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A haplotype at intron 8 of *PTPRT* gene is associated with resistance to *Brucella* infection in Argentinian creole goats

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Highlights

- Five SNPs were found at intron 8 of *PTPRT* caprine gene in Argentinian creole goats.
- A new SNP located at the position 69169526 was identified.
- TTCCT haplotype was associated with absence of *Brucella*-specific antibodies.
- Identification of genetic markers could help of limiting the spread of brucellosis.

Abstract

Brucellosis is the leading zoonosis on a worldwide scale and constitutes a major public health threat in many regions of the world. Several molecular markers associated with natural resistance to intracellular bacterial infection have been identified. Recently seven single-nucleotide polymorphisms (SNPs) located in the *PTPRT* gene were associated with resistance to *Mycobacterium bovis* infection in cattle. Here, we perform a case-control study to test if polymorphisms at *PTPRT* intron 8 might influence the resistance or susceptibility to *Brucella* infection in goats. DNA samples from 22 seropositive (cases) and 22 seronegative (controls) for brucellosis, unrelated female creole goats, were included in the present study. Four previously reported polymorphisms (SNP1: rs643551276, SNP2: rs651618967, SNP3: rs662137815 and SNP4: rs657542977) and a new SNP (SNP5: chr13: 691695526) were detected by PCR-DNA sequencing method. Genotypic and allelic frequencies differed significantly between cases and controls at SNPs 1, 2, 4 and 5 ($p \leq 0.001$). Indeed, the SNP1 TT, SNP2 TT, SNP4 CC and SNP5 TT genotypes were associated with absence of *Brucella*-specific antibodies (ORs = 0.019 to 0.045). Moreover, haplotype association analysis revealed a significant association of the TTCCT haplotype with protection to *Brucella* infection ($p \leq 1 \times 10^{4}$; OR=21), including the major allelic variants associated with resistance. These results represent the first evidence of genetic association between polymorphisms in the *PTPRT* gene and absence of brucellosis in goats.

Keywords: *PTPRT* gene; goats; brucellosis; genetic resistance; polymorphisms; haplotype

Introduction

Brucellosis is a zoonotic disease with a high socioeconomic impact caused by facultative intracellular bacteria of the genus *Brucella* spp. Transmission of brucellosis from animals to humans occurs mainly through direct contact with infected animals, or ingestion of contaminated foods such as unpasteurized dairy products. It is estimated that more than 500,000 humans are infected with *Brucella* spp. annually (de Figueiredo et al., 2015). *Brucella melitensis* is the etiological agent of brucellosis in small ruminants (goats and sheep) and is the main cause of human brucellosis. Caprine brucellosis is clinically characterized by middle to late gestation abortion in pregnant females, and orchitis in males (Alton, 1990), while human brucellosis is a chronic debilitating disease with occasional complications such as arthritis, spondylitis, endocarditis and neurologic signs (Dean et al., 2012). Although caprine brucellosis has been controlled in most industrialized countries, it remains a major problem in the Mediterranean region, the Middle East, Central Asia, sub-Saharan Africa, and parts of Latin America (FAO, 2009). Control strategies of caprine brucellosis are associated with vaccination, serodiagnostic and culling of seropositive animals (Blasco, 2010). However, these measures are not always effective or practical for controlling or eradicating the disease.

A relatively new approach with great potential to control and eradicate diseases and improve the health status of the herd, is the identification of animals with natural resistance to pathogenic agents' infection (Iacoboni et al., 2014; Mapholi et al., 2016; Periasamy et al., 2014). Recently, a genome-wide association study (GWAS) in dairy cattle identified seven closely located single-nucleotide polymorphisms (SNPs) on chromosome 13, with significant association with resistance to *Mycobacterium bovis* infection (Bermingham et al., 2014). The SNP rs132841890 is located close to the 3' end of intron 7 of *PTPRT* bovine gene, while the other six significant SNPs (rs110465273, rs109809949, rs42494357,

