Unsuccessful Identification of Mutations in Genes Involving the RAS-MAPK Pathway among 30 Patients with Kabuki Syndrome

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Abstract

Kabuki (Niikawa-Kuroki) syndrome (KS) is a multiple congenital anomaly/mental retardation (MCA/MR) syndrome characterized by long palpebral fissures with eversion of the lower eyelids, skeletal anomalies, persistence of fingerpads, short stature, joint laxity, and occasional immune abnormalities. Previous molecular cytogenetic approaches including fluorescence in situ hybridization and whole-genome CGH microarray analysis failed to find copy-number changes in the genome of KS patients. Recently, germline mutations in *PTPN11/KRAS/SOS1/RAF1*, *HRAS*, and *KRAS/BRAF/MEK1/MEK2* were shown to be causes of Noonan syndrome, Costello syndrome and cardio-facio-cutaneous syndrome, respectively. Since KS patients share some phenotypical manifestations with the syndromes above, we hypothesized that KS may be associated with mutations in genes involving the RAS-MAPK pathway. Sixteen genes (*PTPN11, GRB2, SOS1, HRAS, ERAS, NRAS, KRAS, ARAF, BRAF, RAF1, MEK1, MEK2, RASA1, RASA2, RASA3,* and *RASA4*) in the pathway were screened for mutations. DNA from 30 KS patients (14 females and 16 males) was sequenced for entire coding regions and splice junctions of the 16 genes. We identified 29 base substitutions in the genes, including 9 nonsynonymous changes, 18 synonymous changes, one in 5' untranslated region and one at position "-4" in splice acceptor site. But they were almost all confirmed as SNPs listed in the NCBI database or found in 82-89 normal Japanese individuals, while two of them were rare variants with nonsynonymous changes.

INTRODUCTION

Kabuki (make-up) syndrome (KS, OMIM 147920) also known as Niikawa-Kuroki syndrome, is a multiple congenital anomaly/mental retardation (MCA/MR) syndrome characterized by a peculiar facial appearance, 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. 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. Studies with microarray comparative genomic hybridization (CGH) on karyotypically normal patients did not find any pathological genome-copy changes [Hoffman et al., 2005; Schoumans et al., 2005; Miyake et al., 2006]. Disrupted *C20orf133* gene with a patient with KS was reported, but the mutations or copy number changes were not detected in other 19 patients with KS [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 syndromes; i.e. *PTPN11, KRAS, SOS1* and *RAF1* in Noonan syndrome [Tartaglia et al., 2001; Schubbert et al., 2006; Roberts et al., 2006; Tartaglia et al., 2006, Pandit et al., 2007; Razzaque et al., 2007], *HRAS* in Costello syndrome [Aoki et al., 2005], and *KRAS, BRAF, MEK1* and *MEK2* in cardio-facio-cutaneus (CFC) syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Since KS patients share some clinical manifestations with Noonan/Costello/CFC syndromes, we hypothesized that KS may be associated with mutations in a gene involving the RAS-MAPK pathway. To test the hypothesis, we screened in 30 KS patients for mutations in 16 genes involving the RAS-MAPK pathway.

MATERIALS AND METHODS

Subjects

The subjects involved in this study consisted of 30 KS patients (14 females, 16 males). 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. Two phenotypically and karyotypically normal individuals were also subjected to the study as normal controls. Experimental procedures were

approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis in Nagasaki University.

The 16 genes selected for mutation analysis were the following: *NRAS* (located to 1p13.2), *SOS1* (2p22.1), *RASA2* (3p23), *RAF1* (3p25), *RASA1* (5q13.3), *RASA4* (7q22.1), *BRAF* (7q34), *HRAS* (11p15.5), *KRAS* (12p12.1), *PTPN11* (12q24.13), *RASA3* (13q34), *MEK1* (15q22.31), *GRB2* (17q25.1), *MEK2* (19p11.3), *ERAS* (Xp11.23) and *ARAF* (Xp11.23). Genomic sequences were retrieved from the UCSC genome browser (assembly: Mar, 2006; http://genome.ucsc.edu/).

Mutation Search

We sequenced the entire coding region and splice junctions of the genes in the 30 patients and two controls. Primer sequences are available on request. PCR was carried out in a final volume of 10 μl, containing 10 ng of genomic DNA, 0.5 μM each primer, 200 μM dNTP, 0.05 Units TaKaRa ExTaq HS version (TaKaRa, Kyoto, Japan) and 1x PCR buffer with conditions of initial incubation at 94 °C for 2 min followed by cycles of denaturation at 94 °C for 30 sec, annealing at 56-64 °C for 30 sec, extension at 72 °C for 30-45 sec, and final extension at 72 °C for 5 min. Amplification of the first exon of *MEK1* required use of special buffer, containing DMSO, $MgCl_2$, NH_4SO_4, and β-mercaptoethanol. To amplify the first exon of *BRAF*, we performed nested PCR with the use of above-mentioned special buffer. A 100-fold diluted first-round PCR product was served as a template in the second-round PCR.

