Uncovering the molecular basis of syndromic intrahepatic bile duct paucity in local patients

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Abstract

Alagille Syndrome (AGS) is an autosomal dominant rare disease caused by mutations in *JAG1* gene which codes for a protein regulating cell fate determination. It is characterised by cholestasis with paucity of interlobular bile ducts and anomalies of the cardiovascular system, skeleton, eyes, and face. This disease is caused by mutations in *JAG1* gene which produces a dysfunctional protein. There is wide variation in clinical symptoms making clinical diagnosis challenging. Due to the rarity of this disease and the high incidence of new mutations, uncovering the molecular basis of patients with such clinical symptoms is important to provide a definite basis for the disease. In this study, four patients with AGS presenting signs were analyzed and mutations were found in three of four patients, with another being a reported polymorphism. Two of these mutations are novel, while other mutation had previously been reported at least thrice. Polyphen analysis of the missense mutation screened predicts it to be severely damaging. The results of this study confirms the diagnosis of AGS in three out of four patients screened, confirming diagnosis and offering possible carrier screening for the family. The main objective of this proposal is to identify *JAG1* mutations in patients and determine the predicted effect on the protein. DNA analysis was carried out by melt curve mutation screening. One of the detection mutations, a missense mutation, was analyzed by *in silico* tool, PolyPhen. The effect of these mutations on the clinical presentations in the patients was analyzed against reported phenotypes and known mutation databases for this disease.

Introduction

Alagille Syndrome (AGS, MIM118450) is a rare autosomal dominant disease with an incidence of one in every 100,000 live births. It can be diagnosed by the paucity of bile duct on liver biopsy and the presence of at least three out of five major clinical signs, namely chronic cholestasis, vertebral deformities (butterfly-like hemivertebral), anterior chamber ocular defects including posterior embryotoxon, malformations of the pulmonary arteries and valves, and a typical facial appearance [1]. AGS is caused by mutations in *JAG1* gene

(MIM601920) located on chromosome 20p12 [2]. It spans approximately 36kb, contains 26 exons and encodes a messenger RNA of 5.9kb, which turn translates a 135 kDa protein. JAG1 protein consists of several evolutionary conserved domains; which includes a signal peptide, the conserved Delta-Serrate-Lag2 Domain (DSL), a 16 epidermal growth factor (EGF)-like repeats, a cystein rich region and a transmembrane domain. JAG1 protein acts as a ligand for the NOTCH1 receptor involved in the evolutionary conserved signaling pathway implicated in tissue patterning, cell fate determination and morphogenesis [3].

There is considerable variable expression and the true incidence of the disease may be under-estimated due to the difficulties in accurate diagnosis [4]. Family studies have also shown phenotypic differences expressed even between affected family members carrying the same mutation. There have also been reports of individuals who carry *JAG1* mutations but missed being diagnosed as AGS using the standard clinical criteria [5]. These patients actually manifest the mild end of the phenotypic spectrum of this disease. Hence, molecular analysis of *JAG1* gene can contribute towards improved diagnosis of this disorder.

This project aims to study the molecular basis of four local Chinese patients who show symptoms of intra-hepatic bile duct paucity and syndromic features of Alagille's syndrome. Precise identification of mutations in *JAG1* gene would help confirm the diagnosis of AGS and provide options of carrier screening and genetic counseling for affected families. In this study, a rapid and cost-effective method based on high resolution melt (HRM) or thermal denaturation will be used for mutation screening. The experimental strategy consists of an HRM assay followed by direct sequencing. More than 230 unique mutations in JAG1, including point mutations, microdeletions and small insertions, have been described so far [6, 2, 7]. Since there are no mutation hot-spots and all 26 exons of the *JAG1* gene have to be screened, a rapid and cheap method would allow improved clinical diagnosis over existing laborious screening methods like single strand conformation polymorphism (SSCP) or denaturing high performance liquid chromatography (DHPLC) methods or direct DNA sequencing. Although direct sequencing method is accurate, it is considerably more expensive than HRM and requires numerous steps of PCR amplification, product purification and sequence amplification for all 30 reactions corresponding to the 26 exons of the gene. In HRM analysis, a single closed-tube assay is performed in which PCR is carried out followed by melt analysis. The exon showing aberrant melt profile can then be sequenced to confirm presence of mutation. This reduces the number of reactions to be sequenced and analyzed.

Materials and Methods

A total of 4 anonymized DNA patient samples were used in the study, and provided by National University of Singapore, Department of Paediatrics. These samples were previously collected with informed consent. These patients had previously been suspected of a diagnosis of Alagille Syndrome due to the clinical manifestation of two or more related symptoms associated with AGS.

Mutation analysis was carried out by PCR amplification using pre-existing primers in the lab for all 26 exons adopted from [4]. PCR reactions were carried out in 96 well plates using the CFX96 real time PCR detection system Bio-Rad, USA). The reaction volumes consist of 1.2µl of 2.5µM forward primer and 1.2µl of 2.5µM reverse primer, 2µl of 5ng/µl DNA, 3µl of ddH_2O, 1µl of 10x PCR Buffer, 0.3µl of 50mM $MgCl_2$, 0.2µl of 10mM dNTPs, 1µl of 10x EvaGreen, and 0.1µl of 5U/µl Platinum Taq. Melt analysis were carried out on the post-PCR products in the same plate. The PCR cycling conditions are: 40 repeated cycles of a denaturing step at 95 ºC, an annealing step at 58 ºC, and an extension step at 72 ºC, each lasting 20s, 20s and 45s respectively. Subsequently, melt analysis was carried out by heating the plates from 65 ºC to 95 ºC with increments of 0.2 ºC of intervals 10s each (Appendix A). The plates were then held at 30 ºC for 30s. Using the Bio-Rad Precision Melt Analysis Software (Bio-Rad, USA), melt curves were then generated and plotted into difference curves. Normalization and temperature shifting was applied to the curves in order to standardize the analysis for all the samples (Appendix A). The results were then analyzed with the Precision Melt Analysis Software (Bio-Rad, USA). The samples were analyzed together with four normal controls. Products which showed melt curve shifts anomalous to the normals were subsequently analyzed. The corresponding exons showing the melt curve shifts were amplified again and the products were sequenced using the BigDye Terminator V3.0 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing reaction product was then electrophoresed on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA)and analyzed using Chromas software.

