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Meta Gene



Polymorphism analysis and new *JAG1* gene mutations of Alagille syndrome in Mexican population[☆]



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Abbreviations: AA, African American; ALGS, Alagille syndrome; CEU, Utah Residents with Northern and Western European Ancestry; CHB, Han Chinese in Beijing, China; CI, confidence interval; dbSNP, The Single Nucleotide Polymorphism Database; DHPLC, Denaturing high performance liquid chromatography; DSL, Delta-Serrate-Lag2 domain; EA, European American; ESP, Exome Sequencing Project; HGMD, The Human Gene Mutation Database; HWE, Hardy–Weinberg Equilibrium; *JAG1*, Gene coding for *JAGGED1* protein; JPT, Japanese in Tokyo, Japan; kb, kilobase(s) or 1000 bp; LOVD, Leiden Open Variation Database; MAF, minor allele frequency; MEX, Mexican population; MIM, Mendelian Inheritance in Man; mutDB, mutDB Polymorphism Database; NA, not applicable; ND, not determined; NMD, Nonsense Mediated mRNA Decay; *NOTCH2*, gene coding for *NOTCH2* protein; OR, odds ratio; PCR, polymerase chain reaction; YRI, Yoruba in Ibadan, Nigeria

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ABSTRACT

Alagille syndrome is a multisystem disorder with an autosomic dominant pattern of inheritance that affects the liver, heart, eyes, kidneys, skeletal system and presents characteristic facial features. Mutations of the *JAG1* gene have been identified in 20–89% of the patients with Alagille syndrome, this gene encodes for a ligand that activates the Notch signaling pathway. In the present study we analyzed 9 Mexican patients with Alagille syndrome who presented the clinical criteria for the classical presentation of the disease. By using the denaturing high performance liquid chromatography mutation analysis we were able to identify different mutations in 7 of the patients (77.77%), importantly, we found 5 novel mutations in *JAG1* gene. The allelic frequency distribution of 13 polymorphisms in Mexican population is also reported. The overall results demonstrated an expanding mutational spectrum of *JAG1* gene in the Mexican population.

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Introduction

Alagille syndrome (ALGS (MIM 118450), **OMIM**) is a multisystem disorder with an autosomic dominant pattern of inheritance that affects the liver (particularly presenting bile duct paucity diagnosed by liver biopsy), heart (peripheral pulmonary artery stenosis), eyes (posterior embryotoxon), kidneys (renal dysplasia, renal tubular acidosis, among others), and skeletal system (butterfly vertebrae), and presents characteristic facial features (broad forehead, deep-set eyes, pointed chin and a triangular face) (Kamath et al., 2010, 2011). ALGS shows a variable expression that complicates the diagnosis, which is established with the identification of at least three out of five main clinical characteristics (Emerick et al., 1999; Kamath et al., 2003). The frequency of the ALGS is about 1:100 000 newborns and the reported mortality is between 10% and 20% (Krantz et al., 1998).

The phenotype of the ALGS patients is due in 20–89% of the cases to mutations in the *JAG1* gene, which product participates in Notch signaling pathway as a ligand (JAGGED1) (Guegan et al., 2012). *JAG1* gene is located at 20p12.2, has 26 exons and 25 introns, and codifies for three different transcripts depending on alternative splicing. The transcript *JAG1-001* (ENST00000254958) expands 5.901 kb, encodes a 1218 amino acid protein with 11 different domains and presents so far more than 300 mutations (Ensembl).

Our institution, Hospital Infantil de México Federico Gómez, is a pediatric medical center with the largest and most successful pediatric liver transplant program in the country. Since 1998, 83 liver transplant procedures have been performed and this experience has been reported elsewhere (Castañeda-Martínez et al., 2010; Varela-Fascinetto et al., 2005, 2011). ALGS has been one of the main reasons for referral for transplantation and has been the 4th more frequently transplanted disease (9 cases, 11%), only after biliary atresia, metabolic disorders and fulminant liver failure. However, no molecular studies regarding the mutational profile of Mexican patients with ALGS have been investigated. For this purpose, a group of patients clinically diagnosed with ALGS were screened for *JAG1* gene mutations by denaturing high performance liquid chromatography (DHPLC) and sequencing techniques.

