Assessment of disease-associated missense variants in *RYR2* on transcript splicing

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Abstract

Heterozygous *RYR2* missense variants cause catecholaminergic polymorphic ventricular tachycardia. Rarely, loss of function variants can result in ventricular arrhythmias. We used splice prediction tools and an *ex vivo* splicing assay to investigate whether *RYR2* missense variants result in altered splicing. Ten *RYR2* variants were consistently predicted to disrupt splicing, however none altered splicing in the splicing assay. In summary, missense *RYR2* variants are unlikely to cause disease by altered splicing.

Catecholaminergic polymorphic ventricular tachycardia-associated heterozygous *RYR2* missense variant assessment

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare genetic arrhythmogenic condition affecting approximately one in 10,000 individuals.[1] It is characterized by episodic ventricular dysrhythmia triggered by exercise or emotion. CPVT is genetically heterogeneous with both autosomal dominant and recessive forms. Heterozygous variants in *RYR2* (MIM 180902, ID: 6262)[2] and *CALM1* (MIM 114180)[3] result in autosomal dominant forms of CPVT, whereas biallelic variants in *CASQ2* (MIM 114251),[4] *TRDN* (MIM 603283)[5] and *TECRL* (MIM 617242) result in recessive forms.[6] A number of cases of CPVT are molecularly unexplained, however approximately 50% of cases can be accounted for by gain of function missense variants in the cardiac ryanodine receptor (RYR2).[1,7] The classification of genetic variants identified in the *RYR2* genes pathogenic or benign is important for the accurate diagnosis, treatment and counseling of affected individuals and their relatives. Increased genetic testing of individuals with arrhythmias and advances in sequencing technology has resulted in a rapid increase in the number of *RYR2* variants identified. Application of the guidelines from the American College of Medical Genetics (ACMG) for sequence variant classification,[8] results in the majority of variants defined as variants of unknown significance (VUS) due to factors including, incomplete penetrance, a lack of functional data and as the majority of putative variants have only been described in a single family. Comparison of allele frequency with databases of sequence variation in healthy controls, including gnomAD has facilitated variant classification.[9,10]

As many as 9% of disease-associated single nucleotide variants in the Human Gene Mutation Database (HGMD) result in splice alterations.[11,12] A subset of these variants disrupt the function of exonic splicing elements.[12] Recent studies have shown that some individuals with ventricular arrhythmias with similarity to CPVT can be attributed to loss of function variants in *RYR2*.[13] Therefore, spliceogenic variants resulting in loss of function may manifest as CPVT. Indeed a spliceogenic *RYR2* variant c.6167-2A>G was recently identified in a 9 year old male with CPVT and no structural cardiac abnormalities.[14] In the present study we proposed that some CPVT associated *RYR2* missense variants cause a loss of function through the disruption of exonic splice elements and altering splicing, resulting in frameshifts and haploinsufficiency. We investigated this using computational splice prediction tools and an *ex vivo* splicing assay. A total of 324 rare or novel variants in *RYR2* classified as pathogenic, likely pathogenic or VUS were collated from a cohort of individuals undergoing genetic testing for CPVT or associated ventricular arrhythmia in the North West Genomic Laboratory Hub, UK. This list was supplemented with *RYR2* variants, reported in the literature and in clinical variant databases, including ClinVar and HGMD (Supplementary Table 1).[15,16] The effect variants are likely to have on splicing was predicted computationally using Alamut version 2.0 (Interactive Biosoftware, Rouen, France). Alamut incorporates five splicing prediction tools (Table 1). Those variants in which a 100% change was seen in the confidence score for the presence or absence of a splice feature in the wild-type (WT) compared to the variant by at least four prediction tools were chosen to be studied in the *ex vivo* mini gene assay (Table 2).

