A SNP in protamine 1: a possible genetic cause of male infertility

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ABSTRACT

Introduction Gene targeting of the sperm nuclear proteins, the protamines, in mice leads to haploinsufficiency, abnormal chromatin compaction, sperm DNA damage, and male infertility. Because of the critical roles the protamines play in spermatid differentiation, aberrations in protamine expression or changes in protein structure could be causes of certain idiopathic human male infertilities.

Method In order to investigate whether changes in amount or structure of the protamines could be a cause of human infertility, we sequenced the protamine genes of infertile men whose sperm appeared phenotypically similar to those of protamine deficient mice.

Result We identified a heterozygous single nucleotide polymorphism (SNP) in the protamine (*PRM1)* gene in three infertile men (10% of the total infertile men analyzed). This SNP disrupts one of the highly conserved arginine clusters needed for normal DNA-binding. To rapidly screen for this SNP in infertile patients, we developed a simple PCR-restriction fragment length polymorphism assay.

Discussion This is the first report of a SNP in the protamine 1 gene that appears associated with human male infertility. Protamine deficiencies and aberrant protamine ratios have been linked to reduced fertilization rates, and PRM1 has been proposed to be a potentially critical factor in post-ICSI human embryonic development. Our PCR-RFLP assay provides an easy means to screen and detect G197T SNPs in idiopathic infertile males.

INTRODUCTION

Approximately 15% of couples are affected by infertility, with the male being responsible for nearly half of the cases.[1] Despite advances in assisted reproduction technologies making paternity possible for many of these men, the genetic causes of the majority of the male infertilities remain unknown. Gene targeting in the mouse has provided valuable insights into the genetic etiology of many human diseases and infertilities.[2]

During spermatogenesis, diploid spermatogonia replicate and differentiate into spermatocytes, which undergo meiosis producing four spermatids. As the haploid spermatids develop into sperm, specialized structures such as the acrosome, tail, and a novel highly condensed nucleus are produced. In sperm nuclei the DNA-protamine complex compacts, stabilizes, and protects the haploid genome. Protamines are small basic proteins widely conserved among species. All mammals have one protamine, PRM1, while some species including human and mouse have a second protamine, PRM2. Both proteins are rich in arginine and cysteine, amino acids needed for DNA-binding and disulfide bridge formation.[3] [4] Disruption of either the *Prm1* or *Prm2* gene in mice leads to haploinsufficiency, abnormal chromatin compaction, sperm DNA damage, and male infertility.[5] [6] Because of the critical roles the protamines play in spermatid differentiation, aberrations in protamine expression or changes in protein structure could be causes of certain idiopathic human male infertilities.

To address this issue, we sequenced the genomic loci of the *PRM1* gene in 30 infertile male patients whose sperm showed phenotypes similar to the sperm of the protamine deficient knockout mouse.

MATERIALS AND METHODS

Patients

All patients and donors participating in this study were recruited with full approval from and oversight by Baylor College of Medicine's Institutional Review Board for Human Subjects (Houston, TX, USA). DNA was extracted from blood samples of thirty unrelated infertile patients, as well as from ten men of pregnancy-proven fertility who served as controls. The sperm of the thirty infertile males were similar to the sperm derived from protamine deficient mice.[5] The sperm counts were normal, but the sperm showed abnormal DNA fragmentation (>27% of the sperm fragmented) and/or abnormal morphology based upon Kruger's strict criteria (<4.4% normal forms).[7] Known causes of infertility were excluded and their physical examination, endocrine profiles, semen parameters (except for strict morphology defects and/or DNA damage) were normal. Because their sperm count was normal, they were not analyzed for Y chromosome micro-deletions.

Genomic analysis of the *PRM1* gene

To analyze the *PRM1* gene locus, PCR-directed sequencing was performed with DNA purified from the blood from ten normal fertile men and thirty infertile males. RT-PCR was performed with a primer set designed to be homologous to the genomic regions for *PRM1* on chromosome 16 [8][9] [10]; Pr597 (forward): 5'-cat agg cag ccc cta cac tc-3' and Pr087 (reverse): 5'-ccc tct caa gaa caa gga gag aa-3'. Using the primer set, we were able to amplify fragments of 644 nucleotides inside of the primer set (from nucleotides –160 to 9 base pairs (bp) after the end of transcription) of the *PRM1* gene locus. The PCR conditions were as follows: 1 cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 45 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. The resultant PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA) and were directly sequenced in both orientations using the PCR primers as sequencing primers. When mutations were detected, the results were confirmed by the direct sequencing of two independently prepared PCR products to rule out PCR artifacts.