Material and methods

This study was approved by the research committee of our hospital and under the Declaration of Helsinki ethical principles for medical research involving human subjects, and informed consent letter form signed by the patients and their families was obtained in all cases. The study included nine patients who met the standard criteria for the diagnosis of ALGS as previously reported (Krantz et al., 1997).

Genomic DNA, from all patients, was extracted from lymphocytes by the Gentra Puregene Blood Kit as specified by the company (Qiagen®, Hilden, Germany). Polymerase chain reactions were carried out as previously described in order to amplify the 26 exons and splicing sites of the *JAG1* gene (Krantz et al., 1998). The PCR products were analyzed to look for mutations with the DHPLC technique (Transgenomic® WAVE-MD, Nebraska, United States). The recommended conditions for the acetonitrile-buffer gradient and

temperature, needed to free the heteroduplex and homoduplex DNA fragments, were used accordingly to the manufacturer specifications (Supplementary Tables 1 and 2). After identifying patients with suspected mutations, their PCR products were analyzed by direct sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation®, California, United States). The sequences were performed in both strands and 100 chromosomes of normal subjects were included as controls to discard mutations and to determine polymorphisms frequencies. Both, mutations and polymorphisms were further analyzed in several databases ([Ensembl](#); [dbSNP](#); [HGMD](#); [LOVD](#); [mutDB](#); [Uniprot](#)).

The splicing mutations were confirmed by cDNA sequencing and exon specific RT-PCR. Briefly, total RNA from peripheral blood lymphocytes was isolated with TRIZol® reagent (Life Technologies Corporation®, California, United States). Then cDNA was synthesized by using a commercial kit and according to the manufactures instructions (Life Technologies Corporation®, California, United States). The cDNA obtained from patient 1 was analyzed by PCR and sequencing, using the following primers (5′–3′, 375 bp): TCCTGCTCGCCTGCTCTGT (sense) and TCCCACGCCCTCCACAAGCAAC (antisense). The corresponding cDNA from patient 3 was analyzed by exon 4 specific RT-PCR, using the following primers (5′–3′, 306 bp): AACGACCGCAACCGCATCGT (sense) that anneals with exon 2 and GCCATTCTGGTCACGGCATAGTGT (antisense) that anneals with exon 4. The RT-PCR products were analyzed by densitometry in a 2% agarose gel stained with GelRed® (Biotium®, California, United States), using the VisionWorksLS Software (UVP®, California, United States). The data obtained was normalized by using the Eukaryotic 18S rRNA Endogenous Control assay (431089E, Life Technologies Corporation®, California, United States) that produces an amplicon of 187 bp.

Results

Identification of *JAG1* mutations in ALGS patients

Using the analysis of dHPLC from DNA heteroduplex after PCR, we have been able to identify mutations in the *JAG1* gene in seven out of nine patients (77.77% of the total, [Fig. 1](#)), which is in line with the frequency reported by [Guegan et al. \(2012\)](#). Patients with a positive *JAG1* mutation were 4 males and

