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LABORATORY STUDY

Mutational screening of *VSX1* in keratoconus patients from the European population

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Abstract

Purpose To perform mutational screening of the visual system homeobox gene 1 (*VSX1*; MIM#605020) in patients with sporadic and familial keratoconus (MIM#148300) in a European population and, for the first time, report the mutational analysis of the two newly identified *VSX1* exons.

Methods *VSX1* sequence variants in patients with keratoconus were evaluated by direct sequencing of the entire coding region, including two novel exons. In familial keratoconus cases, segregation of potentially pathogenic *VSX1* variants was assessed to determine pathogenicity. Transcript analysis was carried out on splice site and synonymous sequence variants not detected in controls.

Results A total of 66 unrelated patients with keratoconus from the European population (27 with familial keratoconus; 39 with sporadic keratoconus) were analysed for *VSX1* mutations. Four sequence variants were not observed in 100 healthy control individuals: c.432C>G (p.D144E), c.479G>A (p.G160D), c.789C>T (p.S263S), and an intronic change c.844-13T>A (numbered with respect to NM_014588). Segregation was not detected for p.D144E and c.844-13T>A. The change in p.G160D was observedin two patients with sporadic keratoconus. Although predicted to alter *VSX1* splicing, p.S263S had no effect on transcript processing. Four known SNPs were detected and the following polymorphic variants were observed in keratoconus patients and controls: c.711T>A (NM_199425; p.P237P), c.844-5_-6insT (NM_014588), c.*28G>T (DQ854811/DQ854812), and c.*50G>A (DQ854809/DQ854810).

Conclusions *VSX1* has a minor role in keratoconus pathogenesis. The pathogenicity of p.G160D remains controversial and this change may represent a rare polymorphism or genetic modifier. Further evidence is provided that the previously reported variant, p.D144E, is a polymorphism.

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Keywords: keratoconus; *VSX1*; mutation; cornea; polymorphism

Introduction

Keratoconus (KCTN; MIM#148300) is a bilateral, non-inflammatory progressive corneal ectasia, manifesting as progressive myopia and irregular astigmatism, and is a major indicator for corneal transplantation in the Western world.[1,2] The estimated prevalence of keratoconus in the general population is ~50–230 of 100 000,[3] depending on the clinical detection method used and the diagnostic criteria applied. Keratoconus is believed to be inherited autosomally because of familial occurrence,[4] with a higher concordance rate of the trait in monozygotic twins than in dizygotic twins,[5] and its prevalence in first-degree relatives is 15–67 times higher than that of the general population.[6] Most studies describe autosomal dominant inheritance, with incomplete penetrance or variable expressivity.[7–9]

The introduction of computerised videokeratoscopy or corneal topography has provided objective indices improving diagnostic accuracy and the ability to detect subclinical or 'forme fustre' keratoconus.[10–13] Mild corneal topographic anomalies have been reported among relatives of keratoconus patients[14,15] and may allow the detection of low

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expressivity forms of keratoconus, expanding pedigrees for mapping studies.[14,16,17] Although most cases have been thought to be sporadic, the ability to detect subclinical cases with corneal topography has caused a revision of familial incidence by 6–8%.[18]

The genetic basis of keratoconus allows the application of linkage mapping and mutational analysis to elucidate its molecular basis and pathogenesis.[19–21] Mapping studies have identified a number of loci for autosomal dominantly inherited keratoconus: 20p11-q11 (KTCN1; MIM#148300),[22,23] 16q22.3-q23.1 (KTCN2; MIM#608932),[9] 3p14-q13 (KTCN3; MIM#608586),[17] 2p24 (KTCN4; MIM#609271),[16] 15q22.32-24,[24,25] and 5q14.3-q21.1.[26] Other potential loci have been reported,[27,28] with likely further genetic heterogeneity.

