*, 2002). Therefore, the difference in concentration between placental mRNA and total mRNA in maternal plasma may be affected by direct uteroplacental transfer of cell-free placental mRNA molecules. The results suggest that cell-free PL mRNA concentration in maternal plasma has the potential to be used as a marker for a group of placenta previa who have hysterectomy, though the sample size was too small to give sufficient strength to the analysis.

Women of Group D whose placenta was located at the anterior wall of the uterus with a history of cesarean sections were diagnosed as placenta previa-increta or -percreta. At the time of attempting placental removal, they had massive hemorrhage, the most common indication for emergent, intra- or postpartum hysterectomy,

with the risk of disseminated intravascular coagulopathy and high mortality. Meanwhile, conservative approaches to placenta previa-percreta or -increta by leaving the affected placenta also bring a significant risk of serious complications, such as unpredictable uterine bleeding and maternal mortality. To reduce these complications, a multidisciplinary team approach and accurate prediction of placenta accreta are desired (Oyelese and Smulian, 2006). The prediction of placenta previa-increta or -percreta prenatally is usually made by ultrasonography, which is readily available in most centers (Finberg and Williams, 1992; Comstock *et al.*, 2004; Comstock, 2005). In this study, the case that had all four ultrasonographic features we examined was a high risk of placenta previa-increta or -percreta that resulted in a hysterectomy. However, as only four out of seven cases that had all four ultrasonographic features belonged to Group D, the diagnostic accuracy of ultrasonography for the placenta previa-increta or -percreta resulting in a hysterectomy was still unsatisfactory. Therefore, molecular analysis of cell-free PL mRNA levels, combined with the imaging results, is expected to give a more accurate prediction of placenta previa-increta or -percreta that results in a hysterectomy, and surgeons can take precautions, e.g., by preparing balloon catheter occlusion, transfusion of blood, and other surgical specialties such as urology and gynecological oncology. Among the groups A, B, and C, there was the possibility that some cases had a placenta accreta or abnormally adherent placenta that was managed without hysterectomy, though their final diagnosis was unknown because pathological examination was impossible. Advent of a noninvasive prenatal diagnostic procedure will undoubtedly be a great advance for the practice. Further studies of cell-free placental mRNA may enable an obstetrician to identify women who are ideal candidates for conservative management. For the prediction of the possibility of cesarean hysterectomy in the cases of posterior placenta previa, the measurement of cell-free PL mRNA concentration in maternal plasma may be superior to ultrasonography.

In conclusion, measurement of the cell-free PL mRNA concentration in maternal plasma has the potential to detect abnormal placentations, such as placenta accretae, that need hysterectomy. As this pilot study was limited by its small sample size, further large-scale studies are necessary to verify our hypothesis. Plasma concentration of cell-free PL mRNA combined with an imaging examination can be used as a noninvasive obstetrical examination for prenatal prediction of placental invasion in pregnant women with placenta previa (Mazouni *et al.*, 2007). This may help reducing perinatal maternal mortality from not only placenta previa but also other placental abnormalities.

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Precision of High-Throughput Single-Nucleotide Polymorphism Genotyping with Fingernail DNA: Comparison with Blood DNA

To the Editor:

Recently, high-throughput single-nucleotide polymorphism (SNP) genotyping arrays have been used for genome-wide association study. Blood samples are commonly used for such analyses because they provide genomic DNA of high molecular weight and in high quantities. Blood sampling is often difficult, however, when patients are located a great distance from the laboratory and the sample requires careful handling for transportation. Instead, buccal swabs or urinary epithelial cells have been used as noninvasive sources of DNA (*1*). Fingernail clippings are also obtained noninvasively and more easily. Such samples need no special attention for transportation and can be preserved dry at room temperature for long periods; however, because fingernail clippings yield fragmented DNA that may be contaminated with certain PCR inhibitors (*2*), this sample type has seemed unsuitable for analyses requiring high-quality genomic DNA. In this study, we compared the precision of Affymetrix GeneChip™ array-based SNP genotyping with fingernail DNA vs blood DNA and demonstrated the usefulness of fingernail DNA in genotyping and genome copy-number analysis.

