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Mutations in *RPSA* and *NKX2-3* link development of the spleen and intestinal vasculature

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Abstract

Idiopathic intestinal varicosis is a developmental disorder defined by dilated and convoluted submucosal veins in the colon or small bowel. A limited number of families

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with idiopathic intestinal varices has been reported, but the genetic cause has not yet been identified. We performed whole exome and targeted Sanger sequencing of candidate genes in five intestinal varicosis families. In four families mutations in the *RPSA* gene were found, a gene previously linked to congenital asplenia. Individuals in these pedigrees had intestinal varicose veins and angiodysplasias, often in combination with asplenia. In a further four generation pedigree that only showed intestinal varicosities, the *RPSA* gene was normal. Instead, a nonsense mutation in the homeobox gene *NKX2-3* was detected which co-segregated with the disease in this large family with a LOD score of 3.3. *NKX2-3* is a component of a molecular pathway underlying spleen and gut vasculature development in mice. Our results provide a molecular basis for familial idiopathic intestinal varices. We provide evidence for a relationship between the molecular pathways underlying the development of the spleen and intestinal mucosal vasculature that is conserved between humans and mice. We propose that clinical management of intestinal varices, should include assessment of a functional spleen.

Introduction

The presence of dilated and convoluted submucosal veins in the colon or small bowel, referred to as intestinal varices is a rare clinical entity with a poorly understood aetiology(Speicher, et al., 2014). Intestinal varices may cause recurrent bleeding of the lower gastrointestinal tract or may be noticed in an asymptomatic individual upon colonoscopy. The most prevalent cause of varices in the digestive tract is portal hypertension. About one quarter of reported cases of varices coli are idiopathic, and 30% of these are familial.(Han, et al., 2006) Thus far, at least 12 families with idiopathic intestinal varices have been reported in literature but no genetic cause has been identified.(Atin, et al., 1993; Beermann, et al., 1988; Bernardini, et al., 1998;

Boland, et al., 2014; el-Dosoky, et al., 1994; Hawkey, et al., 1985; Iredale, et al., 1992; Kori, et al., 2000; Morini, et al., 1993; Solis-Herruzo, 1977; Zaman, et al., 2008). In some families siblings are affected(Atin, et al., 1993; Boland, et al., 2014; Kori, et al., 2000), suggestive of autosomal recessive inheritance, while in other families the intestinal varices occur in two generations(el-Dosoky, et al., 1994; Solis-Herruzo, 1977; Zaman, et al., 2008), consistent with autosomal dominant inheritance. Here we describe five families with autosomal dominant intestinal varices, and identify mutations in the *RPSA* gene or *NKX2-3* gene as genetic cause Although evidence for a direct interaction between RPSA- and NKX2-3-related pathways is currently lacking, we show evidence from multiple sources that links the molecular programs underlying development of the intestinal vasculature and spleen, both in humans and mice.

Materials and Methods

Whole exome sequencing

After obtaining written informed consent, whole exome sequencing was done using DNA isolated from blood, as described previously(Lelieveld, et al., 2016). Briefly, exome capture was done using the Agilent SureSelect v4 kit (Agilent, Santa Clara, CA). Exome libraries were sequenced on an Illumina HiSeq instrument (Illumina, San Diego, CA) with 101 bp paired-end reads at a median coverage of 75X and with >95% of exons having coverage >30X. Sequence reads were aligned to the hg19 reference genome using BWA version 0.5.9-r16. Variants were subsequently called by the GATK unified genotyper, version 3.2-2 and annotated using a custom diagnostic annotation pipeline. Variants were filtered for having less than 1% frequency in dbSNP, having less than 1% frequency in our in-house database and having less than 1% frequency in the ExAC database (www.exac.broadinstitute.org).

Sanger sequencing

Sanger sequencing was done according to standard procedures, using M13 tailed forward and reverse primes for each exon of the *RPSA* gene or *NKX2-3* gene. Primer sequences are given in Supp. Table S1.

Patients gave informed consent for the genetic studies, which were done in a routine diagnostic setting, and for inclusion of the data in this manuscript.

Variants detected in the *NKX2-3* gene and *RPSA* gene were reported to the Global Variome shared Leiden Open Variation Database, at respectively https://databases.lovd.nl/shared/genes/RPSA and https://databases.lovd.nl/shared/genes/NKX2-3.