Amplification of the first exon of *RASA2* and the first exon of *MEK2* required DNA polymerase special for high GC content sequence (AccuPrime^{TM}, Invitrogen, Carlsbad, CA). PCR products were cleaned up with ExoSAP-IT (Amersham Biosciences, NJ) following the instruction manual, and sequenced directly using BigDye sequencing kit ver.3.1 (Applied Biosystems). Sequenced samples were purified with SephadexG-50 (Amersham Biosciences) and run on an automated sequencer Model 3100 (Applied Biosystems). Resultant electropherograms were analyzed by DNA Sequencing Analysis software version 3.7 (PE Applied Biosystems), and were aligned using ATGC v3.0 (Software Development, Tokyo, Japan) and inspected visually to find DNA alterations.

RESULTS AND DISCUSSION

In the 16 genes analyzed in 30 patients with KS, we found 29 base substitutions (Table I). Nine base substitutions in the coding regions lead to nonsynonymous amino acid changes. Two of them were confirmed as SNPs listed in the NCBI database, and five of them were found in 85 or 88 normal Japanese individuals. Two of them were not found in the normal controls, but each mutation was detected in only one patient. Eighteen base substitutions in the coding regions lead to synonymous amino acid changes. Thirteen of them were found in dbSNP, and three of them were found in 85 or 89 normal Japanese individuals, while two of them were found in only one patient and registered as SNPs. One is base substitution in the

5'untranslated region and another is around the splice donor site.

It is less likely that a large scale genomic rearrangement is the common cause of KS, since 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 Maas et al. [2007] reported a *de novo* deletion of the exon 5 region in *C20orf133* gene with a patient with KS, they failed to detect mutations or copy number changes of the gene in 19 additional patients with KS. The deleted region might be a rare copy-number variation because the region was in common with a normal loss [Pinto et al., 2007] registered in Database of Genomic Variants (http://projects.tcag.ca/variation/).

Recently, germline mutations in the RAS-MAPK pathway have been shown to a cause of Noonan, Costello, and CFC syndromes [Tartaglia et al., 2001; Aoki et al., 2005; Schubbert et al., 2006; Niihori et al., 2006; 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, and we picked up the genes.

Mutations in *RASA1*, most of them results in premature termination codon, are known as a cause of capillary malformation-arteriovenous malformation (CM-AVM) [Boon LM, et al., 2005]. Although we found two missense mutations in *RASA1* gene in two KS patients, it

is not a definitive evidence to confirm *RASA1* gene is the responsible for KS. We also cannot find copy number change in *RASA1* gene by quantitative PCR (data not shown). In conclusion, no pathogenic mutations were detected in any of the genes analyzed and in any of the patients examined.

KS is a clear clinical entity with a recognizable facial gestalt, it also has a variety of other manifestations, such as vertebral, visceral, ectodermal and connective tissue, and sometimes immunological abnormalities [Matsumoto and Niikawa, 2003; Hoffman et al., 2005]. So many various clinical manifestations make it difficult to speculate rationally a candidate gene. Some KS patients have characteristic lower lip pits [Niikawa et al., 1988; Makita et al., 1999; Matsumoto and Niikawa, 2003] which is a common and specific finding of Van der Woude syndrome associated with haploinsufficiency of the *IRF6* gene [Kondo et al., 2002]. The IRF-6 has an SMIR domain interacting with Smads that act as a component of transcriptional factor complex activated by serine/threonine phosphorylation by transforming growth factor β receptors (TGFBRs) [Shi and Massague, 2003]. It is speculated that TGF-β signaling may regulate IRF-6 as does for IRF-7 [Qing et al., 2004]. These findings suggested that *IRF6* was a candidate gene causative for KS [Armstrong et al., 2005]. Bottani et al. [2006] added transforming growth factor β receptors (*TGFBR*) 1 and *TGFBR2* to candidate genes. They screened for mutations and copy number changes of the two genes among 14 KS patients, but they did not detect any abnormalities in the genes.

It remains to be seen whether mutations are found in KS patients in genes involving the TGF-β and INF signaling pathways. We may need to perform an intensive PCR-based mutation search in these genes.

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TABLE I. Nucleotide changes found in 15 genes involving in the RAS-MAPK pathway in 30 patients with Kabuki syndrome

	Gene	Change of		Number of	SNP ID	Allele
		Nucleotide	Amino Acid	patients		Frequency
Non-	RASA1	c.73G>A	A26T	1	NR	0.000*
Synonymous		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.1929A>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	
Others	RASA4	IVS17+4C>T	intron17	2	NR	0.057**
	NRAS	-208T>A	5’UTR	4	rs2273267	

A, Allele frequency among normal Japanese; NR, not registered in NCBI database

*, not found in 86 control Japanese individuals; †, found in the 85 controls; ‡, found in the 88 controls; §, found in the 89 controls; #, not found in the 82 controls; **, found in the 87 controls; ††, 5’UTR