The study protocol was approved by the Ethics Committee on Human Genome and Gene Analysis, Nagasaki University, and written informed consent was obtained from every volunteer. Ten milliliters of peripheral blood and 10 fingernail clippings were obtained from 5 healthy volunteers. Blood DNA was extracted with the standard phenol-chloroform method. Clipped fingernails were frozen in liquid nitrogen and crushed into fine powder with Multibeads Shocker™ (Yasui Kikai). The nail powder was dissolved in a urea-containing lysis solution (2 mol/L urea, 5 g/L SDS, 10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L EDTA) containing 1 g/L proteinase K and 40 mmol/L dithiothreitol and was incubated overnight at 55 °C. Nail DNA was extracted by the phenol-chloroform method and suspended in 30 μL 1× Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0). Genome-wide SNP genotyping was performed with the GeneChip™ Human Mapping 250K Nsp Array according to the manufacturer's assay manual (Affymetrix). SNP data were analyzed with GTYPE software (Affymetrix). This analysis is based on the dynamic modeling algorithm that is included in GTYPE. Genome copy-number analysis was performed with CNAG software (http://www.genome.umin.jp/) (*3*, *4*).

The human nail plate includes soft and hard types of keratin. Hard keratin constitutes >80% of nail keratin (*5*) and contains large numbers of cysteine moieties linked by

Table 1. SNP call rates and concordance rates on Affymetrix 250K Nsp arrays.

Sample no.	DNA source	Total call rate, %	Concordance rate, %	
			Homozygous	Heterozygous
1	Blood	96.46		
	Nail	92.43	99.79	98.15
2	Blood	96.79		
	Nail	95.90	99.85	99.29
3	Blood	96.28		
	Nail	96.99	99.82	99.56
4	Blood	95.57		
	Nail	95.28	99.75	99.02
5	Blood	96.20		
	Nail	93.21	99.83	97.88
Mean	Blood	96.26		
	Nail	94.76	99.81	98.78

multiple disulfide bonds that make the nail insoluble in lysis buffer. We froze fingernail clippings in liquid nitrogen, mechanically crushed them into as fine a powder as possible, and then treated them with 2 mol/L urea. With this procedure, we were able to lyse the nail proteins almost completely without denaturing the DNA. Consequently, 10 mg (2 pieces) of fingernail clippings yielded approximately 1 μg DNA, an amount ample for the GeneChip system.

We assumed in this study that all SNP calls for the blood samples were correct, and these calls were used as baseline values for comparison with the calls for nail DNA. The mean total call rate for the blood samples was 96.26%, and that of the nail samples was 94.76%. The mean concordance rates for homozygous and heterozygous SNPs were 99.81% and 98.78%, respectively (Table 1). The concordance rates for heterozygous SNPs in nail samples decreased as the total call rates decreased. Therefore, the discrepancy in the call rates between the 2 different sources of DNA is most likely due to some incorrect calls for heterozygous SNPs in the nail DNA. In other words, many cases of lower call rates in nail samples reflect erroneous calls as homozygous SNPs instead of as heterozygous SNPs; however, the call rates and concordance values for nail DNA were sufficient for genome-wide association studies. Moreover, we performed SNP genotyping with DNA from old fingernail clippings that had been preserved for >5 years at room temperature. The total call rate for old nail DNA was 95% or higher, showing that results for nail clippings preserved for a long time were equivalent to those obtained with fresh clippings.

We also used copy-number variation analysis to compare signal homogeneity for blood and nail DNA on the GeneChip. In this study, volunteer no. 5 had a duplication polymorphism in chromosome 8 that was clearly demonstrated in both sources of DNA (data not shown). The results of the present copy-number variation analysis showed that the results obtained with nail samples were equivalent to those for blood DNA with respect to the accuracy of detecting such variation.

In conclusion, our study comparing blood and fingernail DNA with respect to SNP-genotyping accuracy on the 250K Affymetrix GeneChip systems revealed that nail DNA was as useful as blood DNA for both genome-wide association studies and genome copy-number analysis. Our results reinforce the merits of using nail samples, because nails can be stored at room temperature for a long period and need not be processed immediately for DNA extraction.

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ORIGINAL ARTICLE

Genome-wide linkage analysis and mutation analysis of hereditary congenital blepharoptosis in a Japanese family