Results

Patients

The index patient in family 1 presented with anaemia from the age of 10, which required blood transfusions on several occasions. Colonoscopy demonstrated intestinal varices in the colon and to a lesser extent in the small bowel. At age 48, he developed bacterial meningitis. Abdominal imaging showed absence of the spleen. The index patient in family 2 had been hospitalized with bacterial meningitis at the age of 3 years. She developed severe anaemia from the age of 27 years. She was hospitalized repeatedly for recurrent gastrointestinal bleedings from varicose veins in the ascending and transverse colon. There was involvement of the small bowel with varicosities in the jejunum. On abdominal imaging, the spleen was described as “multiseptated” or ”fragmented”, possibly representing polysplenia. Family 3 was reported previously as presenting with idiopathic congenital asplenia(Bolze, et al., 2013). We re-evaluated the clinical data for some of the individuals from this family, who were known to have

ectatic blood vessels in their intestines. The index patient presented at 17 years of age with severe fatigue and anaemia managed with repeated transfusions as no cause could be identified at that time. Asplenia was detected on an abdominal CT scan. At age 24 years, an exploratory laparotomy identified abnormal dilated vessels in the wall of the distal duodenum. Since then the patient has undergone repeated argon laser cauterisation of duodenal blood vessels approximately every 6 months as they are inoperable. Family 4 has been reported previously(Wurfel, et al., 2011). The index patient had a history of iron-deficiency anaemia necessitating repeated erythrocyte transfusion from the age of 2 years. At 16 years of age, gastroduodenoscopy and capsule endoscopy revealed distorted teleangiectatic vessels in the stomach and numerous angiodysplastic lesions in the duodenum and jejunum. A number of bleeding lesions were treated by argon plasma coagulation. Abdominal imaging confirmed asplenia. The index patient of family 5 (patient IV:1 in figure 1) had recurrent episodes of rectal bleeding for which she first underwent colonoscopy and upper gastrointestinal endoscopy at age 7 years. This failed to demonstrate a source for the intestinal blood loss. Because of recurring rectal bleeding she underwent a second colonoscopy at age 10 years, which showed prominent, distended blood vessels in the sigmoid (see figure 2). At the age 13 years, a massive bleeding occurred, requiring transfusion of packed red blood cells. Abdominal imaging documented a morphologically normal spleen. Pedigrees of families 1-5 are shown in figure 1. Extended case reports are given in the Supplementary Methods.

Detection of pathogenic variants in the *RPSA* gene or *NKX2*-3 gene

Heterozygous likely pathogenic variants in the *RPSA* gene were detected in families 1-4 by exome sequencing followed by confirmation with Sanger sequencing and/or by targeted Sanger sequencing (figure 1 and figure 3 and Supplementary data). No variants

in the NKX2-3 gene were found in these families. In family 1 a heterozygous c.223dup (p.(Ser75Lysfs*36)) frameshift variant in exon 3 of the *RPSA* gene (NM_002295.5) was found resulting in a premature termination codon in the *RPSA* transcript and most likely leading to haploinsufficiency. Material of the deceased and similarly affected father or of other family members was not available for testing. In family 2, a c.252G>C (p.(Gln84His)) missense variant was detected in both the affected father and daughter, which affects an evolutionairy conserved amino acid residue in the RPSA protein (conserved up to *Saccharomyces cerevisiae*) . A different substitution of the same amino acid residue (p.Gly84Arg) was previously reported in a family with apparently isolated asplenia(Bolze, et al., 2013). In family 3 a c.538C>G (p.(Arg180Gly)) amino acid substitution was found, which was previously discovered and described in the context of a study on isolated congenital asplenia(Bolze, et al., 2013). In family 4, a c.542A>T (p.(Glu181Val)) variant was detected in RPSA, affecting an evolutionairy conserved amino acid residue (conserved up to *Saccharomyces cerevisiae*). None of these *RPSA* variants are present in control individuals from the GnomAD database (gnomad.broadinstiture.org).