The 3.8kb pSpliceExpress minigene splicing reporter vector gifted from Stefan Stamm (Addgene plasmid # 32485 ; http://n2t.net/addgene:32485; RRID:Addgene_32485)[17] was restriction digested with NheI/BamhI and amplified by PCR with Phusion High-Fidelity DNA Polymerase (Figure 1) (ThermoFisher Scientific). The DNA sequences of the exons containing the *RYR2* variants of interest as well as ~100bp of the flanking 5' and 3' intronic sequences were amplified using PCR from genomic DNA using Phusion High-Fidelity DNA Polymerase. Two primer pairs were designed to generate two fragments that overlap with each other and the vector fragment, by approximately 20 bp and 10 bp, respectively (Table 3). Overlapping primer sequences were modified where needed to

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produce CPVT-associated *RYR2* variants (Table 3). The assembly of the *RYR2* fragments and vector fragment was achieved using the Gibson method using the manufacturer's protocol. The resulting plasmid was transformed into competent E.coli. Vector DNA was amplified and purified from selected colonies and the successful assembly of the vectors was confirmed by direct Sanger sequencing. Minigene vector DNA (0.2 µg) was transfected into HEK293 cells at confluence of 40-60% grown in Dulbecco's modified Eagle's medium high-glucose, DMEM (Sigma), supplemented with 10% foetal bovine serum (Sigma) in tissue-culture treated 6-well plates at 37°C and with 5% CO_2. Transfections were per-

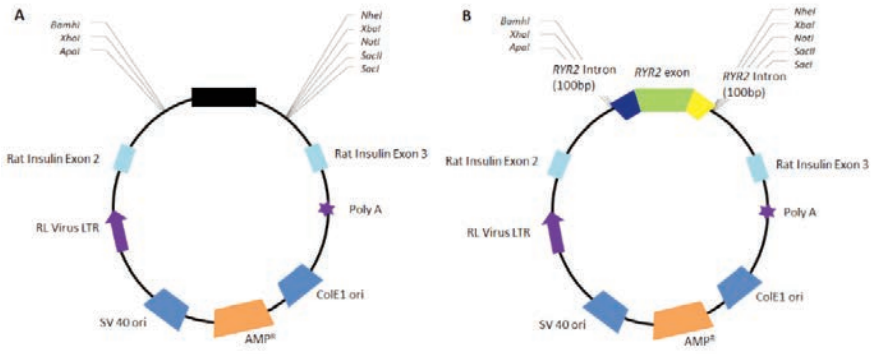

Figure 1. Schematic representation of pSpliceExpress vector before (A) and after (B) the insertion of RYR2 exonic and intronic sequences. The black rectangle in A represents ccdB and Cm^R sequences which are present in the pSpliceExpress vector but were not used for the selection of vectors with the correct insert.

Table 1. Algorithm by which the various tools predict splicing effects.

Splicing tool	Splice prediction algorithm
NNSplice http://www.fruitfly.org/seq_tools/splice.html	NNSplice uses a neural network that identifies motifs with consensus sequences. It also takes into account commonly occurring neighbouring sequences.[20]
SpliceSiteFinder-like https://www.interactive-biosoftware.com/doc/alamut-visual/2.6/splicing.html	SpliceSiteFinder-like uses position weight matrices developed from a database of human exon/intron boundaries for both donor and acceptor sites.[21]
MaxEntScan http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html	The maximum entropy principle is used to model sequence motifs. The short sequence motifs involved RNA splicing are modelled with a maximum energy distribution.[22]
Human Splicing Finder http://www.umd.be/HSF/	Human splicing finder uses position weight matrices supplemented with position based logic. Each nucleotide is assigned a weight, the assigned weight is dependent on the frequency of the nucleotide and the comparative importance of its location within the sequence motif.[16]
GeneSplicer http://ccb.jhu.edu/software/genesplicer/	GeneSplicer uses a combination of a second order Markov model and the maximal dependence decomposition decision tree method. Markov models predict a base by studying the surrounding bases. In this case a region consisting of the 16 and 29 bases is scored for donor sites and acceptor sites, respectively. The MDD aligns a set of sequences of varying sizes and creates a model that incorporates the most vital dependencies from one position to another.[23]

Table 2. CPVT associated RYR2 variants predicted to affect splicing by 4 or 5 of the 5 splice prediction tools.