rs42494342, rs109042660 and rs137562332) are close to the beginning of intron 8 of this gene. Receptor protein tyrosine phosphatase rho (RPTPρ, gene symbol *PTPRT*) is a signaling molecule that contains an extracellular region, a single transmembrane region, and two tandem intracellular phosphatase catalytic domains (Besco et al., 2001). RPTPρ phosphatase activity is crucial in regulating signal transducer and activator of transcription 3 (STAT3) translocation into the nucleus and activation of target genes (Zhang et al., 2007), many of which are involved in cell proliferation, differentiation, cell migration and apoptosis. These processes are essential determinants for the development of immune cells as well as of many other tissues throughout the organism (Böhmer and Friedrich, 2014). It's known that STAT3 promotes regulatory T cells (Tregs) differentiation and expansion by regulating FoxP3 expression (Kane et al., 2014), and that Tregs suppress *B. abortus*-specific CD4+ T cells and favor the maintenance and the progression of *B. abortus* infection in a mouse model (Pasquali et al., 2010).

The goal of this study was to identify polymorphisms at *PTPRT* intron 8 and evaluate the association of these polymorphisms with the presence or absence of *Brucella*-specific antibodies in goats.

Material and methods

Samples

All 44 studied animals were unrelated adult female creole crossbreed goats from eight flocks located in Rivadavia county, Salta Province, in the northwest of Argentina. Cases were goats seropositive for brucellosis by the buffer plate antigen (BPA) test (98.1% SE, 97% SP) and the fluorescence polarization assay (FPA; 94.9% SE, 99.4% SP); while controls were goats seronegative by the BPA test. Tests were performed as previously

explained (Iacoboni 2014) and well known positive and negative goat serum were used as controls. Positive or negative results were determined by the presence or absence of visible agglutination (BPA) and values > or ≤ 85mP (FPA), respectively. Animals included in this study were unvaccinated against *Brucella* spp. and belonged to flocks with a high prevalence of brucellosis (>30% of the animals were serologically positive for brucellosis, **Table 1**) with clinical signs of the disease such abortion. Cases and controls were therefore equally exposed to *Brucella* infection.

DNA isolation

Genomic DNA was isolated from hair follicles, using a previously reported protocol (Iglesias et al., 2011) with minor modifications. Briefly, ten to twenty hair follicles were collected into a 1.5 ml microtube. For digestion, 100 µl of SNET lysis buffer (20 mM Tris HCl, pH 8.0; 5 mM EDTA; 400 mM NaCl; 1 % w/v SDS) and 2 µl of proteinase K (19.6 mg/ml, TermoFisher, Carlsbad, CA) were added. After 30 min incubation at 55ºC for proteinase K inactivation, 80 µl of the supernatant was transferred to a new microtube. Then, 16 µl of sodium chloride (5 mM) and 100 µl of phenol:chloroform:isoamyl alcohol (TermoFisher) were added, gently mixed and centrifuged (10 min, 800x*g*). The aqueous phase was transferred to a 1.5 ml tube and two volumes of 100 % ethanol were added for an overnight DNA precipitation at -20°C. The precipitated DNA was collected by centrifugation (30 min, 9.000x*g*), washed twice with 70% ethanol, air dried at room temperature and re-suspended in 30 µl of TE buffer. Quality and quantity of DNA samples were estimated by NanoDrop® ND-1000 (NanoDrop, Wilmington, DE).

Genotyping

According to the sequence of the caprine *PTPRT* gene (GenBank accession number: NC_022305.1), two primer pairs were designed using Primer3 software (Rozen and Skaletsky, 2000) to amplify two regions of the *PTPRT* intron 8. Primer pair 1 (forward: 5'-AGGACTGTGGGTGTGTGTCAAAG-3', reverse: 5'-AATGGCTTCCCTGTGCTACCC-3') amplified a region A of 399 bp containing the homologous site of the bovine SNP rs110465273. Primer pair 2 (forward: 5'-TCTATTACAGCCCAAGGAGCA-3', reverse: 5'- ACATGAGCCAGGCAGAAGAAT-3') amplified a region B of 300 bp containing the homologous site of the bovine SNP rs109809949 (**Fig. 1**). PCR was carried out with 80 ng of genomic DNA in a total reaction volume of 30 µl containing 1X PCR buffer, 1.5 mM MgCl2, 200 µM dNTPs, 0.5 µM of each primer and 0.75 U of Taq DNA polymerase (Inbio-Highway, Tandil, Argentina). Amplification reactions were performed with an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 45 s at 94°C, 30 s at 56°C and 1 min at 72°C, with a final extension step of 10 min at 72°C. PCR products were electrophoresed on 1.8% agarose gels, stained with ethidium bromide, and purified using ADN puriprep-GP kits (Inbio-Highway).