In family 5 the whole exome sequencing data of two distantly related affected relatives were compared (i.e. individuals IV:1 and III-9; see pedigree in figure 1), using the filter settings detailed above. Among the shared variants (see Supp. Table S2), a heterozygous c.268del (p.(Gln90Argfs*25)) variant in the *NKX2-3* gene (NM_145285.2) was detected thereby, and subsequently confirmed by Sanger sequencing in these two individuals. Given the absence of this variant in the ExAC database and the fact the variant presumably leads to loss-of-function, other family members were investigated. In individuals, III-4, III:5, III:1, III:8, and III:10 from family 5 the presence of this frameshift variant in the *NKX2-3* gene was confirmed by targeted Sanger sequencing. . Between these 2 family branches there are obligate

carriers of this variant. The single nucleotide deletion causes a frameshift resulting in a premature termination codon in the *NKX2-3* transcript, probably leading to haploinsufficiency. The variant co-segregated with the intestinal varices in this family, resulting in a LOD score of 3.3, indicating that the probability that this particular variant is shared in the five affected family members solely by chance is less than 1 in 1000. *NKX2-3* is predicted to be highly intolerant to loss-of-function (LoF) variation, as indicated by absence of LoF variants in the gene in the "Exome Aggregation Consortium" (ExAC) database(Lek, et al., 2016). The gene has a Probability of Loss-of-Function Intolerance (pLI) of 0.95 which is very high for such a small gene and an "observed over expected ratio for LoF variants of 0.00 (gnomad.broadinstitute.org/gene/ENSG00000119919)(Lek, et al., 2016). The Database of Genomic Variants currently lists no copy number losses or gains for the *NKX2-3* gene, further indicating the gene to be intolerant to dosage variation.

Genotype-phenotype correlation

The families with *RPSA* variants (families 1-4) all had a combination of asplenia and intestinal varices. In family 5, several individuals presented with rectal bleeding. The clinical presentation ranged from mild to severe intestinal bleeding requiring surgical intervention (supplementary case reports). However, some obligate carriers of the mutation (family 5, II:9, II:12) have no known history of rectal bleeding or anaemia, suggesting variable involvement, and possibly reduced penetrance. Such individuals might still have asymptomatic intestinal varices as there was no clinical indication to perform a colonoscopy in them. In family 5, only intestinal varices is present in individuals with the *NKX2-3* variant. Two individuals (III:4 and VI:1) were found to have normal spleens on abdominal ultrasound imaging, but other individuals with intestinal varices did not have assessment of the spleen. None of the individuals had

other signs of varicosis or angiodysplasia such as epistaxis, lung or other organ involvement, or varicosis of the legs.

Discussion:

Here we report the first genetic cause for idiopathic intestinal varices, i.e. mutations in either the *RPSA* gene (in 4 unrelated families) or the *NKX2-3* gene (in an extended 4 generation pedigree). The *RPSA* gene encodes the SA ribosomal protein, which has myriad functions, among which are laminin binding, ribosomal functions, nuclear functions, modification of the extracellular matrix, and signal transduction(DiGiacomo and Meruelo, 2016). Our study establishes *RPSA* mutations as an important cause of intestinal varices and expands the clinical phenotypic spectrum associated with this gene. Previous work showed that *RPSA* mutations cause congenital asplenia with variable penetrance in humans(Boland, et al., 2014; Bolze, et al., 2018). Asplenia was also part of the extended phenotype in families described here. Both asplenia and intestinal varices may be occult for many years. Nonetheless, both disorders may have severe consequences, with asplenic patients developing meningitis or other forms of severe septic infections, and intestinal varices sometimes leading to severe bleeding necessitating hospitalizations, transfusions, and in some the removal of sections of the intestine. Several persons with RPSA mutation in these pedigrees developed bacterial meningitis or pneumonia, suggesting that persons with intestinal varices should be examined for the presence of a functional spleen.

A synthetic peptide recapitulating amino acids 161-180 of the *RPSA*-encoded Laminin-receptor protein binds to laminin with high affinity(Castronovo, et al., 1991). Others have argued based on the crystal structure of the laminin receptor protein that of this putative laminin-binding domain, only amino acid R180 is solvent-exposed, with a critical role for a binding face involving Phe-32, Glu-35 and Arg-155(DiGiacomo and

Meruelo, 2016; Jamieson, et al., 2011; Jamieson, et al., 2008). However, as far as we know requirement specifically of the Arg-180 residue for laminin binding properties of *RPSA* has not been experimentally proven, and Griffin et al. showed that this residue is required for pre-rRNA processing(Griffin, et al., 2018). It is therefore unclear at the moment which cellular process exerted by NKX2-3 are at the basis of asplenia pathogenesis.It is striking however that Arg-180 and the adjacent Gln-181 appear to constitute a hotspot for mutations leading to asplenia and intestinal varices(Bolze, et al., 2018)(figure 3).