In silico analysis of the mutation data was carried out by comparison against the known reference sequence of JAG1 (Genbank RefSeq: HSU73639). The positions of mutations were then identified and translated to the position of mutated codon. The FASTA sequence (Appendix F) of JAG-1 was then input into PolyPhen software (http://genetics.bwh.harvard.edu/pph2/) for analysis, with the position of affected codon and amino acid indicated.

Results

Known SNPs were obtained and compiled from NCBI on *JAG1*, and known mutations were also compiled from mutation databases; Zhejiang Mutation Database (http://www.genomed.org/lovd/algs/variants.php?action=search_unique&limit=100&order=Variant%2FDNA%2CASC&page=1) for gene JAG1 and Korean Mutation Database (http://112.169.110.15:9099/search?type=mimid&query=118450). These databases were checked with research reports on mutations.

Primers were checked through Primer-BLAST of NCBI, Primer3 software, and MeltSim (Appendix B). Gel electrophoresis was also conducted as a preliminary test to find out if they worked after PCR. The primers were then optimized using quantitative real-time PCR (qPCR) and high resolution melt analysis.

Prior to use for this project, the lab primers were checked by Primer3, Primer-BLAST, and MeltSim programmes indicated its complementarity of each primer to chromosome 20 and its

suitability fitting to the set criteria for melt primers (Appendix C). Gel electrophoresis showed amplification products of expected sizes corresponding to exons 1 to 26 (Appendix D).

Melt analysis for all four patient samples showed abberant melt profiles for the corresponding exons 1 to 26. In each of the four patients, significant shift in melt curves from the wild types was observed. DNA sequencing analysis for the respective exons confirm the presence of mutations either as a single base substitution or deletion (Appendix E).

Patient Sample No.	Exon	Sequence Change	Amino acid change	Type of Mutation
311	10	c.1753G>A	Cys447Tyr	Missense
140	6	c.1213delC	At412	Deletion
227	8	c.2643C>T	Arg744Stop	Nonsense
406	4	c.588C>T	Cys196Cys	Polymorphism

Genbank RefSeq: HSU73639

Patient Sample No.	Exon	Sequence Change	Amino acid change	Type of Mutation
311	10	c.1856G>A	Cys447Tyr	Missense
140	6	c.1316delC	At412	Deletion
227	8	c.2746C>T	Arg744Stop	Nonsense
406	4	c.1104C>T	Cys196Cys	Polymorphism

Genbank cDNA RefSeq

Patient Sample 311 carried a base change of c.1753G>A (Genbank RefSeq: HSU73639). This corresponded to c.1856G>A using the new sequence nomenclature (Genbank cDNA RefSeq). As most of previous published mutations have been reported using the old nomenclature, the sequence using both DNA sequence nomenclatures will be reported in this study in order to make meaningful comparisons with previously published mutations. This sequence change in exon 10 results in a missense mutation, Cys447Tyr. PolyPhen analysis of this codon change predicts this mutation to be potentially damaging (score 1.00).

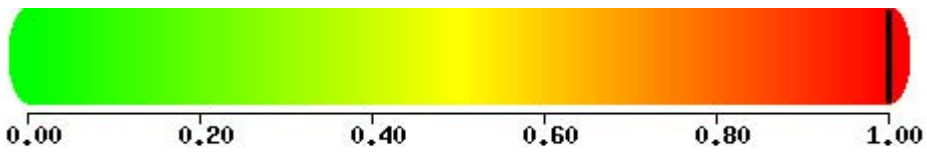

Fig 2 HumDiv Prediction for Missense mutation for Exon 10 of patient 311

Shown are 75 amino acids surrounding the mutation position (marked with a black box). An interactive version of the complete alignment is also available.

Fig 3 Mutiple Sequence Alignment (PolyPhen) for patient 311

A single base deletion in exon 6, c.1213delC (Genbank RefSeq HSU73639) was observed in patient sample No. 140 This resulted in a frameshift in codon 412 leading to a downstream termination codon in codon 414. This is predicted to result in a premature termination of translation of the protein.

In patient sample 227, a base change of c.2643C>T in exon 8 was detected from the sequence analysis. This is results in an immediate termination codon, Arg744Stop, and is classified as a nonsense mutation. It is predicted that it will lead to the formation of a non-functional truncated JAG1 protein.

Patient sample No. 406 had a base change of c.588C>T (Genbank RefSeq: HSU73639), or that of c.1104C>T (Genbank cDNA RefSeq, NM_000214.2), affecting exon 4 of the *JAG1* gene. However, this is considered as a silent mutation as it does not lead to a codon change (Cys196Cys). Further anlaysis on dbSNP database showed this to be a polymorphism (rs1801138) with a reported frequency of 13% for the minor allele T in chinese populations.

Discussion

The results of JAG1 analysis in this study uncovered four sequence changes in all four patient samples in exons 4, 6, 10 and 18. Two of these mutations are novel and have not been previously reported, namely c.1753G>A in patient 311 and c.1213delC in patient 140.