Héon *et al*[22] mapped a major gene for posterior polymorphous corneal dystrophy-1 (PPCD1; MIM#122000) to chromosome 20p11-q11, and subsequently identified mutations in the visual system homeobox gene 1 (*VSX1*; MIM#605020) in PPCD1 and keratoconus (KTCN1; MIM#148300).[23] There has been debate in literature with regard to the significance of *VSX1* in keratoconus development, with some authors questioning the role of *VSX1* in keratoconus.[29,30] *VSX1* was originally characterised as a 5-exon gene, spanning 6.7 kb of genomic DNA on chr20 with two major transcripts, 1 and 2 (NM_014588 and NM_199425), encoding protein isoform A (NP_199425.1) and B (NP_955457), respectively.[31,32] Recently, two novel exons downstream of the original *VSX1* gene sequence were identified, which produce four additional previously unknown *VSX1* transcripts.[33] *VSX1* is now known to span 10.65 kb of genomic DNA and consists of seven exons producing a total of six transcripts (Genbank NM_014588 also called DQ854807, NM_199425 also called DQ854808, DQ854809, DQ854810, DQ854811, and DQ854812).[33] A further elucidation of the *VSX1* transcript structure with two novel exons allows mutational analysis of the complete gene sequence for the first time and may explain the relatively low frequency of the pathogenic mutations reported in *VSX1* resulting in keratoconus.

The purpose of this study to perform mutational screening of the *VSX1* gene in patients with sporadic and familial keratoconus in a European population and, for the first time, report the mutational analysis of the two newly identified *VSX1* exons.

Methods

Patients and clinical assessment

Patients of European decent were recruited from the Royal Victoria Hospital, Belfast, and from the Department of Ophthalmology, University of Toronto. We certify that all applicable institutional and governmental regulations with regard to the ethical use of human volunteers were followed during this research. A diagnosis of definitive keratoconus was based on slit-lamp biomicroscopy, retinoscopy, and corneal topography. A history of penetrating keratoplasty or corneal surgery for keratoconus was considered as evidence of a definitive diagnosis, and pre-surgical clinical data were retrospectively assessed to ensure diagnostic accuracy. Corneal topography (videokeratography) was performed using the Topographic Modelling System (TMS-2) (Computed Anatomy Inc., New York, NY, USA) according to standardised methods previously reported by our group[34] and was analysed for features diagnostic of keratoconus. Subjects with keratoconus were classified as familial cases if at least two or more individuals in one family met the clinical definition of keratoconus. When determining the familial segregation of *VSX1* sequence variants, all available family members underwent a clinical and topographic examination to determine their clinical status before molecular analysis.

DNA extraction and sequencing

Genomic DNA was isolated from peripheral blood leukocytes using a Puregene blood extraction kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Individual DNA samples were quantified using Nanodrop-1000 (Nanodrop Technologies, Wilmington, DE, USA) and a PCR dilution of 50 ng/μl was carried out for each DNA sample.

The coding exons, exon–intron junctions, and the 5′ and 3′ untranslated regions (UTRs) of VSX1 were amplified with at least 50 bp flanking the coding region of each exon. The five previously known exons, including the 3′ end of exon 3 specific to transcript variant 2 (exon 3b), were identical to those previously used,[35] with modifications in PCR conditions. The primer pairs for the two novel exons,[33] exons 6 and 7, were designed using Primer Detective V1.01 (Clontech Labs Inc., Cambridge, UK): exon 6 (5′-GCTTTTTCCTTAGGT TTAATCG-3′ and 5′-TGCTGCATGGGTCCATTTGTC-3′) with a PCR product of 310 bp and exon 7 (5′-TTCACTAA GCCACAGTCTCTCG-3′ and 5′-ACTTCAGCCCAGGAG TTAGAGG-3′) with a PCR product of 738 bp. The following additional sequencing primers were used for exon 7: 5′-GAGTTGAAAGCATTGGTCTCCC-3′ and 5′-AGGATGACACAGGAAAATCGGC-3′. NCBI dbSNP was screened to ensure no known SNPs were located in the primer-annealing site.