The *NKX2-3* gene belongs to the NKX class of homeobox genes which are key regulators of spleen ontogeny in embryogenesis ((Brendolan, et al., 2005; Brendolan, et al., 2007). *NKX2-3* is closely related to the *NKX2-5* gene, which plays a role in cardiogenesis, and spleen development(Koss, et al., 2012). NKX2-3 functions in development and function of the intestinal lymphoid system and intestinal vasculature(Kellermayer, et al., 2016; Yu, et al., 2011). *Nkx2-3* is embryonically and postnatally expressed in the midgut and hindgut of the mouse and the chicken(Pabst, et al., 1997; Wang, et al., 2000). High human NKX2-3 mRNA expression is restricted to the colon, ileum and spleen (gtexportal.org/home/gene/NKX2-3)(Pabst, et al., 1997). *NKX2-3* has hitherto not been linked to a genetic disorder but the gene is a strong candidate gene for intestinal varices since it is expressed in human intestinal microvascular cells (HIMECs) where it regulates VEGFA and MADCAM-1 signalling(Wang, et al., 2000; Yu, et al., 2011). Wang et al.(Wang, et al., 2000) replaced the NK-2 specific domain of *Nkx2-3* with a LacZ construct in mice. They noted positive staining for LacZ in pharyngeal and visceral regions, including the vascular smooth muscle and endothelial cells of capillaries and small blood vessels of the intestine. Many of the *Nkx2-3*$^{lacZ\Delta HD}$ homozygous mice died in the first weeks after birth, due to intestinal malabsorption. A striking observation was the presence of blood in the

intestinal lumen. Mice who survived the neonatal period recovered and were apparently normal. Pabst et al.(Pabst, et al., 1999) studied *Nkx2-3* null mice generated by targeted gene disruption. Homozygous *Nkx2-3* -/- mice were growth retarded, and the majority died before 3 weeks after birth. Reduced proliferation of the epithelium was shown in the fetal small intestine. In adult homozygous knockout mice, the small intestine showed altered villus morphology and increased villus size. Extensive extra vascularization of the small intestine was noted in these mice.. Remarkably, the intestinal changes in *NKX2-3* knockout mice were limited to the jejunum and ileum and absent in the colon, even though *Nkx2-3* is also expressed in the hindgut. Outside of the digestive tract, splenic malformations were noted, with absent spleens in 20% of *Nkx2-3* -/- mice and abnormalities in size or morphology of the spleen in the other mice(Pabst, et al., 1999). Another study found ectopic vessel formation in the spleen of *Nkx2-3* mutant mice, which were described as “high endothelial venule (HEV)-like” (Kellermayer, et al., 2016).

Currently no direct molecular connection between NKX2-3- and RPSA-related pathways is known, but data reviewed here provide hints that both genes may be involved in the same molecular processes in spleen and intestinal vasculature development from mesenchymal tissue during embryogenesis. An anatomical relationship may exist between asplenia and vascular malformation as the former is associated with anomalous venous drainage and possibly subsequent arteriovenous malformation(Arnautovic, et al., 2017). Our finding that mutation of both *RPSA* and of *NKX2-3* can cause intestinal varices complements previous studies showing that RPSA mutations affect spleen development in humans, and that knockout of *NKX2-3* disrupts spleen development in mice. It is currently unclear why asplenia is not a feature associated with NKX2-3 mutations in humans. Possibly, the expression of the spleen phenotype may be variably penetrant as has been described for *RPSA* mutations(Bolze,

et al., 2018) and the condition may be occult. The identification of more patients with NKX2-3 mutations in the future may define a broader clinical phenotypic spectrum linked to NKX2-3 mutations.

A possible molecular link between RPSA and NKX2-3 could be through NKX2-5, since NKX2-3 and NKX2-5 can heterodimerize(Kasahara, et al., 2001) and NKX2-5 is part of a functional module that contributes to the development of the spleen in mouse(Burn, et al., 2008; Czompoly, et al., 2011; Koss, et al., 2012). Moreover, depletion of RPSA in *Xenopus* causes severe reduction of NKX2-5 mRNA expression, that can be rescued by WT human RPSA mRNA but not by mutant p.Arg180Gly RPSA mRNA(Griffin, et al., 2018). Strikingly, a frameshift variant in the NKX2-5 gene was found in sporadic patient with asplenia and heart defects(Izumi, et al., 2014; Koss, et al., 2012).

In summary, we find that mutations in at least two genes can cause familial idiopathic intestinal varices, and hypothesize that there may be links between the molecular pathways involved in development of the spleen and of the intestinal vasculature.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends.

Figure 1.