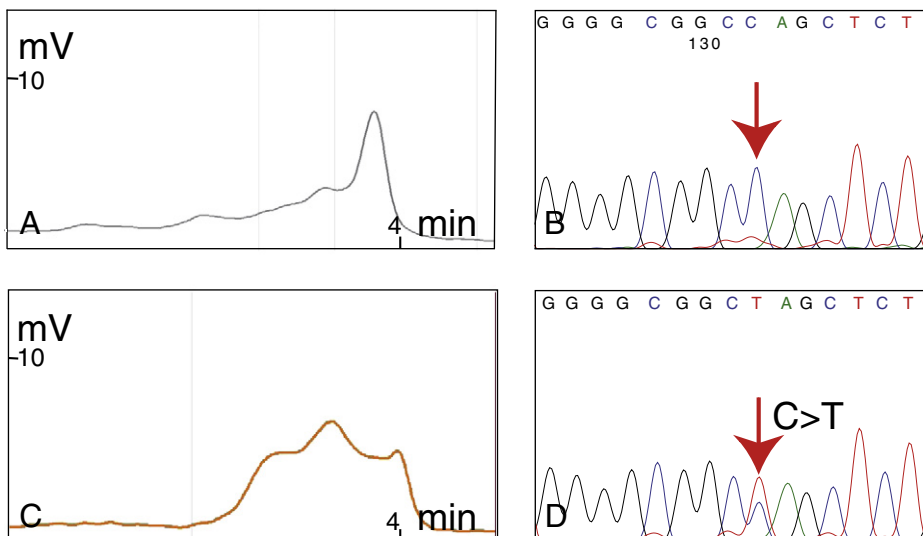


Fig. 1. Nonsense heterozygous mutation identified by DHPLC and sequencing in patient 4. Chromatograms and electropherograms obtained to detect the NM_000214.2 (*JAG1*):c.871C>T mutation. A and C: DHPLC chromatographic patterns of a control and patient 4, respectively. B and D: A and C electropherograms, the sequence change detected is pointed by an arrow. PCR products were analyzed by DHPLC using standard conditions (Buffer A: TEAA [100 mM], EDTA [0.1 mM], pH 7.0; Buffer B: TEAA [100 mM], EDTA [0.1 mM], pH 7.0, Acetonitrile [25%]) at 62.7 °C, and then sequenced in both strands (Supplementary Tables 1, 2).

3 females. The predicted effect of each *JAG1* mutation and the corresponding phenotype are summarized in Table 1, and interestingly, five of the mutations found were novel. The changes observed corresponded to nonsense mutations (57.1%), splicing mutations (28.6%) and one insertion (14.3%). As observed in Fig. 2, the effect of the splicing mutation in patient 1 was confirmed by cDNA sequencing. Moreover, the heterozygous loss of exon 4 in patient 3 due to a splicing mutation was confirmed by exon 4 specific RT-PCR (Fig. 3).

Detection of polymorphisms between ALGS patients and controls

Multiple polymorphisms were identified in patients and controls as depicted in Table 2. Most of the polymorphisms were exonic variants (71.4%) and the rest were intronic. As it has been reported to occur with the mutations, a widely distribution was observed for the polymorphisms (Warthen et al., 2006).

Discussion

Analysis of mutations identified in ALGS patients

The aberrant splicing mutations were identified at introns 1–2 and 4–5. The Alamut 2.0 software predicts that the change in intron 1–2 will generate a new splicing acceptor site which will cause a frame shift mutation by adding four nucleotides in the coding sequence, as confirmed by cDNA sequencing (Fig. 2). This change in turn adds a stop codon in the position p.72. regarding intron 4–5 mutation, the heterozygous loss of exon 4 was confirmed (Fig. 3), since the amplification band obtained from exon 4 specific RT-PCR was about half compared with the control. Interestingly, the affected site is highly conserved among vertebrates, and corresponds to the consensus GA splicing donor site. This mutation has not been previously reported and interestingly, is the only one among the mutations identified in this study that was not linked to a premature stop codon, therefore most of the remaining domains (with the exception of DSL domain) are present in the resulting protein.

On the other hand, the other six mutations identified in this study would produce a transmembrane domain loss, thus, generating a truncate protein unable to translocate and activate Notch signaling pathway. However, as predicted by the Alamut® software, it cannot be discarded that the corresponding mRNA products would be degraded by the Nonsense Mediated mRNA Decay mechanism (NMD). Regardless of the mutations causing a truncated protein, no correlation was observed between the genotype and phenotype of the patients.

In the case of the intron 4–5 mutation described (and as has been previously reported for other abnormal splicing mutation affecting the following downstream base in the donor splicing site), this change could generate a less severe phenotype (Onouchi et al., 1999). However, there is no extensive evidence to support this claim due to the widely variable expressivity in ALGS. With the exception of the intron 4–5 mutation, no other specific phenotype–genotype correlations were established.

Table 1
JAG1 mutations detected in 7 patients with Alagille syndrome.