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Abstract Hereditary congenital ptosis (PTOS) is defined as drooping of the upper eyelid without any other accompanying symptoms and distinguished from syndromic blepharoptosis. Two previous linkage analyses assigned a PTOS locus (PTOS1) to 1p32-p34.1 and another (PTOS2) to Xq24-q27.1. In addition, in a sporadic case with a balanced chromosomal translocation t(1;8) (p34.3;q21.12), the *ZFHX4* (zinc finger homeodomain 4) gene was found to be disrupted at the 8q21.12 breakpoint, but there was no gene at the 1p34.3 breakpoint, suggesting the existence of the third PTOS locus (PTOS1) at 8q21.12. We carried out a genome-wide linkage analysis in a Japanese PTOS family and calculated two-point and multipoint log of odds (LOD) scores with reduced penetrance. Haplotype analysis gave three candidate disease-responsible regions, i.e., 8q21.11-q22.1, 12q24.32-q24.33, and 14q21.1-q23.2. Although the family size is too small to define one of them, 8q21.11-q22.1 is a likely candidate region, because it contains the previously reported translocation breakpoint above. We thus performed mutation, Southern-blot and methylation analyses of *ZFHX4* but could not find any disease-specific change in the family. Nevertheless, our data may support the localization of PTOS1.

Keywords Hereditary · Congenital ptosis · *ZFHX4*

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Introduction

Blepharoptosis is pathological drooping of the upper eyelid. It is classified into myogenic, neurogenic, aponeurotic, and mechanical ptosis according to the primarily affected lesion. Hereditary congenital ptosis (PTOS) distinguished from other syndromic blepharoptosis involves only the upper eyelid. PTOS is genetically heterogeneous, and two modes of inheritance are known: autosomal dominant PTOS1 (MIM %178300) and X-linked PTOS2 (MIM %300245). Autosomal PTOS may be further divided into at least two types. Engle et al. (1997) mapped PTOS1 by linkage analysis to 1p32-p34.1, whereas McMullan et al. (2002) found, by the analysis of a sporadic case of PTOS who had a de novo balanced chromosomal translocation t(1;8)(p34.3;q21.12), that the *ZFHX4* gene at 8q21.12 was disrupted by the translocation but there was no gene at the 1p34.3 breakpoint, suggesting the third locus at 8q21.12. McMullan et al. (2000) mapped PTOS2 by linkage analysis to Xq24-q27.1.

We recently encountered a Japanese family with PTOS in which there are nine affected members in five successive generations. Here we report on a linkage analysis and mutation analysis of a candidate gene, *ZFHX4*.

Materials and methods

Family and patients

A Japanese family consisted of at least nine members affected with PTOS in five generations (Fig. 1). The disease in the family was found in both genders and transmitted (male to male transmissions twice) directly through successive generations, indicating that it is autosomal dominant PTOS. A total of 18 members including five affected and 12 nonaffected members and one of their spouses participated. The proposita (V-5, Figs. 1, 2) suffered from congenital ptosis on her right eye and visited Department of Plastic and Reconstructive Surgery, Nagasaki University Hospital, at age 15 years. She had severe ptosis (drooping >4 mm, levator function = 5 mm) without any other ophthalmic disorders and appeared to have overaction of the frontalis muscle. She had undergone a levator muscle shortening surgery for her ptosis. Three patients (III-13, IV-2, and IV-12) suffered from unilateral congenital ptosis, and all had undergone a repair surgery. The other patient (III-5) with bilateral ptosis has received no surgical treatment. None of the five patients had any associated ophthalmic disorders. All were examined by one or two well-trained plastic surgeons. The study protocol was approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University.