PCR amplification was carried out in a final reaction volume of 10 μl with 50 ng of genomic DNA, 10 × PCR

buffer with 15 mm mgcl2, 5 × Q-solution (Qiagen, Sussex, UK), 200 μm of each dNTPs, 0.3 μm of gene-specific forward and reverse primers, and 2.5 units of HotStar Taq DNA polymerase (Qiagen). Thermal cycling was carried out in a 2700 ABI thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s; 58°C (exon 1), 59°C (exons 2, 4, and 5), 62°C (exon 3), 61°C (exons 6 and 7) for 30 s, and 72°C for 30 s; and a final extension of 72°C for 10 min. The amplified PCR products were directly sequenced in a final reaction volume of 10 μl using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and BetterBase diluent (Microzone Ltd, Sussex, UK). The cycling conditions were 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 60°C for 4 min, with a final holding temperature at 4°C. Amplified DNA fragments were sequenced using both forward and reverse primers and were analysed on an ABI PRISM 3100 DNA sequencer (Applied Biosystems). Sequencing results were analysed manually and using the sequence analysis software Sequencher 4.7 (Gene Code Corporations, Suite, Ann Arbor, MI, USA).

Identified sequence variants were described according to the guidelines published by the Human Genome Variation Society. A total of 100 unrelated individuals (200 chromosomes) without ocular disease from a Northern Irish population were used as normal controls and *VSX1* was sequenced in both directions in all individuals.

Bioinformatics

To determine the pathogenicity of the identified amino-acid substitutions, conservation was analysed using multiple sequence alignment with ClustalX and was visualised using GeneDoc software. Deleterious structural effects of amino-acid substitutions on protein function were assessed using the PolyPhen (*Poly*morphism *Phen*otyping) programme.[36] Intronic changes and sequence variants close to splice sites not seen in controls were assessed to determine their impact of splicing signals using four scoring algorithms: Consensus Sequence, Neural Network, Information Theory, and Maximum Entropy.[37–40] To determine the possible mutational effects of synonymous sequence variants not seen in controls on exonic splicing, enhancers and silencers were modelled using servers ESEfinder[41] and ESRsearch.[42,43]

RNA extraction and RT-PCR

Total RNA was extracted from whole-blood samples from individuals harbouring *VSX1* sequence variants with potential effects on splicing accuracy that were absent in control individuals. *VSX1* gene expression can be detected in normal blood[44] and hence RNA was extracted using a red blood cell lysis solution (Puregene, Gentra Systems) and an RNA extraction kit (TRIzol; Invitrogen, Paisley, UK), according to the manufacturer's instructions. Puromycin was used to inhibit nonsense-mediated mRNA decay[45] and a DNase treatment was performed. To analyse the effect of novel silent mutation p.S263S on alternative splicing in a sporadic keratoconus patient, 1 μg of total RNA was reverse transcribed using random hexamer primers and reverse transcriptase (Sensiscript, Qiagen). RT-PCR was carried out using primers 5′-ATGCTGGCTGTGAAAACTGAGC-3′ and 5′-CTTCCTGGCTTCCTTA-TCATCC-3′ in exons 3 and 5, flanking the region of interest of *VSX1* mRNA harbouring the synonymous p.S263S variant. RT-PCR products from the sporadic keratoconus patient harbouring p.S263S and control samples were assessed for aberrant splicing by gel electrophoresis.

Results

The entire coding region, exon–intron junctions, and 5′ and 3′UTR of *VSX1* including two novel exons[33] were analysed for mutations in a total of 66 unrelated patients with keratoconus from a European population (27 with familial keratoconus; 39 with sporadic keratoconus). Table 1 summarises all *VSX1* sequence variants identified in this European cohort with keratoconus. Four sequence variants were not observed in 100 healthy controls: c.432C>G (p.D144E), c.479G>A (p.G160D), c.789C>T (p.S263S), and an intronic change c.844-13T>A (numbered with respect to NM_014588).