Genotyping was performed by DNA sequencing using version 3.1 of the Big Dye terminator cycle sequencing kit (Applied Biosystems) and the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The length of the capillary was 36 cm and the separation medium was POP-7 polymer (Applied Biosystems). The sequence data were analyzed using BioEdit. We excluded SNPs with a minor allele frequency less than 0.01 or a genotype call rate less than 90%.

Analysis of caprine and bovine *PTPRT* gene sequences

Global and local nucleotide alignments were performed using EMBOSS Stretcher (http://www.ebi.ac.uk/Tools/psa/emboss_stretcher/nucleotide.html) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), respectively. *PTPRT* nucleotide sequences were obtained from the GenBank database: NC_022305.1:c69611614-68781297 (caprine gene), AC_000170.1:c72243309-71390499 (bovine gene), XM_018057885.1 (caprine mRNA) and XM_015474206.1 (bovine mRNA).

Statistical and LD analyses

Allelic and genotype frequencies were calculated using GenePop. Hardy–Weinberg equilibrium was calculated by the Hardy–Weinberg calculator (http://www.oege.org/software/hwe-mr-calc.shtml). Linkage disequilibrium (LD) blocks, haplotypes and haplotype frequencies were estimated with HaploView using the solid spine of LD method (Barrett et al., 2005). Associations between the absence of *Brucella*-specific antibodies and individual SNPs or haplotypes were determined by the Fisher's exact test using GraphPad software (on-line version: http://www.graphpad.com/quickcalcs/) Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using VassarStats (http://www.vassarstats.net/odds2x2.html). Bonferroni-corrected p-values of < 0.01 were considered statistically significant (Clarke et al., 2011).

Results

Comparative analysis of caprine and bovine *PTPRT* gene sequences

Global alignment with EMBOSS Stretcher of caprine and bovine *PTPRT* gene sequences showed 76.2 % identity. The length of intron 8 was conserved between goats (103,204 bp) and cows (112,960 bp), and the local alignment with BLAST showed a high degree of

identity (91%) (data not shown). Alignment of regions A and B (Fig. 1) of the caprine *PTPRT* intron 8 with the equivalent intron 8 regions of cattle showed 94% and 90% identity, respectively. In addition, comparison of caprine and bovine *PTPRT* predicted mRNA sequences showed 93% identity at the nucleotide level. This data suggests that the *PTPRT* gene structure is highly conserved between goats and cows. Therefore, *PTPRT* intron 8 could be a good candidate to be investigated as a brucellosis-associated region in goats.

SNP detection

While region A of the *PTPRT* intron 8 was not polymorphic in our goat population, four previously reported polymorphisms (minor allele frequency -MAF- ≥ 0.05; SNP1: rs643551276, SNP2: rs651618967, SNP3: rs662137815 and SNP4: rs657542977) and a new SNP located at the position 69169526 were identified in region B (**Fig. 1**). The genotype distributions of the five identified SNPs were in agreement with Hardy-Weinberg equilibrium among controls and cases ($p > 0.01$; **Table 2**). The SNPs: 1, 2, 4 and 5 had MAFs that were higher in cases than in controls ($p \leq 0.01$; **Table 2**). For SNP 3, allele distribution didn't show significant differences ($p = 0.06$), although a higher frequency of minor allele 'G' was seen in cases (0.18 vs. 0.05).

Individual SNP-based association analysis

The association between individual SNPs genotypes and *Brucella*-specific antibodies was evaluated by Fisher's exact test (**Table 3**). The major homozygous genotype at SNP1, 2, 4 and 5 sites were associated with absence of *Brucella*-specific antibodies ($p \leq 0.01$). Genotypes at SNP3 locus were not associated with absence or presence of *Brucella*-specific

antibodies, although a higher frequency of major homozygous CC genotype was detected in controls (0.91 vs. 0.70).