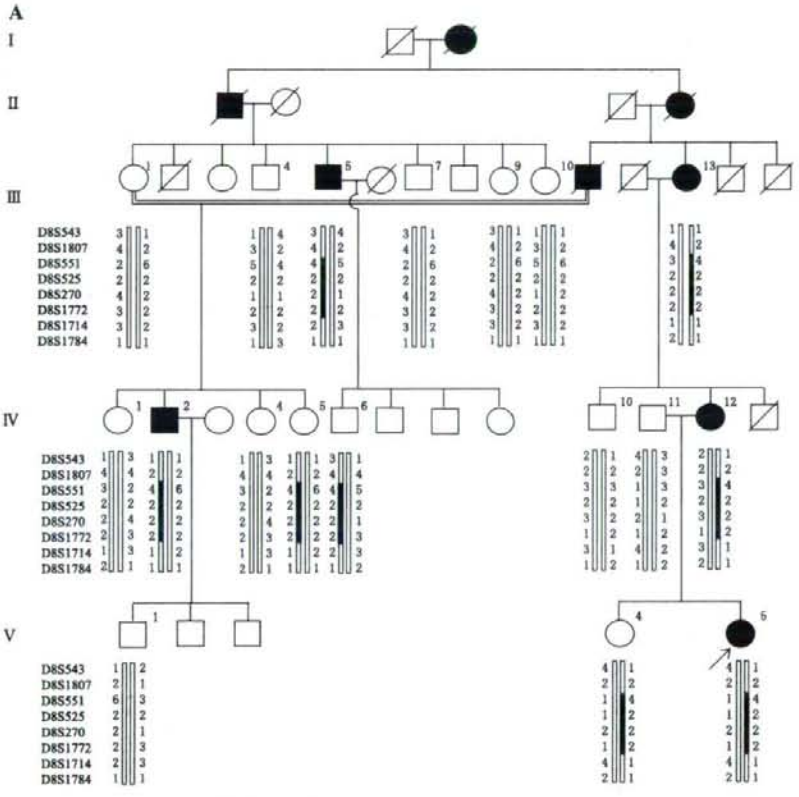

Fig. 1 Family tree with haplotypes at **a** 8q21.11-q22.2, **b** 12q24.32-qter, and **c** 14q21.1-q23.2 regions. *Closed, open and slash symbols* indicate affected, unaffected, and deceased individuals, respectively. *Double horizontal line* depicts consanguineous marriage, and *short bar* above individual symbols indicates individuals examined clinically. *Thick columns* depict disease-associated haplotypes

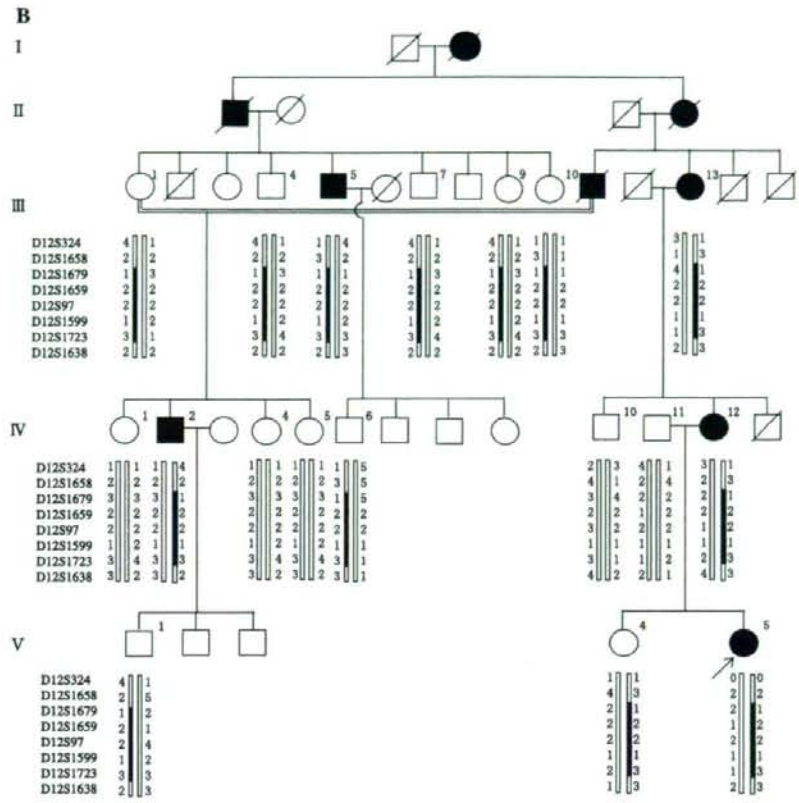

Fig. 1 continued

Linkage and haplotype analyses

After obtaining written informed consent from each participant, DNA was extracted by conventional method from their whole blood, or using ISOHAIR™ (Nippon Gene, Tokyo, Japan) from their fingernail clippings, concentrated, and then purified by phenol–chloroform method (Matsuzawa et al. 2006) For whole-genome scanning, we used the ABI Prism Linkage Mapping Set-MD10 (AppliedBiosystems, Foster City, CA, USA) that consists of 386 microsatellite markers from whole chromosomes with average distance of about 10 cM. Polymerase chain reaction (PCR) was performed in a 10-μl mixture containing 5 ng genomic DNA/0.25 U ExTaq DNA polymerase HS-version (TAKARA Bio Inc., Kyoto, Japan)/200 μM dNTP/0.3 μM primer/1× PCR buffer on the Dual 384-well GeneAmp PCR System 9700 Thermal Cycler (AppliedBiosystems). The PCR condition was composed of initial denaturation at 94°C for 3 min, followed by 37 (blood samples) or 44 (nail samples) cycles of amplification at 94°C, 30 s/55°C, 30 s/72°C, 30 s, and final extension at 72°C for 7 min. PCR products were analyzed on an Autosequencer Model 3100. Genotyping was carried out using GeneScan and Genotyper software (AppliedBiosystems).