Previous reports have shown a diverse spectrum of mutations in *JAG1* such as missense, splice site and chain terminating mutations [6, 2, 7]. Although it is widely acknowledged that there is no mutation hotspots in AGS, Spinner et al (2001) [7] had noted that 65% occurrence of mutations in 11 exons inclusive of exons 4 and 6 in which mutations were found in this study. Literature search shows that the mutations in (Cys196Cys) of exon 4 and Arg744Stop of exon 18 of patients 406 and 227 have been previously reported [8, 9, 10, 11, 6]. According to the dbSNP database (http://www.ncbi.nlm.nih.gov/) the polymorphism (rs1801138) observed in patient sample 406 has an allele (C or T) frequency ranging from 13 to 15% for minor allele T for chinese populations. This SNP has also been reported in at least 3 previous studies Krantz et al 1998; [8], Warthen et al (2006) [9], and Kohsaka et al (2002) [10]. These studies had analyzed large samples involving 54 families, 247 families and 102 families respectively. The c.2643C>T mutation found in patient sample No. 227 had also been reported at least three times, by Onounchi et al in 1999 [11], Krantz et al in 1998 [8], and

Colliton et al in 2001 [6]; indicating it to be a probable common mutation. The mutations in patient samples patients 311 and 140 are novel. However, there are reported mutations near that of mutation c.1753G>A of patient 311 as reported by Warthen et al that researched a 247 sample size [12] , and near that of mutation c.1213delC of patient 140, as reported by Ropke in 2003 [13]. The lack of previous reports of mutations in these exons could possibly be attributed to its relatively shorter exons (10 and 6) of 114 and 131 base pairs respectively, in comparison to patient 406's known polymorphism located in exon 4 of 255 base pairs in length.

Analysis of the protein domains of JAG1 showed that mutations of three patients, namely 311, 140 and 227, occurred in the EGF portion of the JAG-1 protein. The EGF regions are part of the critical extracellular domain of the JAG-1 protein [14] that has shown high interspecies conservation [13]. These regions play an essential role in the interaction between the extracellular domains of the ligand JAG-1 and the NOTCH receptors, as demonstrated in a mouse model by [15], triggering proteolytic cleavages within the receptors and the subsequent activation of the Notch signaling pathway [16]. This ultimately results in the translocation of the membrane-tethered intracellular domain of NOTCH1 to the nucleus and complexing with protein RBPSUH, leading to the activation of NOTCH target genes [17]. As such, the mutations and polymorphisms occurring in the EGF regions of protein JAG-1 may possibly result in the disruption of notch signaling pathway. Previous reports [8, 6, 18] supports the importance of the EGF region for protein stabilization and protein-protein interaction.

In patient number 406, only a polymorphism (Cys196Cys) was found, occuring in the evolutionary conserved Delta-Serrate-Lag2 (DSL) region of the JAG-1 protein. Although this SNP does not affect the production of a full-length protein or change its conformation, it is noted that it occurs in the highly conserved Delta-Serrate-Lag2 (DSL) region of the JAG-1 protein, showing structural similarities with all Notch ligands; Delta and Serrate in *D. melanogaster* and LAG-2 in *C. elegans*, and is responsible for Notch receptor binding [13]. A study by Yuan et al [17] had also previously determined the essential role of DSL domain in determining the severity of liver defects in AGS. The polymorphism of patient 406 was the only other that occurred in the evolutionary conserved domain occurring at codon 196 resulting in the polymorphism Cys196Cys.

All four patients manifest cholestatic jaundice; however, patient 311 and patient 227 reported of three of the five symptoms of Alagille Syndrome, and hence deducible to be a more severe form. Patient 311 and patient 227 had both reported of a missense mutation and nonsense mutation respectively. Though the effects of missense mutations are still unclear, some missense mutations (R184H, G274D, L37S) have been discovered to generate proteins with abnormal glycosylation patterns which fail to reach the cell surface [13], due to defective intracellular transport [16], resulting in the lack of notch signaling; this may possibly have occurred in Cys447Tyr of patient 311 that have resulted in the more severe symptom as described. This is in line with PolyPhen's 1.00 damaging prediction of missense on protein structure. As for patient 227, the nonsense mutation resulted in a truncated protein lacking in a portion of the vital EGF repeats known to play an important role in notch signaling with

receptor NOTCH1 [18], on top of the cysteine rich, transmembrane domain and intracellular domains also missing as a result of the protein truncation. The lack of the vital EGF region as well as the transmembrane domain may have resulted in the disruption of the interaction between the JAG-1 ligand and NOTCH1 ligand, as well as the proteolytic process, thus resulting in more numerous symptoms as described. However, patient 140 with a deletion mutation of c.1213delC in exon 6 only reported of one symptom of Alagille Syndrome, reinforcing the concept of the lack of genotype to phenotype correlations for Alagille Syndrome as well as its varied expressivity [2], as reported in previous papers. A possible explanation for this may include the existence of genetic modifiers of this disease, such as genes *NOTCH2* [17] and *HEY2* [19], as proposed by Gridley et al (2003). Kamath et al's (2002) [20] research had previously yielded identical mutations in exon 6 of monozygotic twins but reported of different phenotypes; this further suggests the possible existence of other genetic modifiers that could possibly justify the milder symptoms described in patient 140.

Apart from this, there are nevertheless, areas where further work can be done, to further examine Alagille Syndrome. The utilization of other silico tools such as SIFT can also be used to examine mutations of *JAG1* and its effect on the three dimensional structure of the JAG-1 protein to confirm results found in this study. The design of a mutation assay for gene *NOTCH2* and *HEY2* may prove useful, with an increase in research suggesting its roles as a modifier for the Alagille Syndrome disease [21]. Also, for patient 406, mutations could have occured in non-exonic regions which were not screened in this study. Future studies could analyze the flanking intronic regions, promoter and untranslated 5' or 3' ends which could affect protein processing leading to non or partially functional JAG1 protein. For patients in whom mutations were found, the future application of genetic counseling and carrier screening can be offered to family members. The results of this study show that HRM analysis and DNA sequencing can help detect mutations by screening all 26 exons of *JAG1* gene; however, this can only be reaffirmed with a larger sample size, or after the screening of other suggested modifiers as described.

Conclusion

In this study, three mutations and one SNP was found in the four patients suspected of AGS. The three mutations, missense, deletion and nonsense, of patients 311, 140 and 227 respectively, are predicted to be disease-causing and affect the important EGF repeat region of the JAG1 protein.