A restriction fragment length polymorphism (RFLP) assay that detects the G197T SNP

In the analyses of the *PRM1* gene, a heterozygous G to T single nucleotide polymorphism (SNP) at nucleotide 197 was identified in three men. The G197T mutation disrupts the recognition site of the restriction enzyme *Bse*RI (changing GAGGAG to GAGTAG). To develop a restriction fragment length polymorphism (RFLP) assay for this SNP, the PCR products were digested with *Bse*RI for 1h at 37^{o}C and separated on the 1.5% agarose gel. The DNA fragments were visualized by ethidium bromide staining.

RESULTS

One heterozygous single nucleotide polymorphism (SNP) at nucleotide 197 was identified in the DNA of three of the thirty infertile men examined (10%). To rule out PCR artifacts, the SNP was confirmed by the direct sequencing of at least two independently prepared PCR products. A single wave of G was seen at nucleotide 197 in samples from fertile control men, while two overlapping patterns, one common type (G) and one variant (T), were seen in the three unrelated infertile men indicating a polymorphism of one *PRM1* allele (Fig. 1). This SNP causes an amino acid change of arginine to serine at codon 34 in a highly conserved arginine cluster (Fig. 2A, B). No identical SNP was observed in a total of 779 men [the 37 men tested in the current study (10 pregnancy-proven fertile control and 27 infertile), in previous studies a total of 522 men (270 fertile, 226 sterile, and 26 unselected) or in 220 individuals from the Ensembl human GeneSNPView and NCBI SNP databases].[10] [11][12] Although two other SNPs in the coding region of *PRM1*, at codons 45 (SNP ID; rs11544792) and 47 (SNP ID; rs737008), have been reported [12], and three other SNPs at codons 14, 23, and 46 were reported in a previous study [10], all 5 SNPs are synonymous, causing no amino acid change.

By sequence analysis, we found the SNP disrupts the cutting site of the restriction enzyme *Bse*RI (GAGGAG). PCR products were digested with *Bse*RI and separated on agarose gels. One rapidly migrating band, major-type 197G, containing 2 fragments of 369 and 315 bp, was detected with DNA from a fertile control and an infertile patient with two alleles carrying the G in the *PRM1* gene. Analysis of DNA from three patients carrying a G197T SNP (patients 143, 199 and 599) revealed one slower migrating DNA band of 684 bp (Fig. 2C). Thus, this RFLP assay provides a simple and rapid screening method to detect this polymorphism.

DISCUSSION

We have identified a novel SNP, G197T, in the PRM1 gene in three from our selected thirty infertile men. This SNP causes an amino acid change from arginine to serine (R34S) in a highly conserved arginine cluster. Based upon the absence of this SNP in more than 770 individuals (220 from Ensemble human GeneSNP database; 522 from previous reports and 10 proven fertile controls, 27 infertile men from this study), it appears to be a uncommon SNP.[10] [11][12] In addition to disrupting an arginine core essential for DNA-binding, the SNP creates a new RS sequence, a putative phosphorylation site for the enzyme, SR protein-specific kinase 1, known to phosphorylate serines in the RS motifs of PRM1 (Fig.1B).[13] [14] Such phosphorylations are associated with the deposition of protamines on sperm chromatin and the subsequent chromatin condensation.[13] [14] Thus, improper phosphorylation could substantially alter both DNA-binding and protamine-protamine interactions in the sperm nucleus. Furthermore, Robson's secondary structure prediction program (GENETYX, GENETYX CORPORATION, Tokyo, Japan) suggests the R34S amino acid substitution disrupts a beta sheet structure in PRM1 (data not shown), an event likely to change protein conformation. The high incidence of DNA fragmentation in the sperm from the infertile patients carrying the SNP supports our hypothesis of altered protamine-DNA interactions. Taken together, the mutant protein caused by the G197T SNP may impact its binding to DNA and affect chromatin compaction. Although uncommon, we cannot exclude the possibility that this SNP may not directly cause the infertility.

PRM2 defects have been reported to be associated with human male infertility (0.4% of 226 infertile males) [10], on the other hand, the SNP that we identified is the first case to link an amino acid substitution in PRM1 with human infertility. The fact that the sperm of all mammals investigated to date contain protamine 1, but only a subset contain protamine 2 may in part explain the small number of SNPs reported for the *PRM1* gene and is additional evidence for the critical functions of PRM1 in male fertility. As seen with the protamine deficient mice [5], the SNP has a deleterious effect as a heterozygous mutation perhaps because the protamines are first expressed post-meiotically [15] and the germ cells are interconnected in a clonal syncytium by intercellular bridges.[16] [17] [18]