Two-point log of odds (LOD) score was calculated using MLINK program (included in FASTLINK software version 4.0P) (Lathrop et al. 1984), and multipoint LOD score and nonparametric LOD score were calculated using Genehunter software (Kruglyal et al. 1996). To pick up all possibly linked loci, calculation was based on an assumption that the ptosis in the family is an autosomal dominant trait with 90% penetrance ($P = 0.9$) and on the allele frequency of $1/N$, where the number of alleles is N. Haplotypes around loci with positive LOD scores were constructed with four or five microsatellite markers that were set up at intervals of 2–3 cM. Information of these microsatellite markers was referred to the National Center for Biotechnology Information (NCBI) database (Map Viewer: Marshfield and/or decode map).

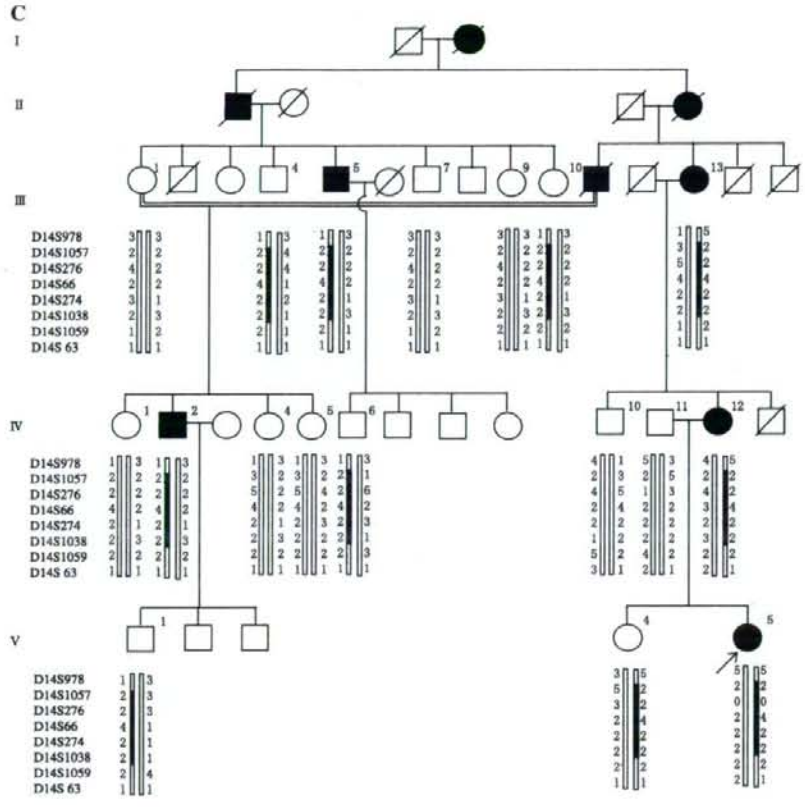

Fig. 1 continued

Mutation analysis

ZFHX4, reported to have been disrupted by a translocation in a patient with PTOS (McMullan et al. 2002), is located at 8q21.1. As its chromosomal localization is one of candidate regions we identified in the study reported here, we performed direct sequencing of all exons of *ZFHX4* using DNA from one affected member (III-5) and one unaffected member (IV-1) in the family. The genomic sequence of *ZFHX4* was retrieved from the University of California, Santa Cruz (UCSC) Genome Browser Home (http://genome.ucsc.edu/) and from the Ensemble Genome Browser (http://ensembl.org/index.html). Primers were designed from sequences of each exon and those around the respective intron. PCR was performed in a 15-μl reaction mixture containing 5 ng DNA/0.4 U ExTaq DNA polymerase/200 μM dNTP/0.67 μM each primer/1× PCR buffer on DNA Thermal Cycler Model 9700 (AppliedBiosystems) with a condition of initial denaturation at 94°C for 3 min, amplification for 37 cycles at 94°C, 30 s/60°C, 30 s/72°C, 30–60 s, and final extension at 72°C for 7 min. PCR products were subjected to cleaning up using Exonuclease I (Epicentre, WI, USA) and shrimp alkaline phosphatase (AmershamBioscience, NJ, USA) for direct sequence reaction. Direct sequencing was carried out using BigDye-terminator sequencing reagent version-3.1 (AppliedBiosystems) on an Autosequencer Model 3100, and sequences were aligned with ATGC software (GENETYX Corp., Tokyo, Japan).