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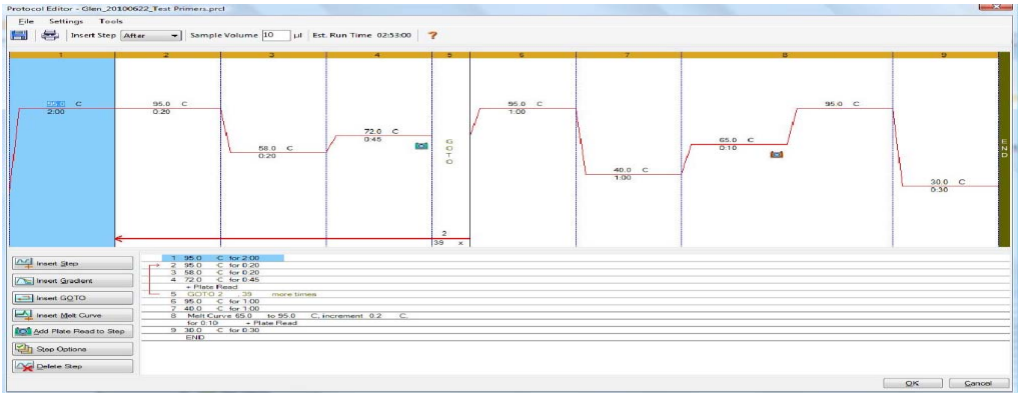
Appendix

Appendix A – Pictorial Illustration of HRM Protocol utilized



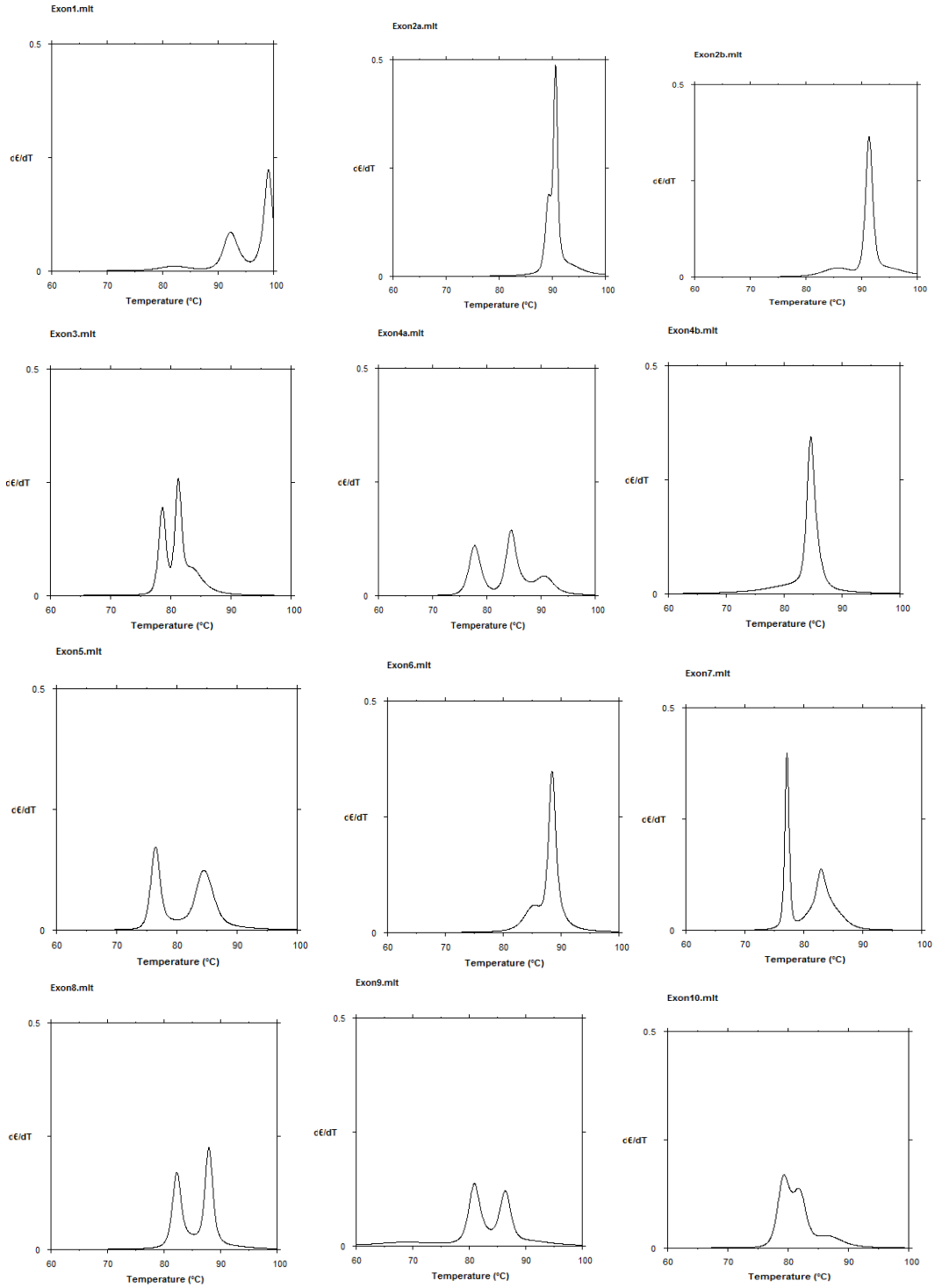
Appendix B – Primer sequences and amplicon sizes of the amplified Exon 1 to Exon 26 of JAG1, checked through Primer3, BLAT and MeltSim

Exon	Forward Primer	Reverse Primer	Product Size	Melting Temperature	GC Content	3' Complementarity	Melt domains / Temperature	Blast Specificity
1	TCCAATCGGC GGAGTATATTA GAGC	agaggacggctggg aggga	251	65.4, 66.8	48.0, 68.4	2.0, 0.0	92C 98C	Specific to Chromsome 20
2a	gcgctgacctacctc cttccct	caCGATGCGGTTG CGGTC	313	67.2, 67.3	63.6, 66.7	0.0, 1.0	90C	Specific to Chromsome 20
2b	GGGGCAACACC TTCAACCTCA	ccaggcgcgggtgtga g	132	66.6, 67.6	57.1, 76.5	1.0, 0.0	91C	Specific to Chromsome 20
3	aaggaaggggagtt ggtttg	gagaaaagtccacaga agcgatac	259	60.3, 60.6	50.0, 45.8	0.0, 2.0	78C 81C	Specific to Chromsome 20
4a	gggaagaaggctgc aatgtgaata	CTGGGGCGCCAG AACTTATTG	221	65.5, 66.0	45.8, 57.1	2.0, 3.0	78C 85C 90C	Specific to Chromsome 20
4b	CCTGTGATGACT ACTACTATGGCT TTGG	gacactaaaagcaaca ggcacacg	188	64.2, 65.2	46.6, 50.0	0.0, 2.0	85C	Specific to Chromsome 20