cDNA change	Protein change	ACMG classification (evidence of pathogenicity)	Predicted effect on splicing	Number of concordant tools	Exon	Domain
c.497C>G	p.(Ser166Cys)	VUS (absent in gnomAD[9])	Introduce 5' Splice Site	4	8	I
c.527G>A	p.(Arg176Gln)	Pathogenic (functional evidence (amino acid change), frequently reported in CPVT cases, absent in gnomAD, computational evidence)	Deletion of 5' Splice Site	4	8	I
c.6272A>G	p.(Gln2091Arg)	VUS (absent in gnomAD, computational evidence)	Introduce 3' Splice Site	4	41	
c.6961G>A	p.(Val2321Met)	VUS (absent in gnomAD)	Deletion 5' Splice Site	4	46	II
c.7169C>T	p.(Thr2390Ile)	VUS(absent in gnomAD, computational evidence)	Introduce 5' Splice Site	5	47	II
c.7181C>G	p.(Arg2394Gly)	VUS (absent in gnomAD, segregation with CPVT phenotype, computational evidence)	Introduce 5' Splice Site	4	47	II
c.7420A>G	p.(Arg2474Gly)	VUS (absent in gnomAD)	Deletion 3' Splice Site	5	49	II
c.7813A>G	p.(Met2605Val)	VUS (absent in gnomAD)	Introduce 5' Splice Site	4	51	
c.11399G>T	p.(Cys3800Phe)	VUS (computational evidence)	Deletion 5' Splice Site	5	83	III
c.12371G>A	p.(Ser4124Asn)	VUS (absent in gnomAD, computational evidence)	Deletion 5' & 3' Splice Site	5	90	III

formed using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer's protocol. RNA was extracted from HEK293 cells after a 48hour incubation period in at 37°C with 5% CO_2, using phenol/chloroform precipitation using Trizol. RNA was purified using the RNeasy column clean up kit (Qiagen), which included a DNase digestion step. Superscript Reverse Transcriptase (ThermoFisher Scientific) was used to synthesise cDNA. The cDNA produced was amplified using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) using *minigene RT PCR-for* and *minigene RT PCR-rev* primers (Table 3). The resulting PCR products were electrophoresed on an agarose gel (1-3%), to establish the sequence of the spliced products the purified DNA was sequenced by direct Sanger sequencing performed by Eurofins Genomics.

RYR2 c.6167-2A>G was used as a positive control in the minigene assays.[13] This variant activated a cryptic splice site resulting in an 11bp frameshift that introduces a premature stop codon within exon 41 (p.Ser2056Serfs*5) (Figure 2). Ten of the disease-associated*RYR2*variants were predicted to affect splicing by four or more

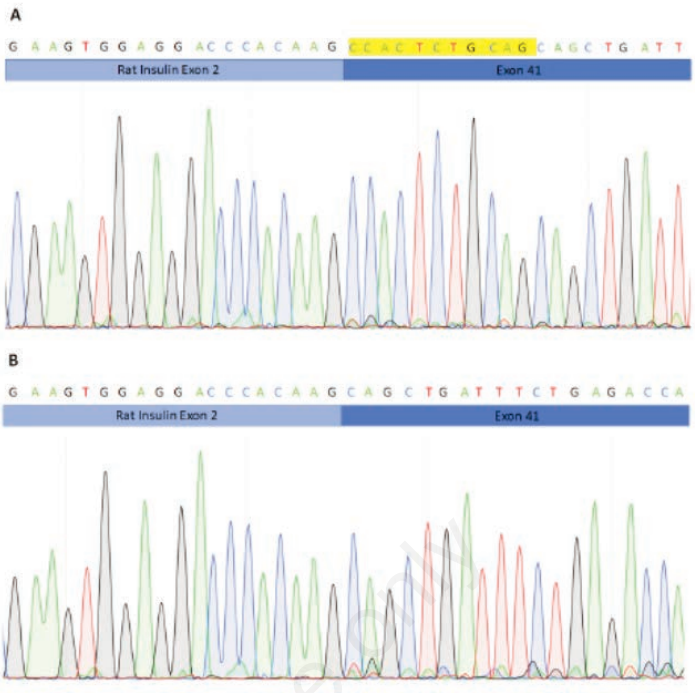

Figure 2. Electropherogram of cDNA sequences at the exon border between vector sequence (rat insulin exon 2) and RYR2 exon 41, for *RYR2* WT (A) and the splice variant RYR2 c.6167-2A>G (B). Regions highlighted in yellow represent sequences skipped in *RYR2* c.6167-2A>G.