Haplotype-based association analysis

Pairwise LD tests indicated that SNPs 1, 2, 4 and 5 at *PTPRT* intron 8 were in strong linkage disequilibrium (LD) with each other (D′= 0.90 - 1.00; r^2 = 0.71 - 1.00). Using the solid spine of LD method in Haploview (Barrett et al., 2005), we constructed a five-marker haplotype block (**Fig. 2**), which contained three common haplotypes (frequency ≥ 0.05, **Table 4**): TTCCT, CCCTC and TTGCT. The TTCCT haplotype carried the major allele at every marker site, and was associated with absence of *Brucella*-specific antibodies ($p \leq 1 \times 10^{-4}$; OR=21.00; 95% IC=4.52 - 97.67). The CCCTC haplotype was composed by the minor alleles of SNPs 1, 2, 4 and 5, and the mayor allele of SNP3. This haplotype was present in 38% of all cases and 2% of all controls, which conferred significant susceptibility against brucellosis infection ($p \leq 1 \times 10^{-4}$; OR=0.037; 95% IC=0.005 - 0.294). The TTGCT haplotype that carried the minor allele G at SNP3 site had a higher frequency in the *Brucella*-seropositive group (0.17) than in controls (0.05), but the difference was not significant (p = 0.067).

Discussion

Recommended caprine brucellosis control and eradication measures consist on test-and-slaughter program combined with vaccination (Blasco, 2010). However, test and slaughter policy is not always possible to implement and vaccination strategies don't achieve desired outcomes sometimes (Minas, 2006).

A promising new tool with great potential to complement classical brucellosis control measures is the identification of animals genetically resistant to *Brucella* infection. Original studies found pigs and cows naturally resistant to brucellosis (Cameron et al., 1942; Templeton and Adams, 1990); later on, this phenotypic characteristic was linked to polymorphisms at 3' untranslated region (UTR) of the solute carrier family 11 member A1 (*SLC11A1*; ex *NRAMP1*) gene (Barthel et al., 2000; Capparelli et al., 2007). In a previous paper, our group showed that alleles of the caprine gene *SLC11A1* were associated with absence of *Brucella*-specific antibodies in goats (Iacoboni et al., 2014). Here, we report a new molecular marker in the caprine genome significantly associated with resistance to *Brucella* infection.

Receptor protein tyrosine phosphatase rho is the most frequently mutated tyrosine phosphatase in human cancer. Several inactivating *PTPRT* mutations were identified in cancer patients, which include missense, 3' UTR, and splice site mutations (Zhao et al., 2015), and SNPs variants of *PTPRT* have been associated with autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis (Armstrong et al., 2009). RPTPρ is also a key inhibitor of STAT3 which mediates transcriptional activation in response to several cytokines involved in the inflammatory response, such as IL-6 and IL-2 (Armstrong et al., 2009), but there is no report about the functional importance of RPTPp during bacterial infection.

Alternative splicing variants of *PTPRT* mRNA were found in mouse and human brain as results of a 57 nucleotides (nt) alternatively spliced region of exon 14, an additional 30 nt alternatively spliced region of exon 16, and an alternatively spliced exon (22a) between exons 22 and 23 (Besco et al., 2001). We may speculate that the haplotype block sequence might affect PTPRT pre-mRNA splicing given its location relative to the 5' intron/exon

border of the eighth intron. Using ESEfinder software (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) (Cartegni et al., 2003), we observed that the SNP4 was located within overlapping exonic splice enhancer (ESE) motifs, and that the minor allele T abolished potential ESE binding sites for the splicing factors SRSF1 and SRSF6 (**Table 5**). SRSF1 and SRSF2 are members of the phylogenetically conserved arginine/serine-rich splicing factor (SR) protein family. The SR proteins are essential splicing factors required for constitutive pre-mRNA splicing, and are important regulators of alternative splicing (Long and Caceres, 2009). It was reported that a SNP at intron 8 of bovine CD46 gene causes the elimination of two ESE motif affecting the production of an alternative splicing variant that retains a 48 bp intronic sequence (Wang et al., 2014). This data suggest a possible functional role of SNP4 in alternative splicing of caprine *PTPRT* gene and resistance to *Brucella* infection. However, the association of SNP4 genotypes with absence or presence of *Brucella*-specific antibodies should not be considered by definition functional since the polymorphism may only represent an indirect genetic marker of *Brucella* infection resistance.