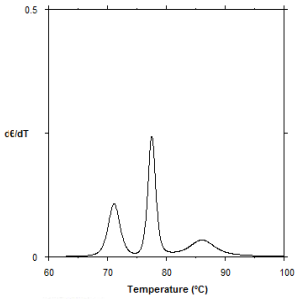
5	gcagtgtgctgacac gccct	aagaggcatagtcaca ataaagtcagttcc	181	67.3, 64.9	65.0, 40.0	3.0, 0.0	76C	Specific to Chromsome 20
6	aaggctaacctgga ggtgtgctg	tcccaccctgggtctcat cc	198	65.9, 66.8	56.5, 65.0	2.0, 1.0	88C	Specific to Chromsome 20
7	tgggttcgccatcttc acagg	tcagcatcacccaaaaa actttagaga	358	67.7, 65.1	57.1, 37.0	0.0, 2.0	78C 83C	Specific to Chromsome 20
8	catccctctctgactg ccatcc	acctctccccaacgtgg tatctt	217	65.5, 64.2	59.1, 52.2	1.0, 0.0	82C 88C	Specific to Chromsome 20
9	tgaattaaattgtca acccccctcctt	ttggtataaaaattaca gtcacagggatg	190	65.6, 63.3	65.6, 63.3	0.0, 3.0	81C 86C	Specific to Chromsome 20
10	ctcatgctcatcccc atctcctt	cagcaagtcggctaccc aagttt	197	65.9, 65.8	52.2, 52.2	0.0, 4.0	79C 82C	Specific to Chromsome 20
11	cactgtaaattacct ctttaaaatgatgac	gagctctcctagtgtcg cacaaatct	170	60.0, 65.4	30.0, 50.0	2.0, 2.0	72C 77C 87C	Specific to Chromsome 20
12	tgaagccctgtgtttg tggaatac	gaaaagtaaagggaag cggaggag	318	64.4, 64.6	45.8, 50.0	2.0, 0.0	81C 84C	Specific to Chromsome 20
13	ccctcccccttttcgc tgtt	aagtggggacaaaagg agcaagt	243	66.7, 64.4	60.0, 47.8	0.0, 1.0	82C 88C	Specific to Chromsome 20
14	gaatgccgcatctgt gggtg	aggctggggagcactg gtc	263	67.1, 65.1	60.0, 68.4	0.0, 5.0	85C	Specific to Chromsome 20
15	aggagggagccatg aaaactgc	caacatgacccatacat cccagag	251	66.0, 64.7	54.5, 50.0	2.0, 1.0	80C 86C	Specific to Chromsome 20
16	gtgaatggtcctgga tctcgtctt	gccccctcccacagaag acag	232	64.9, 67.9	50.0, 66.7	0.0, 1.0	81C 84C	Specific to Chromsome 20
17	ggggccactgggact cacac	ccaggggccaaccagc aga	223	67.5, 69.1	70.0, 68.4	3.0, 0.0	86C	Specific to Chromsome 20
18	gatatttctctgggcc tggttcttg	ccgacagccctgggag agtt	242	65.2, 66.1	48.0, 65.0	0.0, 1.0	85C 88C	Specific to Chromsome 20
19	ggctaagaccgcttt ccctgtt	acgatagtggatgagtg ctggctt	128	64.9, 65.4	54.5, 50.0	0.0, 0.0	81C	Specific to Chromsome 20
20	agagtaatggactg ggaggttggtaa	aggcatggaatgaagc ggtaaag	192	64.2, 65.5	46.2, 47.8	1.0, 0.0	84C 88C	Specific to Chromsome 20
21	catcagtccctaaac ttgaactccatt	cgctcaccccagaaga cccat	196	63.8, 67.9	40.7, 61.9	2.0, 2.0	79C 85C 88C	Specific to Chromsome 20
22	ggcacaggcataac catttcataa	gaactgcggcagccatc at	230	64.0, 64.7	41.7, 57.9	2.0, 3.0	85C	Specific to Chromsome 20
23a	tggctgccgcagttc acct	TCTCCTTGTTAAAT GTGATGTTC	295	67.7, 56.0	63.2, 34.8	3.0, 1.0	86C 91C	Specific to Chromsome 20
23b	AGTGTCGGTCTT CCAGTCTCCAG	caagcagacatccacc attcaaaa	154	64.2, 65.3	56.5, 41.7	2.0, 1.0	78C	Specific to Chromsome 20
24	tctcaatcttacacgt gtgtgggttt	atcgaataatgaggtgt gaatgggtc	210	64.9, 65.2	42.3, 42.3	6.0, 1.0	79C	Specific to Chromsome 20
25	aattttgaagaaagg ctgctttgagtat	cctcgacctgatggctttt attgaa	296	63.7, 65.5	32.1, 45.8	2.0, 3.0	77C 80C 85C	Specific to Chromsome 20
26a	tcttggagagttaatt ggttttgtgc	CCTTGATGGGGAC CGTGTTG	250	63.7, 65.8	38.5, 60.0	2.0, 0.0	84C 89C	Specific to Chromsome 20
26b	GCTGAACCAGA TCAAAAACCCC A	TTGTCCAGTTTGG GTGTTTTGTCG	239	66.4, 67.2	47.8, 45.8	0.0, 2.0	80C 90C	Specific to Chromsome 20

26c	GCGTATACGCT GGTAGACAGAG AAGA	GACAGTTTAAAGA ACTACAAGCCCTC AGA	196	64.1, 64.3	50.0, 41.4	2.0, 3.0	85C	Specific to Chromsome 20