Southern-blot analysis

Genomic DNA from a patient (III-5) as a representative from the family was digested with three different enzymes (*Bam*HI, *Eco*RI, *Hin*dIII) and electrophoresed on 0.8% agarose gel in 0.5× TBE buffer containing 0.25 μg/ml ethidium bromide at 35–40 V for 6–10 h. DNA was then

denatured with 0.4 N NaOH for 15–20 min and capillary-transferred overnight onto nylon membrane (HybondN+, AmershamPharmacia Biotech, Buckinghamshire, UK) using alkaline transfer buffer, and the membrane was washed twice with 2 x SSC. DNA fixed to the membrane by UV crosslinking was hybridized to fluorescein-labeled probes using Gene Images Random Prime Labeling Kit (AmershamBiosciences). Hybridized signal was detected using Gene Image CDP-Star Detection Kit (Amersham-Biosciences). Genomic DNA from phenotypically normal individuals were used as controls.

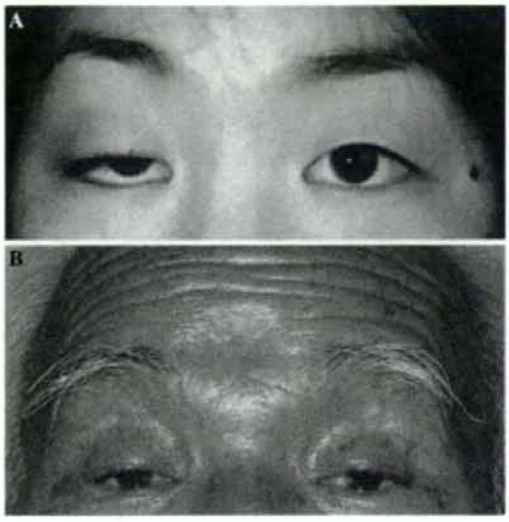

Fig. 2 Blepharopotosis of two affected members (V-5 and III-5). The proband (V-5) has unilateral blepharoptosis (**a**), and III-5 has bilateral ptosis (**b**). Both patients have overaction of the frontalis muscle

Methylation analysis of *ZFHX4*

DNA (1 μg) from patients and a control individual was modified with sodium bisulfite using CpGenome DNA modification Kit (CHEMICON, CA, USA). PCR primers specific for methylated or unmethylated DNA (Herman et al. 1996) were designed from sequences of two CpG islands of *ZFHX4* (Table 1), at which one island is located 3 kb upstream and the other 0.4 kb downstream of exon 1, respectively (Hemmi et al. 2006). Methylation-specific PCR was performed in a 20-μl reaction mixture containing 50 ng bisulfite-modified DNA/0.5 U AmpliTaq Gold (AppliedBiosystems)/200 μM dNTP/0.5 μM each primer/ 1× PCR buffer with a condition consisting of initial denaturation at 95°C for 10 min, 37 cycles of amiplification at 94°C, 30 s/60°C, 30 s/72°C, 30 s, and final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on 2.5% agarose gel. Because aberrant signals on Southern blot were observed, the bisulfite-modified DNA was also amplified with specific primers for the *Eco*RI site around exon12 of *ZFHX4*. PCR products were cloned using TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA), and then cloned DNA was sequenced (Clark et al. 1994).

Results

Linkage and haplotype analyses

As the number of affected members in this family was small and penetrance might be considerably low, we set a cutoff LOD score ≥ 0.4 in all calculations. The two-point and multipoint analyses gave nine markers (D1S255, D1S484, D5S630, D7S669, D9S1776, D11S4046, D19S221, D19S226, and D14S276) that show such scores,

Table 1 Polymerase chain reaction (PCR) primers for methylation-specific PCR and bisulfite sequencing

Primer	Sequences 5′→3′	Anneal temperature	Products size (bp)
CpG1-MF	GTTTAGTCGTTCGGATCGCGTTCGT	60	182
CpG1-MR	AACTTAACCTCGAAACGCGCCAACG		
CpG1-UF	GTTAGGTTTAGTTGTTTGGATTGTGTTTGT	60	192
CpG1 UR	TCCAAAACTTAACCTCAAAACACACCAACA		
CpG2-MF	GGGTTTTGTTTTTTTCGCGAGTTTC	60	139
CpG2-MR	TACGAAAAACCAATCATCCCAATCG		
CpG2-UF	TTTTTGGGTTTTGTTTTTTTTGTGAGTTTT	60	149
CpG2-UR	ATAAATACAAAAAACCAATCATCCCAATCA		
Ex12-upF	GGAAAGTAAAGAAGTTGTTTTAAAA	56	180
Ex12-upR	ATAACAAAACAATACAACACAAATA		
Ex12-dwF	TGATAATGGTAGAAGGTAAAGTATT	56	200
Ex12-dwR	AACATATAAAACAAAAAAACCTCTA		