Pedigrees of families 1-5 described in this study. The proband in each family is indicated by an arrow. Filled (black) symbols indicate intestinal varices. An asterisk indicates the presence of a heterozygous variant in either the *RPSA* or *NKX2-3*, i.e. in family 1 a c.223dup (p.(Ser75Lysfs*36)) variant in *RPSA*, in family 2 a a c.252G>C (p.(Gln84His)) variant in *RPSA*, in family 3 a c.538C>G (p.(Arg180Gly)) variant in *RPSA*, in family 4 a c.542A>T (p.(Glu181Val)) variant in *RPSA* and in family 5 a c.268del (p.(Gln90Argfs*25)) variant in *NKX2-3*.

Figure 2.

Macroscopical presentation of intestinal varices as assessed by colonoscopy. Arrows indicate examples of intestinal varices as observed in the proband of family 1, family 3 and family 5 (from left to right respectively).

Figure 3.

Schematic representation of the *RPSA* gene (NM_002295.5) with published exonic non-synonymous RPSA mutations. Exons: squares, coding exons 2 through 7, introns: lines. Below exons: exon and amino acid numbering . Red rectangles: proposed laminin binding sites at aa.161-180 and aa.205-229 and blue rectangle: predicted transmembrane domain at aa.86-101. Above the gene schematic: novel mutations identified in this publication. Below dotted line: previously published mutations(Bolze, et al., 2018; Bolze, et al., 2013).