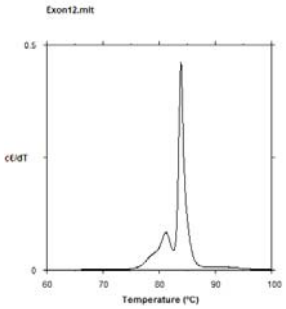
Appendix C – Checking of lab primers via MeltSim to check melt peaks



Exon11.mlt



Exon12.mlt



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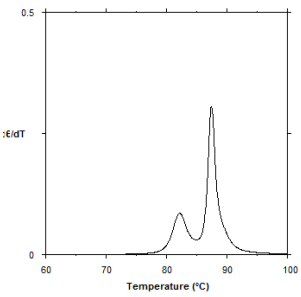
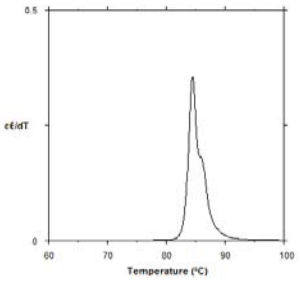
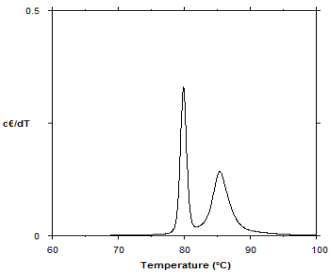