M methylated-specific, *U* unmethylated-specific, *F* forward primer, *R* reverse primer, *up* upstream restriction site, *dw* downstream restriction site

and the nonparametric analysis gave four candidate regions (3q22.1, 8q21.11, 12q24.32, and 20p13). The haplotype analysis at these loci revealed that all but 8q21.1, 12q24.3, and 14q22.3 regions (Fig. 1) were excluded, because some patients do not have putative disease-linked haplotypes (data not shown). Thus, we finally left these three regions commonly shared by the patients as candidates. Further refinement was impossible, because any more family samples were not available.

Mutation analysis of the *ZFHX4* gene

The direct sequencing of *ZFHX4* revealed one missense alteration (G12411T or L4137F) in exon 12 in the affected member and some normal control samples (data not shown), indicating that it is actually a single nucleotide polymorphism (SNP). Likewise, another mutation analysis for homologous sequences between the mouse and human in their promotor/enhancer region of *ZFHX4/Zfhx4* revealed no disease-associated mutation in the patient. Southern-blot analysis using a probe for exon 2 or exon 12 of *ZFHX4* detected a respective extra *Eco*RI-fragment in a patient (III-5) (Fig. 3) and an extra band for exon 12 in two other patients (III-13 and IV-12).There were no aberrant signals with other restriction enzymes. However, sequence analysis of these regions did not identify any genomic rearrangements and confirmed no restriction fragment length polymorphism (RFLP) around exon 2 or 12. This led us to try to detect the differential methylation status in the regions between patients and normal controls. However, methylation-specific PCR following a bisulfite treatment revealed no change of the methylation status in the patient's two CpG islands (data not shown).

Discussion

In this study, we performed linkage and haplotype analyses of a Japanese PTOS pedigree. Consequently, three regions, 8q21.11-q22.2, 12q24.32-qter, and 14q21.1-q23.2, were shown to be candidates. The two-point maximum LOD scores were considerably low, being 1.16, 0.47, and 0.72 at *D8S551*, *D12S1659*, and *D14S276* (Table 2), respectively, because some unaffected family members also had disease-linked haplotypes around the markers. It was obvious that the disease in the family shows incomplete penetrance. Although we diagnosed carefully many members of the family at the beginning of this study, we might have overlooked very mildly affected members.

Two regions responsible for autosomal PTOS were reported previously, i.e., 1p34.1-p32 for PTOS1 by linkage analysis (Engle et al. 1997) and 1p34.3/8q21.12 by analysis of a chromosomal translocation (McMullan et al. 2002). Three candidate loci we have detected include the translocation breakpoint 8q21.2 (McMullan et al. 2002) but do not contain 1p34.1-p32 (Engle et al. 1997). Therefore, the disease locus for our family could be associated with PTOS1. However, we failed to identify in our family any point mutation, genomic rearrangement, or methylation aberration involving *ZFHX4* that was disrupted at the 8q21.12 breakpoint of the translocation in a PTOS patient (McMullan et al. 2002).

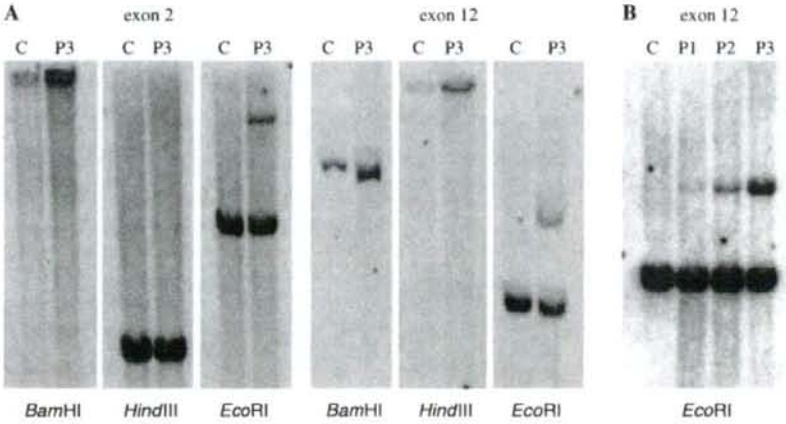

Fig. 3 Southern-blot analysis of patients (*P3*) and a control individual (*C*) after digestion with three different enzymes, using probes for exons 2 and 12 of *ZFHX4*. An extra band is seen in *Eco*RI fragments, including *ZFHX4* from a patient (III-5, P3) (**a**) and three patients (P1, III-13; P2, IV-12; and P3) (**b**)