Patient	Exon/ Intron	Mutation ^a	Domain	Predicted effect ^b	Phenotype ^c
1	I 1–2	<i>NM_000214.2(JAG1):c.82-6C>A</i>	NA	Aberrant splicing (truncated protein, (p. Val28Alafs*46))	L, H, S, K
2	E 2	CM030050 (Röpke et al., 2003)	DSL	Truncated protein (p. Trp128*)	L,H,F.
3	I 4–5	<i>NM_000214.2(JAG1):c.694 + 1G>C</i>	NA	Aberrant splicing (loss of exon 4)	L, H
4	E 6	<i>NM_000214.2(JAG1):c.871C>T</i>	EGF2	Truncated protein (p. Gln291*)	L, H, E, S, K
5	E 13	CI062270 (Krantz et al., 1997)	EGF10	Insertion (p. Cys572Leufs*2, truncated protein)	L, H, E, K
6	E 13	<i>NM_000214.2(JAG1):c.1615C>T</i>	EGF9	Truncated protein (p. Gln539*)	L, H, K
7	E21	<i>NM_000214.2(JAG1):c.2565C>A</i>	EGF16	Truncated protein (p. Cys855*)	L, H, S, F, K

NA. Not applicable.

^a HGMD accession number/"Mutalyzer 2.0" based nomenclature in novel mutations (Wildeman et al., 2008).

^b Predicted by Alamut 2.0 software.

^c Affected systems: liver (L), heart (H), eye (E), skeleton (S), face (F), and kidney (K).

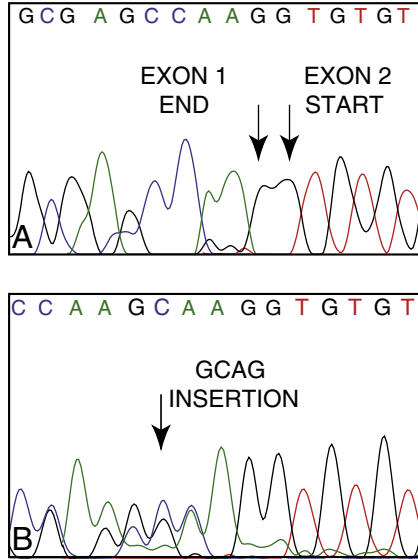


Fig. 2. Effect of the NM_000214.2 (*JAG1*):c.82-6C > A mutation detected in patient 1. As a product of the creation of a new splicing acceptor site, a heterozygous insertion of four bases is observed in the spliced region between exons 1 and 2. A. Electropherogram obtained from a control, the corresponding bases of each exon are pointed by an arrow. B. Electropherogram obtained from patient 1, the insertion is pointed by an arrow.

In all the cases it was possible to clinically examine the parents and ALGS was discarded. The analysis of the parental DNA was performed only in one case, and the mutation has been discarded, thus confirming the *de novo* event. However, we cannot assume this *de novo* event in the other cases, due to the high degree of variable expressivity of ALGS.

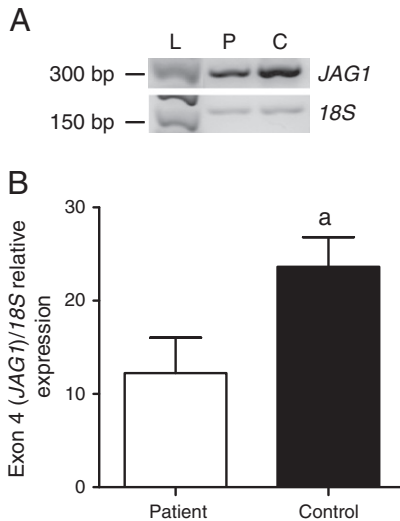


Fig. 3. Effect of the NM_000214.2 (*JAG1*):c.694 + 1G > C mutation detected in patient 3. Heterozygous loss of exon 4 was confirmed by exon 4 specific RT-PCR. A. RT-PCR representative image for *JAG1* (exons 2–4) and ribosomal *18S* mRNA from patient 3 (P) and a control (C); the DNA ladder is represented by L. B. Analysis of exon 4 (*JAG1*) relative expression intensity in patient 3 and a control. Data are expressed as mean ± SD (n = 5), a: p < 0.05.

Table 2

Comparison frequencies of identified polymorphisms between populations.