Table 3. Primer list.

Primer set	Forward	Reverse
c.497 C>G (exon8) fragment 1	GGGCCCCTCCGGATTTCTGAAAGTTGTGTGTTGG	GCTGCTTACAGGCAGGGTGT
c.497 C>G (exon8) fragment 2	CTGCCTGTAAGCAGCGATCAGAA	GCTGGATGGCATTTTCATAGATAATTTACAATATAAACCTTAAAGAGATCATTTTATTG
c.527 G>A (exon8) fragment 1	GGGCCCCTCCGGATTTCTGAAAGTTGTGTGTTGG	ATCTCCAACTTGTACTTTTTCTCCTTCTGA
c.527 G>A (exon8) fragment 2	AAAAAGTACAAGTTGGAGATGACCTCATCT	GCTGGATGGCATTTTCATAGATAATTTACAATATAAACCTTAAAGAGATCATTTTATTG
c.6167-2A>G (intronic) fragment 1	GGGCCCCAGAAATACCAATTTGGGGGTACAGGA	GCAGAGTGGCCGCAAAGTCATTATG
c.6167-2A>G (intronic) fragment 2	TGACTTTGCGGCCACTCTGCAGCA	CTGGATGGCGACAATATATTTTTATCAATGTAGTTAATGTACTGCTCTATAGG
c.6272 A>G (exon 41) fragment 1	GGGCCCCAGAAATACCAATTTGGGGGTACAGGA	CGTCATACCGCCGATGGAGCA
c.6272 A>G (exon 41) fragment 2	CATCGGCGGTATGACGGCATTG	CTGGATGGCGACAATATATTTTTATCAATGTAGTTAATGTACTGCTCTATAGG
c.6961 G>A (exon 46) fragment 1	CGGGCCCCTAGTTATTCTTATACATAGGAAATGATTA GTATAACATTTATTTGTTCAG	CAATCTCATCACGACATTTGCATTTTCC
c.6961 G>A (exon 46) fragment 1	TGTCGTGATGAGATTGCTCATTCG	TGGATGGCACCACAAGTTATATTACAATTCATAGGATGCAGA
c.7169C>T (exon 47) fragment 1	GGGCCCCTGTGTTACCTAGTAGTCCCTTTCCTCGG	TGAATAGAAGATCATGATCGCGTTCC
c.7169C>T (exon 47) fragment 2	CGATCATGATCTTCTATTCAGCTTTGATTGA	CTGGATGGCGAAGATTATTGGTTTTGGATGCTGTTATGCT
c.7181 C>G (exon47) fragment 1	GGGCCCCTGTGTTACCTAGTAGTCCCTTTCCTCGG	GGTCAATCAAACCTGAATAGAAGGTCATGA
c.7181 C>G (exon47) fragment 2	TCTATTCAGGTTTGATTGACCTCTTGGGA	CTGGATGGCGAAGATTATTGGTTTTGGATGCTGTTATGCT
c.7420 A>G (exon 49) fragment 1	GGGCCCCAAACTGTGTTTAAAATGTAAGAAGTCTAGAAAGCAG	CATAGACCCCGTCAAGGAATAAAACCATGG
c.7420 A>G (exon 49) fragment 2	TCCTTGACGGGGTCTATGGGATTGA	CTGGATGGCTTCATTGTCAATAAATTAATGAATGGATATATAAAAAAGAACATCA
c.7813 A>G (exon 51) fragment 1	CGGGCCCCATAGATTCAGGTCCTTGGCTGATATAATTTATTCTAAT	AAGAGGCACCTTTGCGTGTTCATTTAATAATG
c.7813 A>G (exon 51) fragment 2	CGCAAAGGTGCCTCTTAAAGTAAGTATAGGAAA	TGGATGGCAGCGTCAAGCATGATGTATCTAAGAAAT
c.11399 G>T fragment 1 (exon 83)	GGGCCCCCTGTCTATTCTAGAATGGAAAGCCTGTTT	CCTTACCTAAATGACTGCATCAGGC
c.11399 G>T fragment 2 (exon 83)	GCAGTCATTTAGGTAAGGACTCACT	CTGGATGGCAACTATTCTTCTATGTGCAATTATCGTCAGAGT
c.12371 G>A fragment 1 (exon 90)	GGGCCCCTCCTTGATTCAGATGTTATTAAAGATCTACATTGTTATCTTCTG	TCAGGACGTTCTCTGCTAATTCCA
c.12371 G>A fragment 2 (exon 90)	CAGAGAACGTCCTGAATTATTTCCAGC	TGGATGGCAACACCGTTCTGGCACTAGC
Minigene	TGCTGGCCCTGCTCATCCTCTG	TGGACAGGGTAGTGGTGGGCCT