Table 2 Log of odds (LOD) scores of candidate loci of the third hereditary congenital ptosis locus (PTOS1)

Recombination fraction (θ)							Multipoint
Locus	0.00	0.05	0.10	0.20	0.30	0.40	LOD score
D8S551	0.95	1.12	1.16	1.01	0.68	0.29	1.46
D8S525	0.15	0.12	0.09	0.05	0.02	0.00	1.51
D8S270	−2.53	−1.33	−0.86	−0.36	−0.13	−0.02	1.39
D8S1772	0.33	0.31	0.29	0.23	0.15	0.08	1.22
D12S1679	−0.03	0.31	0.45	0.47	0.34	0.17	0.59
D12S1659	0.15	0.12	0.09	0.05	0.02	0.00	1.39
D12S97	−0.20	−0.11	−0.05	−0.00	0.00	0.00	1.61
D12S1599	−0.78	−0.43	−0.27	−0.11	−0.04	−0.01	1.63
D12S1723	0.15	0.25	0.27	0.23	0.14	0.06	1.79
D14S1057	0.62	0.64	0.61	0.51	0.37	0.20	−1.00
D14S276	0.46	0.65	0.72	0.65	0.44	0.19	1.31
D14S66	−1.65	−1.15	−0.83	−0.45	−0.25	−0.12	2.01
D14S274	−1.16	−0.30	−0.07	0.07	0.07	0.02	1.97
D14S1038	−2.15	−1.64	−1.27	−0.76	−0.43	−0.19	1.94

The human *ZFHX4* gene spans about 180 kb, contains 12 exons, is mapped at 8q13.3-q21.11, and has >90% homology to the mouse *Zfh4* and 52% to the human *ATBF1* (Hemmi et al. 2006). The *Zfh4* gene encodes a member of the zinc finger homeodomain family, which has four homeodomains and 22 zinc fingers (Sakata et al. 2000). *Zfh4* was highly expressed in developing muscle and brains of the mouse, especially in the midbrain and hindbrain (Kostich and Sanes 1995), and was also detected in adult rat oculomotor nucleus, which controls the levator palpebrae superioris (LPS) muscle (Nogami et al. 2005). The oculomotor nucleus exists in the mesencephalon. The oculomotor nerve innervates to LPS and the extraocular muscles, except for the lateral rectus and the superior oblique muscle, and also supplies parasympathetic preganglionic fibers to the ciliary ganglion through which postganglionic nerve controls the ciliary muscle and the sphincter muscle of the pupil. It was reported that *ZFHX4* was upregulated in postmitotic neurons and suggested that *ZFHX4* was influenced on neural differentiation including migration and axon outgrowth (Nogami et al. 2005; Hemmi et al. 2006). All these lines of evidence strongly suggest that *ZFHX4* is the responsible gene for a type of PTOS.

There are two opinions concerning the pathological change in LPS from congenital ptosis. Three reports approved that congenital ptosis is caused by primary dysgenesis or myodystrophy of LPS (Berke and Wadsworth. 1955; Isaksson and Mellgren 1961; Isaksson 1962; Stula 1988). On the other hand, Edmunds et al. (1998) argued against such a mechanism because there was no significantly different histology between LPS from PTOS patients and normal individuals. A light-microscope study of an LPS specimen from the proband in our family showed that the muscle fibers were displaced by fibrous and fatty tissues, and scanty atrophic striated muscles remained. This histological finding is consistent with dysgenesis of LPS. Therefore, if *ZFHX4* is relevant to congenital blepharoptosis, its abnormal expression might cause a failure of neuronal differentiation of the oculomotor nerve, leading to LPS dysgenesis.

In conclusion, we identified three possible regions candidate for PTOS1—8q21.11-q22.2, 12q24.32-qter, and 14q21.1-q23.2—by a whole-genome linkage analysis. These regions do not definitely overlap with a 1p34.1-p32 segment to which PTOS1 was mapped (Engle et al. 1997), and one of the regions we assigned includes a segment containing *ZFHX4* (McMullan et al. 2002). Thus, our data may support the existence of the PTOS1 locus. It remains to be seen whether other PTOS1 families have mutations in *ZFHX4*, another gene, or in a putative *ZFHX4* enhancer located at 8q21.11-q22.2.

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