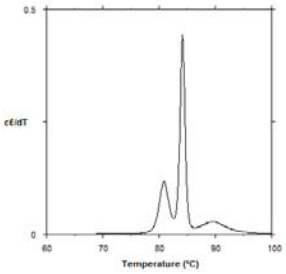
Exon14.mlt



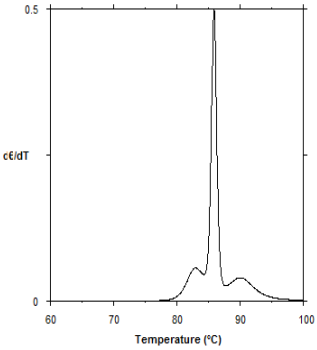
Exon15.mlt



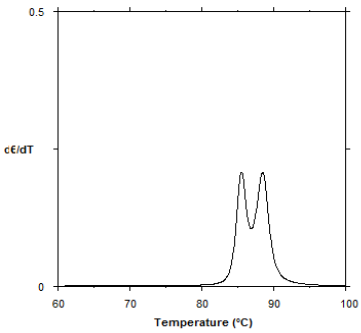
Exon16.mlt



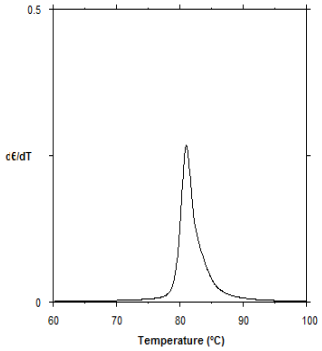
Exon17.mlt



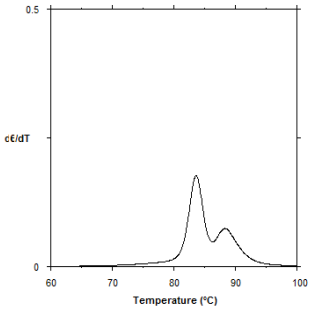
Exon18.mlt



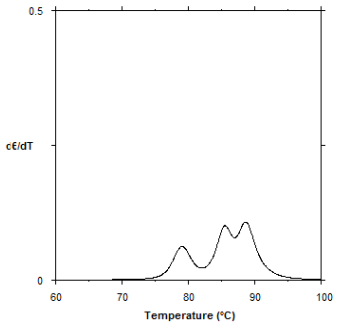
Exon19.mlt



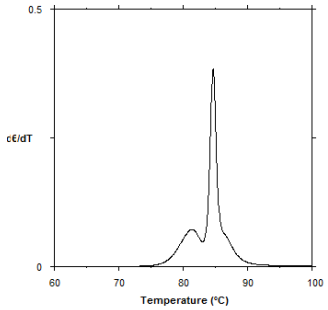
Exon20.mlt



Exon21.mlt

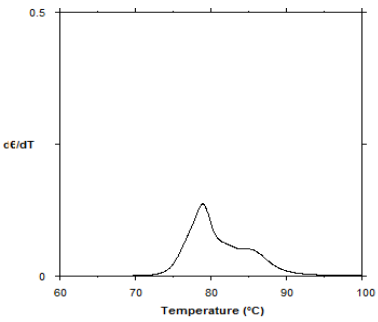


Exon22.mlt

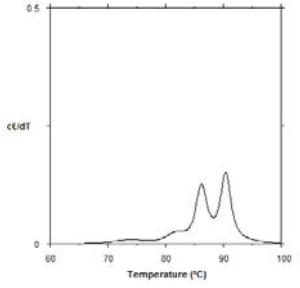


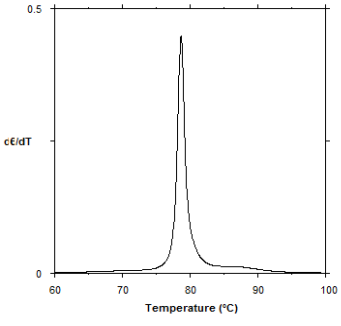
Exon24.mlt

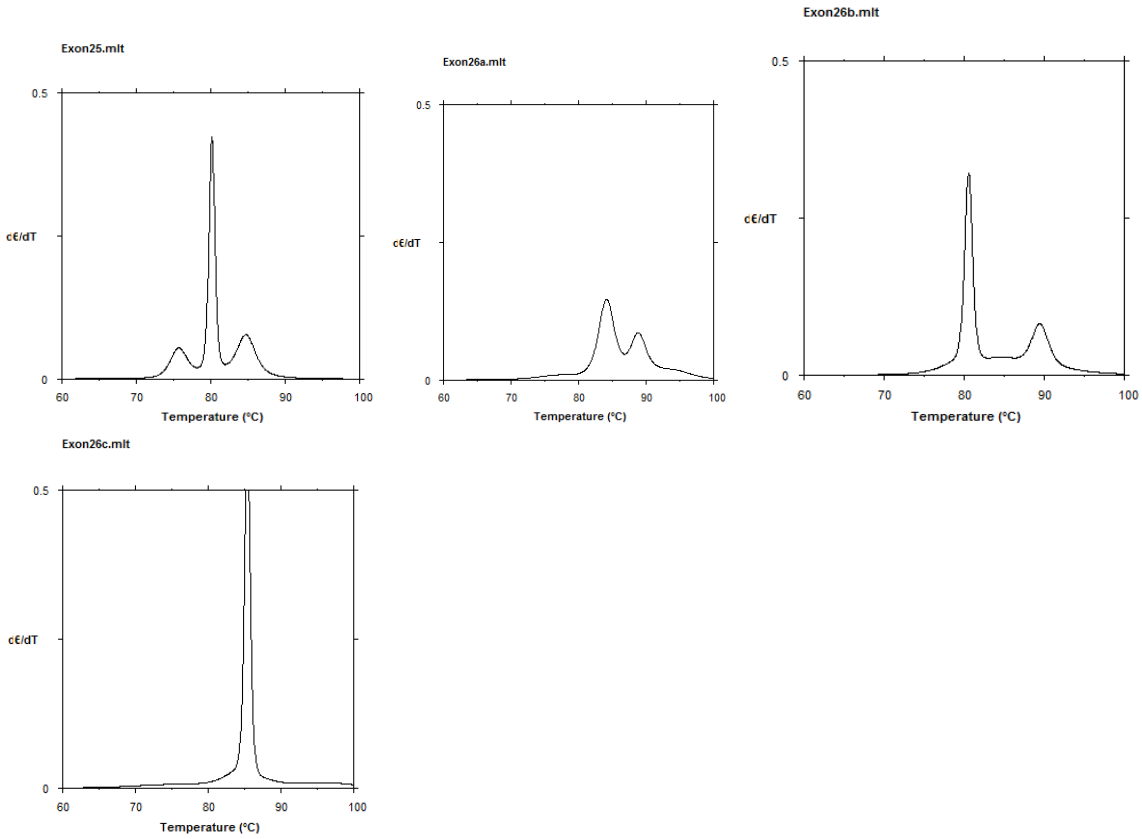
Exon23b.mlt



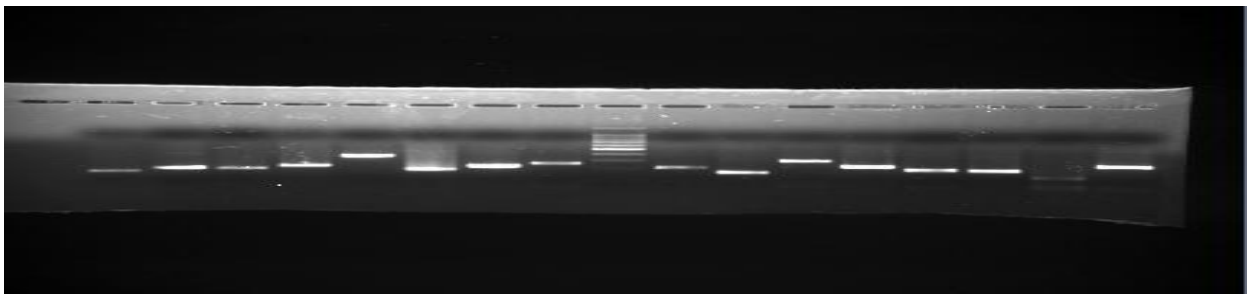
Exon25a.mlt

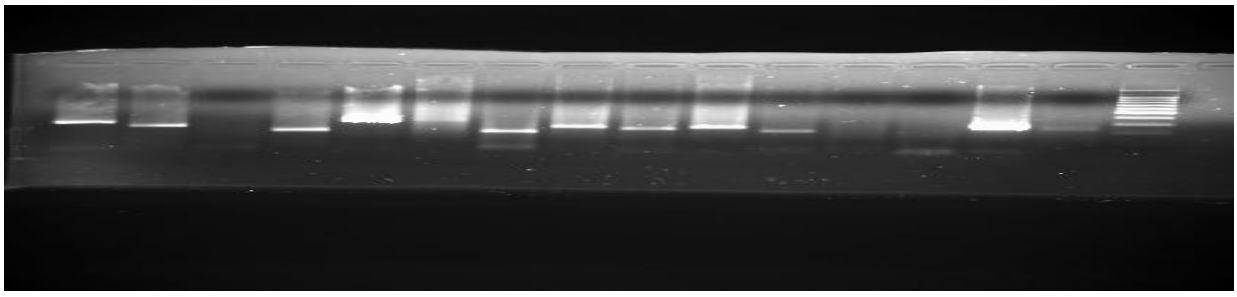






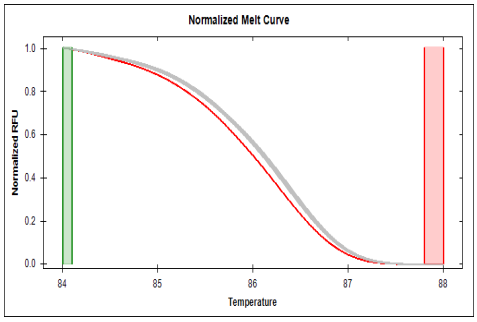
Appendix D – Results of gel electrophoresis of lab primers

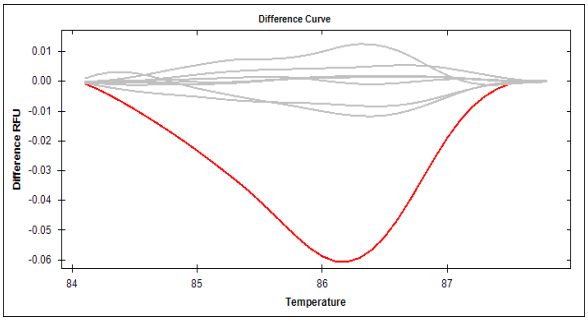


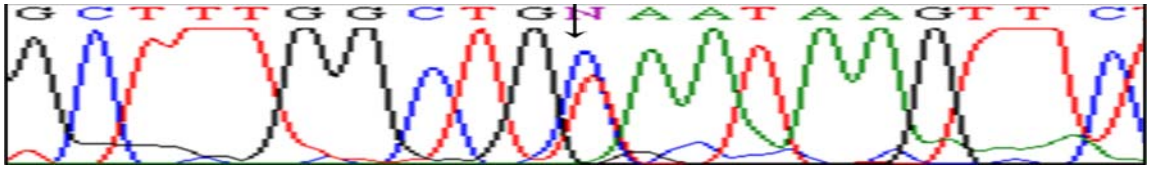


Appendix E – High resolution normalized temperature shifted melt curve and temperature shifted difference curves of exons of JAG1, with sequencing results.