To develop a rapid assay to detect this SNP in infertile patients, we have taken advantage of the fact that the G197T SNP disrupts the cutting site of the restriction enzyme, *Bse*RI. A PCR fragment of 197T was not digested by *Bse*RI, while the fragment of major-type 197G was. Because murine embryonic development is unsuccessful following intracytoplasmic sperm injection (ICSI) with DNA damaged sperm resulting from a protamine insufficiency [6], we speculate that an arrest of embryo development may contribute to the infertility of men carrying the DNA damaged sperm of the G197T SNP. Protamine deficiencies and aberrant protamine ratios have been linked to reduced fertilization rates [19] [20] [21], and PRM1 has been proposed to be a potentially critical factor in post-ICSI human embryonic development.[22] Our PCR-RFLP assay provides an easy means to screen and detect G197T SNPs in idiopathic infertile males.

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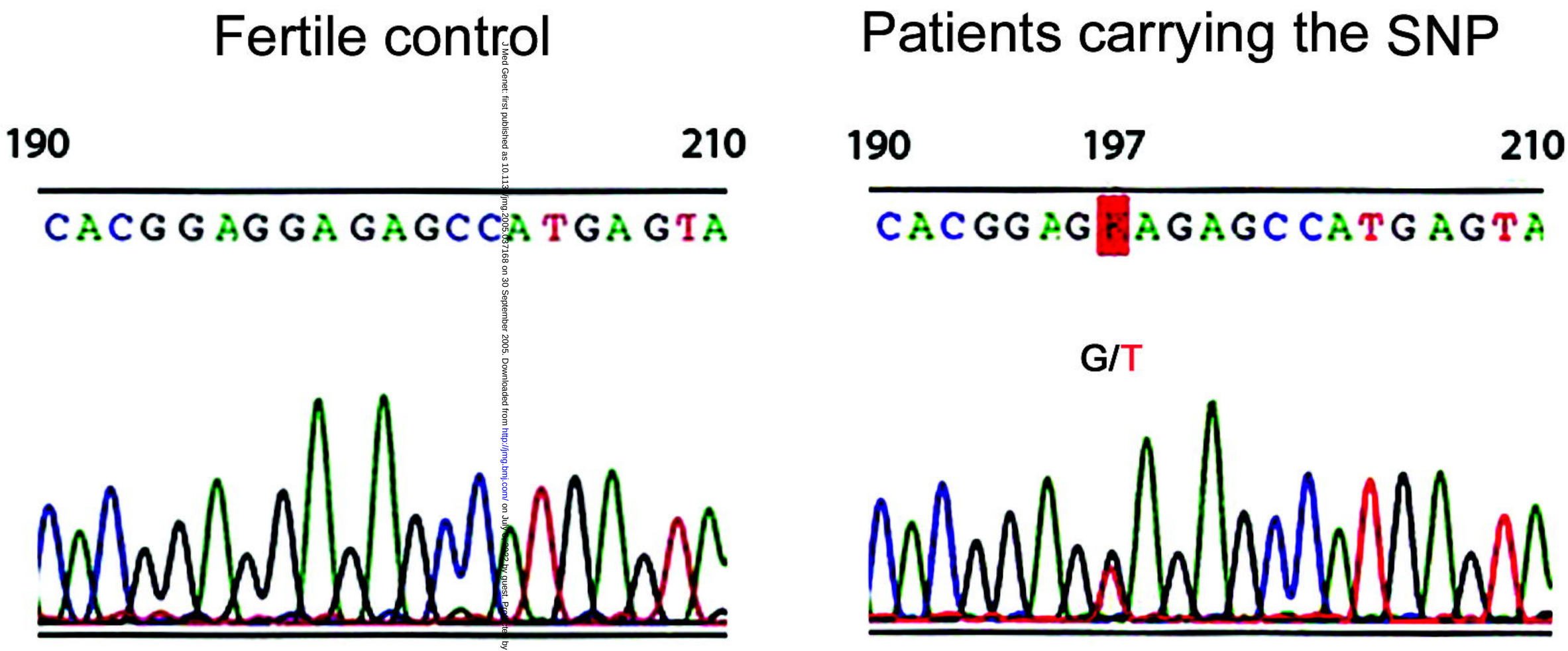
LEGENDS

Figure 1. Sequence profiles of *PRM1* (190 to 210 nt) from a normal fertile man and the three infertile patients carrying the SNP.