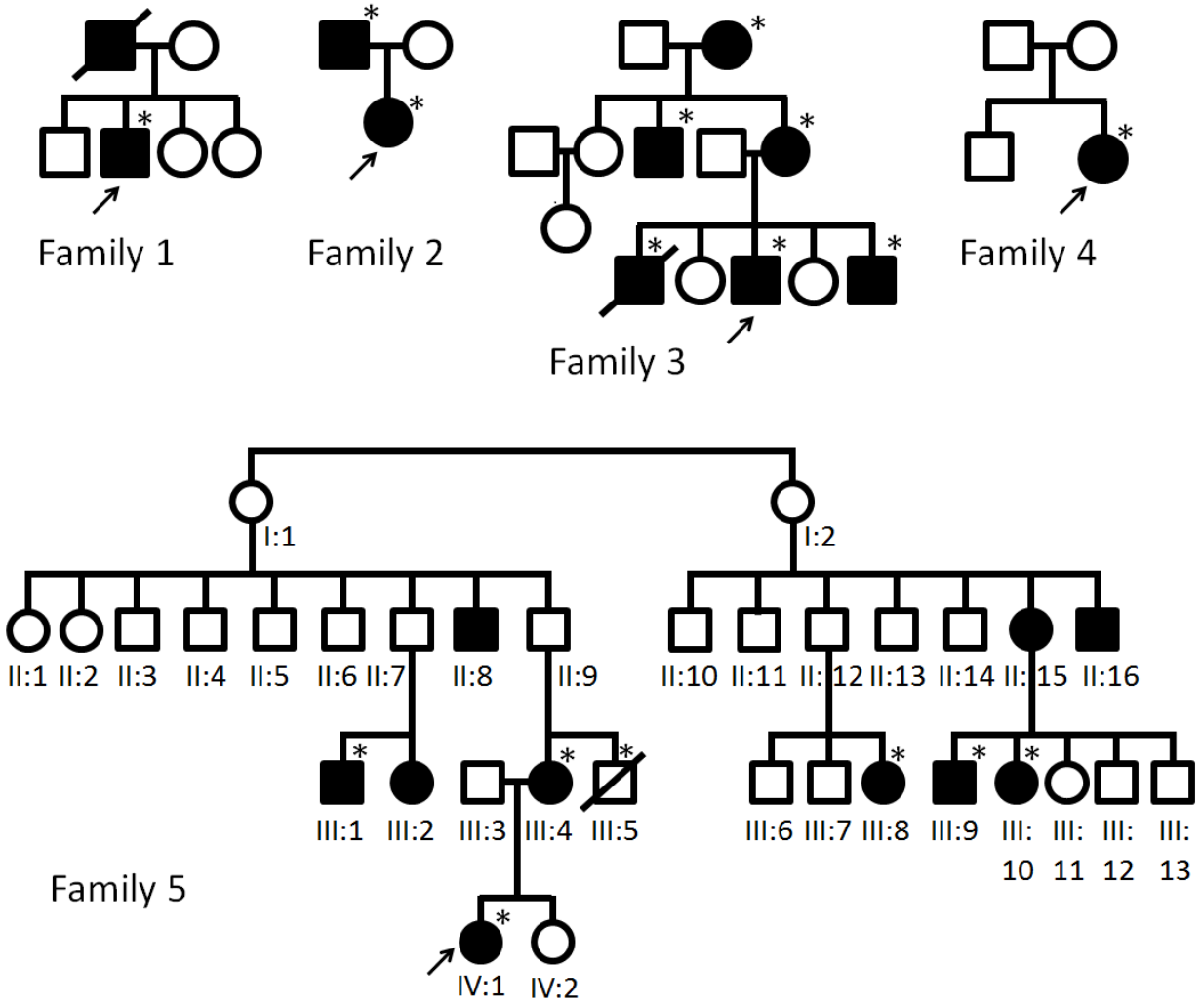

FIGURE 1

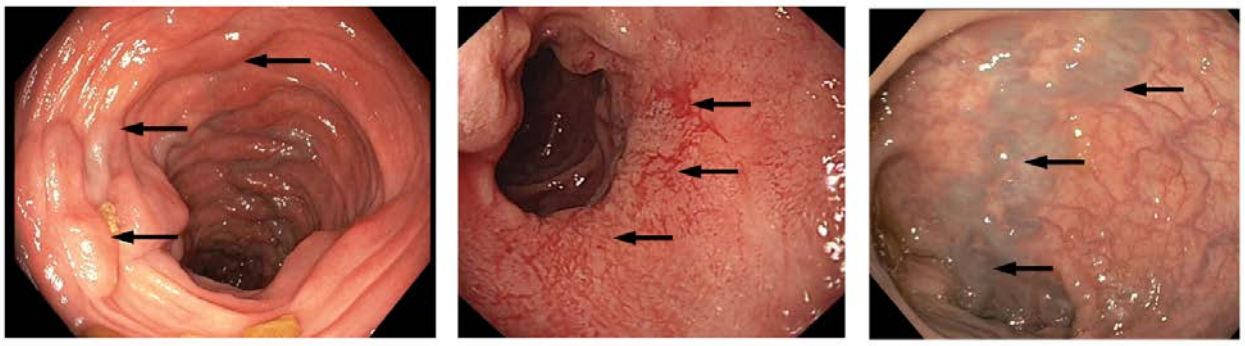

FIGURE 2

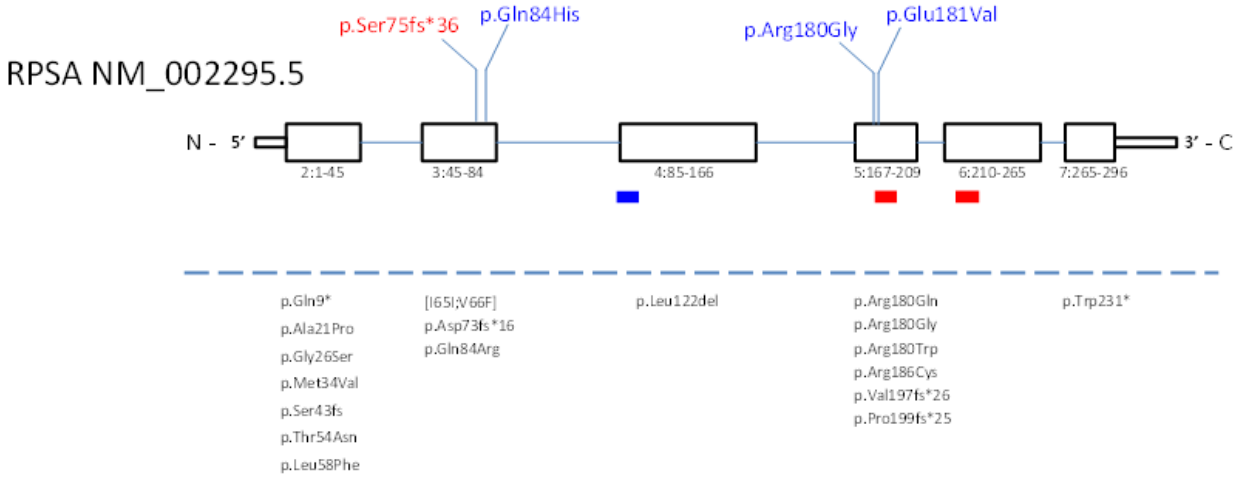

FIGURE 3