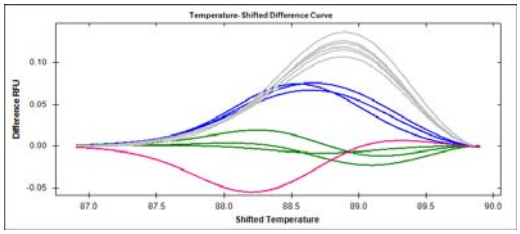
Patient 406

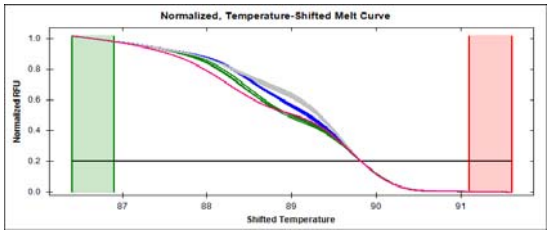




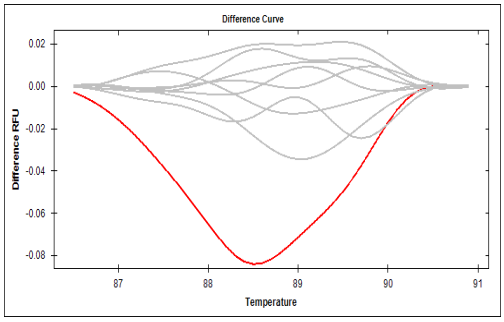


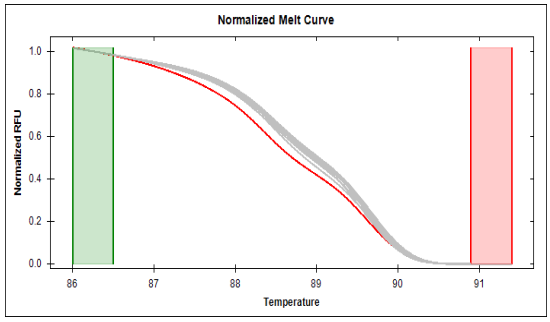
Patient 140

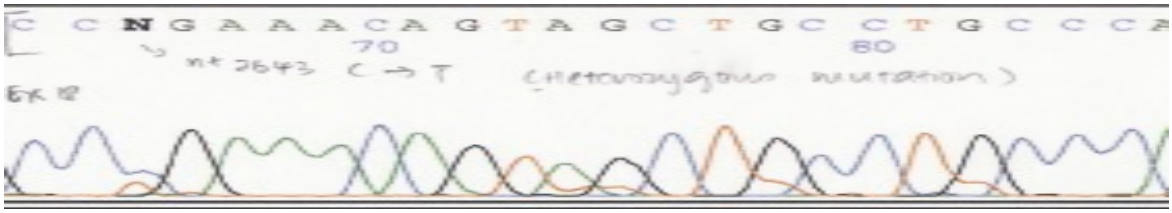




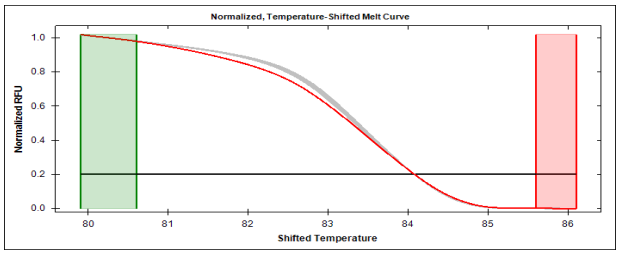
Patient 227

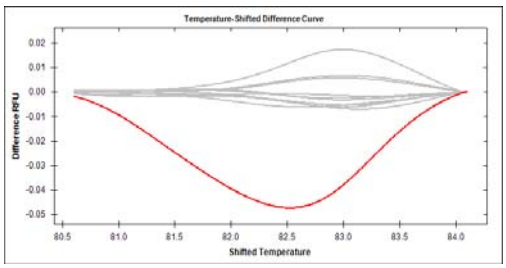


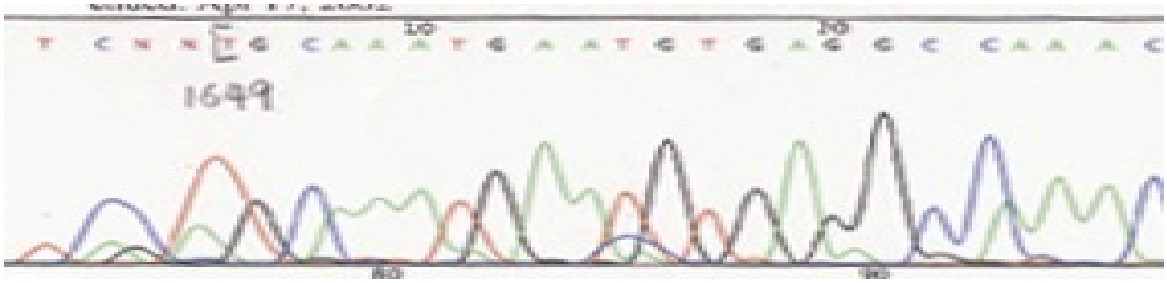




Patient 311







Appendix F – FASTA Sequence input in PolyPhen Protein Prediction Software

```
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