computational prediction tools. Eight out of 10 of the tested variants were present in known disease associated variant hotspot regions of *RYR2* and were reported to have resulted in sudden death, syncope or arrhythmias (Supplementary Table 2). All 10 variants were at least 100 bp away from the canonical splice site and were mostly present in different exons ranging from exon 8 to exon 90 and had no effect on splicing in the minigene assay, this was confirmed by agarose gel electrophoresis and direct sequencing of the resulting bands.

The majority of sequence variants in *RYR2* in patients with a clinical diagnosis of CPVT or ventricular arrhythmia are classified as variants of uncertain significance. It is therefore difficult to molecularly confirm a diagnosis of CPVT and therefore to use genotype data to facilitate cascade testing to clarify the risk to close relatives of an affected individual. Functional studies to determine the pathogenicity of *RYR2* variants are challenging due to the size of the gene and encoded protein and its expression which is limited to cardiac and brain tissue. Recently, loss of function variants in *RYR2* have been reported to result in ventricular arrhythmias.[13]

Although computational splice prediction tools have been shown to be reasonably accurate in predicting the effects of intronic splice variants less is known about their ability to predict the effects of exonic variants on splicing.[18] Théry *et al*. (2011) investigated the effects of 53 coding and non-coding VUS in *BRCA1* and *BRCA2* on splicing.[18] Computational splice prediction tools indicated that none of the exonic variants would be spliceogenic. However, all 53 variants were tested using an *ex vivo* splicing assay and four of the ten non-coding variants, predicted to affect splicing by computational tools, were confirmed and five exonic variants resulted in exon skipping in the *ex vivo* assay in this study.[18] Their data would indicate that the effect on splicing of exonic single nucleotide variants is underestimated.[12] For the exonic variants that altered splicing in the minigene assay Théry *et al*. (2011) were able to confirm the results by analyzing lymphocyte derived RNA from the individual carrying the variant.[18] This validation demonstrated the reliability of the assay, which is particularly useful for conditions like CPVT where relevant RNA from an affected individual is often unavailable due to the expression of RYR2 being limited to the heart and brain.

Here, we tested the ten *RYR2* variants where *in silico* predictions indicated a potential effect on transcript splicing. The vast and rapidly growing number of VUS being identified means that functional testing of each disease-associated variant is impractical. Thus, a reliable means of selecting those variants most likely to affect splicing for *ex vivo* testing is required. Computational splice prediction tools can be helpful, but the reliability of these tools for predicting the effects of exonic splice variants requires further validation. We proposed that by applying more stringent parameters by testing only those variants in which an effect on splicing was predicted by at least four of five available prediction tools may reduce the number of false positives. It is important to note that splicing minigene assays may not be able to detect very rare splicing events. To reduce the chances of such transcripts going undetected it may be beneficial to perform multiple splicing assays using a variety of cell types.