Exon(E)/ Intron(I)	Variant (dbSNP accession number) ^a	HWE ^b	MAF MEX ^c	MAF MEX ^d	MAF CEU ^d	MAF JPT ^d	MAF YRI ^d	MAF EA ^e	MAF AA ^e	MAF CEU ^e	MAF JPT + CHB ^f	MAF YRI ^f
E2	Sense (rs1051415, (p.=)): <i>NM_000214.2(JAG1):c.267G>A</i>	Y	0.08	ND	ND	ND	ND	0.11	0.03	0.14	0.08	ND
I2–3	Intronic (rs74464347): <i>NM_000214.2(JAG1):c.388-149C>T</i>	Y	0.10	ND	ND	ND	ND	ND	ND	ND	ND	0.09
I3–4	Intronic (rs2273060): <i>NM_000214.2(JAG1):c.440-15T>C</i>	Y	0.56	0.62	0.36	0.42	0.44	0.41	0.45	0.31	0.38	0.42
E4	Sense (rs1801138, (p.=)): <i>NM_000214.2(JAG1):c.588C>T</i>	Y	0.18	0.18	0.03	0.24	0.23	0.04	0.22	0.03	0.22	0.20
E5	Sense (rs10485741, (p.=)): <i>NM_000214.2(JAG1):c.744A>G</i>	Y	0.09	0.10	0.11	0.05	0.04	0.07	0.06	0.11	ND	0.05
E6	Sense (rs1131695, (p.=)): <i>NM_000214.2(JAG1):c.765C>T</i>	Y	0.53	0.61	0.56	0.29	0.34	0.47	0.34	0.42	0.27	0.32
E7	Sense (rs45575136, (p.=)): <i>NM_000214.2(JAG1):c.924C>T</i>	Y	0.08	ND	ND	ND	ND	0.01	0.02	ND	ND	ND
E17	Sense (rs1801140 (p.=)): <i>NM_000214.2(JAG1):c.2214A>C</i>	Y	0.13	ND	0.12	0.06	0.04	0.10	0.03	0.13	ND	0.03
E20	Sense (rs56225585 (p.=)): <i>NM_000214.2(JAG1):c.2382C>T</i>	Y	0.02	ND	ND	ND	ND	<0.01	0.28	ND	ND	0.25
I20–21	Intronic (rs2273059): <i>NM_000214.2(JAG1):c.2458 + 30A>G</i>	Y	0.11	ND	ND	ND	ND	0.02	<0.01	ND	ND	ND
I23–24	Intronic deletion (rs71334418): <i>NM_000214.2(JAG1):c.2916 + 27delT</i>	Y	0.43	ND	ND	ND	ND	ND	ND	ND	ND	ND
E26	Sense (rs1051419, (p.=)): <i>NM_000214.2(JAG1):c.3417T>C</i>	N	0.75	ND	0.63	0.43	0.96	0.63	0.90	0.63	0.40	0.97
E26	Sense (rs1051421 (p.=)): <i>NM_000214.2(JAG1):c.3528C>T</i>	NA	ND	0.39	0.24	0.21	0.07	0.29	0.12	0.20	0.16	0.03

HWE—Hardy–Weinberg Equilibrium. MAF—Minor allele Frequency. MEX—Mexican Population. CEU—Utah Residents with Northern and Western European Ancestry. JPT—Japanese in Tokyo, Japan. YRI—Yoruba in Ibadan, Nigeria. EA—European American. AA—African American. CHB—Han Chinese in Beijing, China. NA—not applicable. ND—not determined.

^a “Mutalyzer 2.0 β-8” according nomenclature (Wilderman et al., 2008).

^b Hardy–Weinberg Equilibrium, $P > 0.05$, 1 freedom degree.

^c Present study.

^d Reported in HapMap.

^e Reported in NHLBI GO Exome Sequencing Project (ESP).

^f Reported in 1000 GENOMES.

The present study further supports the idea that the molecular mechanisms involved in the clinical effect of the mutations in ALGS is the haploinsufficiency mainly due to the loss of the transmembranal or the DSL domains (or NMD), and is in line with the previous report of [Heritage et al. \(2002\)](#).

Finally, in our study we observed that in 2 patients with ALGS clinical diagnosis there were no mutations identified in *JAG1*, however, we cannot discard the presence of mutations in other regions of the gene (such as the promoter) or in *NOTCH2* gene, as has been previously described ([Kamath et al., 2012](#); [Mc Daniel et al., 2006](#)).