Our results are in concordance with a previously reported association between *PTPRT* polymorphisms and resistance to infection with another facultative intracellular pathogen. Bermingham *et al.* (2014) identified two *PTPRT* gene haplotypes blocks associated with resistance to *Mycobacterium bovis* infection in cattle. Other gene encoded for a protein tyrosine phosphatases (PTP) family member associated with resistance or susceptibility to facultative intracellular bacterial infection is *PTPN22*, whose product is an intracellular lymphoid-specific phosphatase involved in the inflammatory response with negative regulatory effect on B and T cell activation (Cohen et al., 1999). Gomez *et al.* (2005) and Boechat *et al.* (2013) reported an association between *PTPN22* gene variants and

susceptibility to human tuberculosis; however Bravo *et al.* (Bravo et al., 2009) failed to associate the C1858T polymorphism in the *PTPN22* gene and susceptibility to human brucellosis.

In conclusion, the results reported here represent the first evidence of genetic association between polymorphisms in the *PTPRT* gene and absence or presence of *Brucella*-specific antibodies in goats. Analysis of higher number of samples along with other molecular markers is in progress to validate these results and identify new genetic markers associated with natural resistance to *Brucella* spp. infection in goats.

Conflict of interest: none.

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Figure legends

Figure 1. Sequence comparison of regions B of the goat (*C. hircus*) and the bovine (*B. taurus*) *PTPRT* intron 8. The figure shows the alignment of partial nucleotide sequences of the intron 8 of *PTPRT* gene of goats (*C. hircus*; NC_022305.1) and cows (*B. taurus*; AC_000170.1). The five identified SNPs are highlighted in light grey (a new reported SNP is also in bold) and the homologous site of the bovine SNP rs109809949 in dark grey. Primers hybridization sites are indicated in bold.