In our study, we provide no evidence to support the hypothesis that missense variants in *RYR2* result in altered transcript splicing and so lead to loss of function pathogenic variants. However, it is possible that exonic variants that may have less predictive power using current algorithms do result in altered splicing. Such variants should be considered as higher throughput methods to assess splicing, including saturation genome editing by CRISPR-Cas9, are developed.[19-23]

References

1. Napolitano C, Priori SG, Bloise R. Catecholaminergic polymorphic ventricular tachycardia. 2004 Oct 14 [Updated 2016 Oct 13]. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2019.
2. Priori SG, Napolitano C, Tiso N, et al. Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular tachycardia. Circulation 2001;103:196-200.
3. Nyegaard M, Overgaard MT, Søndergaard MT, et al. Mutations in calmodulin cause ventricular tachycardia and sudden cardiac death. Am J Hum Genet 2012;91:703-12.
4. Lahat H, Pras E, Olender T, et al. A missense mutation in a highly conserved region of CASQ2 is associated with autosomal recessive catecholamine-induced polymorphic ventricular tachycardia in Bedouin families from Israel. Am J Hum Genet 2001;69:1378-84.
5. Roux-Buisson N, Cacheux M, Fourest-Lieuvin A. Absence of triadin, a protein of the calcium release complex, is responsible for cardiac arrhythmia with sudden death in human. Hum Mol Genet 2012;21:2759-67.
6. Devalla HD, Gelinas R, Aburawi EH, et al. TECRL, a new life-threatening inherited arrhythmia gene associated with overlapping clinical features of both LQTS and CPVT. EMBO Mol Med 2016;8:1390-408.
7. Liu N, Ruan Y, Priori SG. Catecholaminergic polymorphic ventricular tachycardia. Prog Cardiovasc Dis 2008;51:23-30.
8. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American college of medical genetics and genomics and the Association for molecular pathology. Genet Med 2015;17:405-24.
9. Karczewski KJ, Francioli LC, Tiao G, et al. Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. bioRxiv 2019:531210.
10. Olubando D, Hopton C, Eden J, et al. Classification and correlation of RYR2 missense variants in individuals with catecholaminergic polymorphic ventricular tachycardia reveals phenotypic relationships. J Hum Genet 2020 [In press].
11. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 1992;90:41-54.
12. Anna A, Monika G. Splicing mutations in human genetic disorders: examples, detection, and confirmation. J Appl Genet 2018;59:253-68.
13. Roston TM, Guo W, Krahn AD, et al. A novel RYR2 loss-of-function mutation (I4855M) is associated with left ventricular non-compaction and atypical catecholaminergic polymorphic ventricular tachycardia. J Electrocardiol 2017;50:227-33.
14. Lieve KVV, Verhagen JMA, Wei J, et al. Linking the heart and the brain: Neurodevelopmental disorders in patients with catecholaminergic polymorphic ventricular tachycardia. Heart Rhythm 2019;16:220-8.
15. Landrum MJ, Lee JM, Benson M, et al. ClinVar: public archive of interpretations of clinically relevant variants. Nucleic Acids Res 2016;44:D862-8.
16. Stenson PD, Mort M, Ball EV, et al. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics,

diagnostic testing and personalized genomic medicine. Hum Genet 2014;133:1-9.
17. Kishore S, Khanna A, Stamm S. Rapid generation of splicing reporters with pSpliceExpress. Gene 2008;427:104-10.
18. Thery JC, Krieger S, Gaildrat P, et al. Contribution of bioinformatics predictions and functional splicing assays to the interpretation of unclassified variants of the BRCA genes. Eur J Hum Genet 2011;19:1052-58.
19. Findlay GM, Daza RM, Martin B, et al. Accurate classification of BRCA1 variants with saturation genome editing. Nature 2018;562:217-22.
20. Jian X, Boerwinkle E, Liu X. In silico tools for splicing defect prediction: a survey from the viewpoint of end users. Genet Med 2014;16:497-503.
21. Zhang MQ. Statistical features of human exons and their flanking regions. Hum Mol Genet 1998;7:919-32.
22. Yeo G, Burge CB. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. J Comput Biol 2004;11:377-94.
23. Pertea M, Lin X, Salzberg SL. GeneSplicer: a new computational method for splice site prediction. Nucleic Acids Res 2001;29:1185-90.