Analysis of polymorphisms frequencies in ALGS patients and controls

All polymorphisms detected have been reported in several databases for other populations ([Exome Variant Server](#); [The 1000 Genomes Project Consortium, 2010](#); [Thorisson et al. 2005](#)), interestingly, their frequencies in this Mexican population were similar to those reported for the Mexican-American population in Los Angeles, Ca., USA, as it may be closer in ancestry ([Table 2](#)).

There was no statistical allele frequency difference in the polymorphisms identified between patients and controls, only with one exception (see [Table 2](#)). The exception was the rs2273060 polymorphism that was more frequent in patients ($p < 0.05$) than in controls (0.83 and 0.49, respectively). However, it is difficult to establish an association with the ALGS phenotype, due to our reduced sample size. Interestingly, the polymorphism rs2273061, is suggested to be in linkage disequilibrium with rs2273060 by GLIDERS software ([Lawrence et al., 2009](#)) in other populations ([Supplementary Table 3](#)), which has been associated to bone defects (osteoporosis) ([Kung et al., 2010](#)). Interestingly, in that study, [Kung et al. \(2010\)](#), demonstrated that the rs2273061 polymorphism alters the c-Myc binding site, which correlated to a decreased *JAG1* expression.

The allele and genotype frequency for the rs71334418 polymorphism has not been previously reported and is in Hardy–Weinberg equilibrium. Of the thirteen polymorphisms identified in this study, it was not possible to establish the allele frequency for the polymorphism rs1051421, due to technical limitations. It

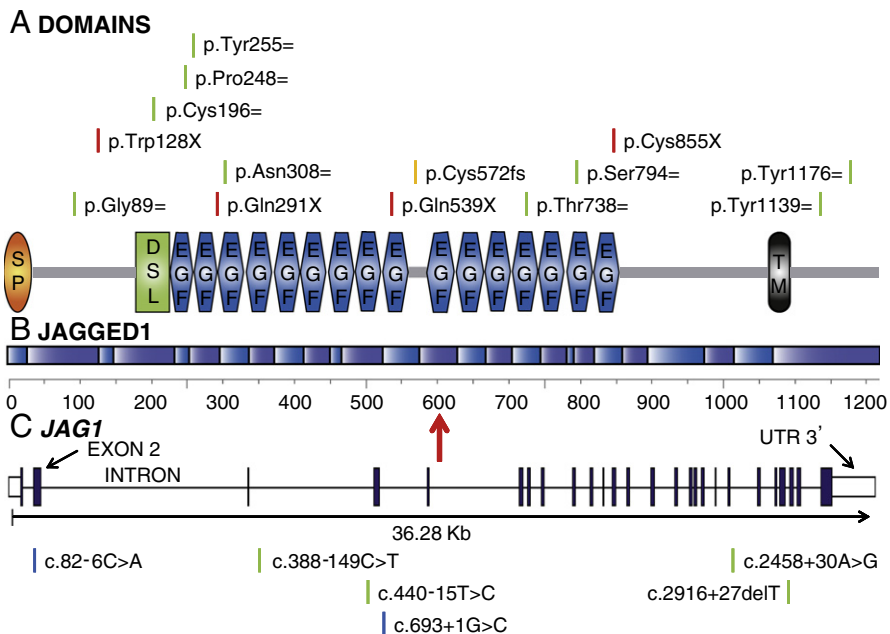


Fig. 4. Mutations and polymorphisms detected along *JAG1* gene in the current study. A—Exonic variations located in domains predicted by Uniprot. B—Aminoacid rule corresponding to 3A. C—Intronic variations located in *JAG1* gene. Variations: nonsense mutation (red), insertion (orange), splicing mutation (blue) and polymorphism (green). The figure is not to scale.

has been demonstrated that polymorphic changes detection is difficult when there are two variations in the same amplicon (Mátyás et al., 2002).

Conclusions

Together with the more than 300 mutations reported to date related with ALGS in other populations, the overall results of this study reflect an expanding mutational spectrum of the *JAG1* gene in the Mexican population (Fig. 4), and indicate that it may have important implications for both the clinical follow up of the patients and for the genetic counseling.

Conflicts of interest statement

The authors declare no conflict of interest or disclosures.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mgene.2013.10.002>.

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