Figure 2. Pairwise SNP LD map. The black triangle indicates the LD blocks identified by the solid spine of LD method. The D' values are shown inside each diamond; diamonds without a number represent D'=1. Black diamonds: r2 = 1.00; grey diamond: r2 = 0.71 - 0.84, white diamonds: r2 = 0.02 - 0.03. Map was drawn based on the genotype data of all case and control samples using HaploView.

```
C. hircus 69169263 ctctattaca gcccaaggag cagccaaaaa attacatgaa gttttcttac aatagctatt
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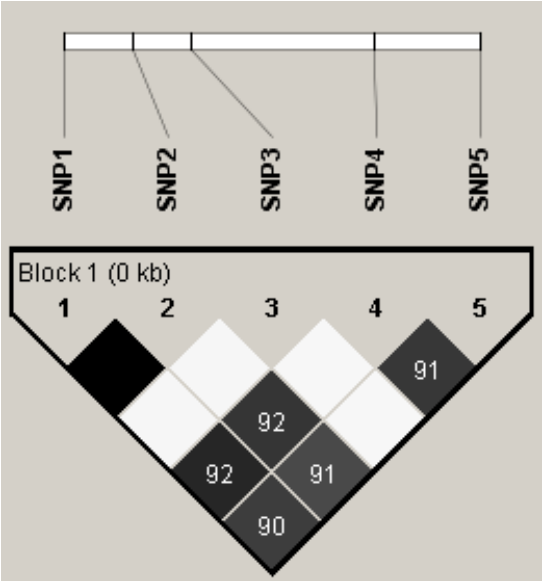

Table 1. Prevalence data, sample size and serological test results.

Farm number	Flocks' prevalence of brucellosis (%)	Number of genotyped samples	
		BPAT (-)	BPAT(+)/FPA(+)
1	53	3	3
2	34	3	3
3	32	2	2
4	20	3	3
5	45	2	2
6	72	2	3
7	47	4	4
8	40	3	2
Total		22	22

Table 2. Minor allele frequencies (MAF) and Hardy–Weinberg equilibrium (HWE) for the polymorphic SNPs identified in region B of the *PTPRT* intron 8. Minor alleles are indicated in bold.

SNP	Accession number	Location (Chr: pb)	Alleles	HWE (p-values)		MAF			
				Control	Cases	Total	Control	Cases	p-value
1	rs643551276	13:69169315	T/**C**	0.823	0.084	0.21	0.05	0.38	3×10^{-4}
2	rs651618967	13:69169350	T/**C**	0.824	0.058	0.21	0.05	0.38	2×10^{-4}
3	rs662137815	13:69169380	C/**G**	0.825	0.548	0.11	0.05	0.18	6×10^{-2}
4	rs657542977	13:69169473	C/**T**	0.920	0.791	0.23	0.02	0.45	2×10^{-6}
5	new	13:69169526	T/**C**	0.920	0.578	0.21	0.02	0.43	7×10^{-6}

Table 3. Genotype frequencies and individual SNP-based association analysis.

SNP	Genotype	N° of:			Association study	
		Total	Control	Cases	OR (95% CI)	p-value
1[a]	TT	25 (0.61)	19 (0.90)	6 (0.30)	TT vs (TC + CC) 0.045 (0.008 - 0.258)	≤ 1 x 10^{-4}
	TC	15 (0.37)	2 (0.10)	13 (0.65)		
	CC	1 (0.02)	0 (0.00)	1 (0.05)		
2[b]	TT	25 (0.60)	19 (0.90)	6 (0.28)	TT vs (TC + CC) 0.042 (0.007 - 0.239)	≤ 1 x 10^{-4}
	TC	16 (0.38)	2 (0.10)	14 (0.67)		
	CC	1 (0.02)	0 (0.00)	1 (0.05)		
3[c]	CC	34 (0.81)	20 (0.91)	14 (0.70)	CC vs (CG + GG) 0.233 (0.041 - 1.330)	1.23 x 10^{-1}
	CG	7 (0.17)	2 (0.09)	5 (0.25)		
	GG	1 (0.02)	0 (0.00)	1 (0.05)		
4[d]	CC	27 (0.63)	21 (0.95)	6 (0.29)	CC vs (CT + TT) 0.019 (0.002 - 0.175)	≤ 1 x 10^{-4}
	CT	12 (0.28)	1 (0.05)	11 (0.52)		
	TT	4 (0.09)	0 (0.00)	4 (0.19)		
5[e]	TT	27 (0.64)	21 (0.95)	6 (0.30)	TT vs (TC + CC) 0.020 (0.002 - 0.189)	≤ 1 x 10^{-4}
	TC	12 (0.29)	1 (0.05)	11 (0.55)		
	CC	3 (0.07)	0 (0.00)	3 (0.15)		

Samples number not genotyped on each SNP: a: 19, 34 and 43; b: 19 and 39; c: 34 and 39; d: 27; e: 27 and 29.

Table 4. Haplotype frequencies and haplotype-based association analysis.

Haplotype					Haplotype frequencies			Association study	
SNP1	SNP2	SNP3	SNP4	SNP5	Total	Control	Cases	OR (95% CI)	p-value
T	T	C	C	T	0.64	0.91	0.36	18.00 (5.39 - 60.15)	≤ 1 x 10^{-4}
C	C	C	T	C	0.20	0.02	0.38	0.038 (0.005 - 0.302)	≤ 1 x 10^{-4}
T	T	G	C	T	0.11	0.05	0.17	0.238 (0.046 - 1.221)	6.64 x 10^{-2}

Table 5. Exonic splice enhancer (ESE) motif threshold scores related to SNP4 genotypes.

"-": scores below the threshold for the ESE motifs

Splicing factor	Binding site	Score (C / T)
SRSF1	GTGAGGA	3.04 / 3.04

SRSF1	CUCAU**G**U	2.59 / -
SRSF1	GUCAGGA	3.28 / 3.28
SRSF2	GACUCAU**G**	4.13 / 4.58
SRSF5	AUACAUG	2.94 / 2.94
SRSF6	UGAGGA	2.81 / 2.81
SRSF6	CAU**G**UC	3.14 / -
SRSF6	TACATG	3.15 / 3.15