Figure 2. A. Schematic representation of the *PRM1* gene and the site of the nucleotide and predicted amino acid sequences adjacent to the SNP. The transcription start site is defined as +1. White boxes indicate the 5' and 3' untranslated regions. The solid bar indicates the genomic region of *PRM1* and contains the two exons and an intron of the gene. The gray boxes indicate the coding region of *PRM1* with the SNP position marked by an arrow. The amino acid sequence is shown in capital letters under the nucleotide sequence. The numbers in the right margin indicate the positions of nucleotides and amino acids. The altered sequences are shown in bold italicized letters. B. Comparison of protamine 1 protein sequences among species. The amino acid substitution from the G197T SNP disrupts one of the conserved arginine clusters. Asterisks indicate the amino acid positions, previously reported as synonymous SNPs. C. PCR-restriction fragment length polymorphism analysis of genomic DNA from a fertile man (control) and four infertile patients. Patient 149 is infertile but lacks a G197T SNP, while patients 143, 199 and 599 have the G197T SNP. M indicates DNA size markers. Samples were incubated with (+) or without (-) the restriction enzyme, *Bse*RI.



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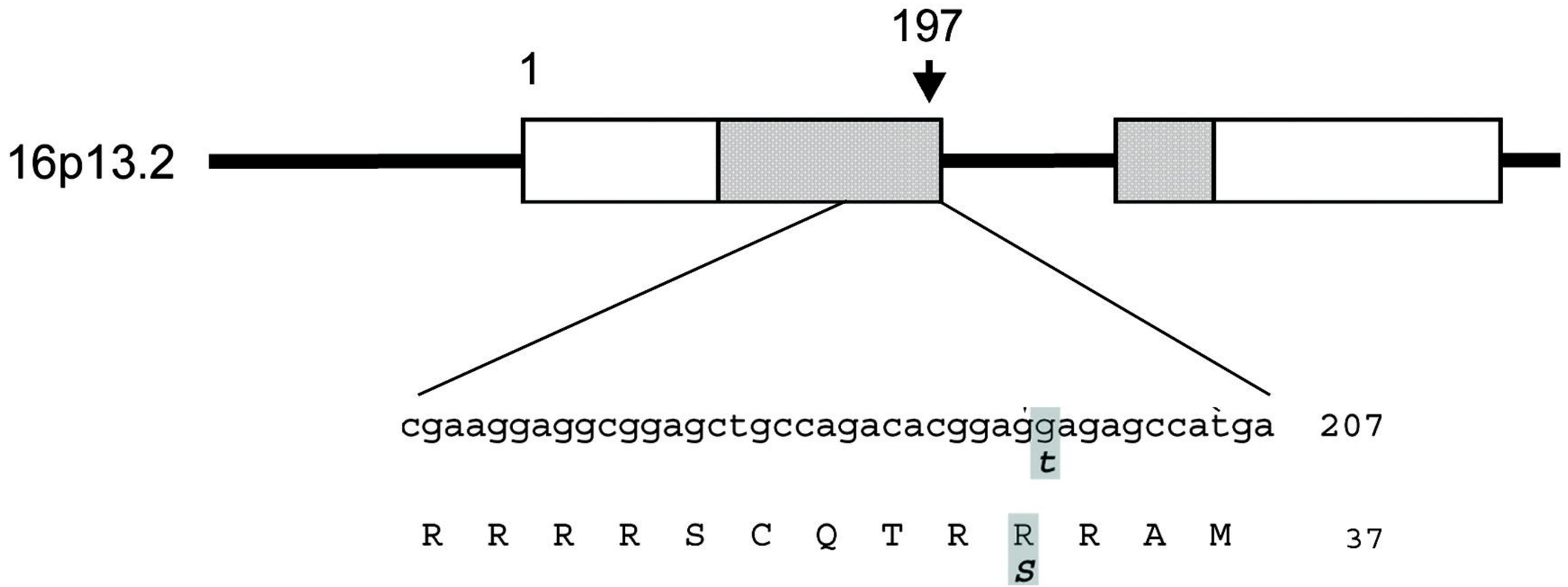

B

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Salmon   1 MPRR--RRSSSRPVRRRRRPRVSRRRRRRGGRRRR--------------------   33
Mouse    1 MARYRCCRSKSRSRCRRRRRR-CRRRRRRCCRRRRRR--CCRRRRSYTI-RCKKY   51
Rabbit   1 MARYRCCLTHSRSRCRRRRRRRCRRRRRRFGRRRRRR-VCCR-R--YTVVRCTRQ   51
Sheep    1 MARYRCCLTHSRSRCRRRRRRRCRRRRRRFGRRRRRR-VCCR-R--YTVVRCTRQ   51
Human    1 MARYRCCRSQSRSRYYRQRQRS-RRRRRRSCQTRRRAMRCCRPR--YRP-RCRRH   51
Mutant                 *         *          S        ***
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C