The variant p.D144E was detected in a family with keratoconus, although segregation was not observed (Figure 1). This sequence variant (p.D144E) was not observed in two siblings affected with keratoconus on the basis of clinical examination and topography, but was observed in a normal sibling and parent, although it was absent in 100 control individuals. Two sporadic keratoconus patients had the non-synonymous p.G160D change, which was not seen in controls (Figure 2a). The G160 residue is not well conserved across species (Figure 2b) and is predicted to be a benign change using the PolyPhen algorithm.

One sporadic keratoconus patient showed a novel silent mutation (p.S263S) in exon 4, which was not seen in 200 control chromosomes. The absence of this synonymous sequence variant in controls prompted a further analysis to assess its potential pathogenicity. Specifically, the ability of p.S263S to initiate mis-splicing events by altering putative ESE-binding motifs in the *VSX1* coding sequence was modelled using ESEfinder[41]

Table 1 Results of mutational screening of *VSX1* including two newly identified exons in 130 European keratoconus patients

VSX1 transcript	*Exon/UTR/intron*	*Nucleotide alteration*	*Protein alteration*	*Segregation in familial KC*	*Controls (n = 100)*	*Polyphen predictions*
NM_014588	Exon 1	c.18 G>T, (rs8123716)	p.S6S	Absent	Not done	NA
NM_014588	Exon 2	c.432C>G	p.D144E	Absent	Absent	Benign
		c.479G>A	p.G160D		Absent	Benign
NM_014588	Exon 3	c.546A>G (rs12480307)	p.A182A	Absent	37/100	NA
NM_199425	Exon 3	c.650G>A, (rs6138482)	p.R217H	Absent	22/100	Benign
NM_199425	Exon 3	c.711 T>A	p.P237P	Absent	36/100	NA
NM_014588	Exon 4	c.789C>T	p.S263S	Absent	Absent	NA
NM_014588	Intron 4	c.844-13T>A		Absent	Absent	
NM_014588	Intron 4	c.844-5_-6insT		Present	30/100	
DQ854809, DQ854810	Intron 6	rs743018, DQ854809: c.843 +140 C>T; DQ854810: c.662 +140 C>T		Absent	40/100	
DQ854811, DQ854812	3′UTR	c.*28G>T		Present	2/100	
DQ854809, DQ854810	3′UTR	c.*50G>A		Absent	6/100	

Identified sequence variants were described according to the guidelines published by Human Genome Variation Society (http://www.hgvs.org/mutnomen/) numbered according to the 'A' of the translation initiation codon (ATG) as nucleotide +1. The NCBI RefSeq curated nucleotide sequence records (NM) or the Genbank ID are given for the VSX1 transcripts analysed (see Hosseini *et al*.[32]).

and ESRsearch.[42,43] The introduction of p.S263S (c.789C>T; NM_014588) completely abolishes a potential motif for the SR protein, SF2/ASF, and causes a reduction in the binding motif for the SR protein, SC35. RT-PCR failed to show aberrant splicing around exon 4 in the presence of p.S263S (data not shown). One patient with familial keratoconus showed a sequence variant, c.844-13T>A, in intron 4 within the 3′ splice junction, which was not observed in 200 control chromosomes. This sequence change was predicted using bioinformatics to have an impact on splice site strength; however, segregation was not seen in the family (data not shown).

The known SNPs, rs8123716 (c.18 G>T; p.S6S), rs12480307 (c.546A>G; p.A182A), rs6138482 (c.650G>A; p.R217H), and rs743018 (c.843+140 C>T for DQ854809 or c.662+140 C>T for DQ854810 transcripts), were also found in keratoconus patients and failed to show segregation in familial cases. Two sequence variants in the 3′UTR were seen in sporadic and familial keratoconus patients. The sequence variant c.*28G>T segregated in two keratoconus families but was also seen in two healthy controls. The c.*50G>A change segregated in two keratoconus families but not in a third, and was also seen in six controls. The following polymorphic variants were seen in keratoconus patients and controls: c.711T>A (NM_199425; p.P237P), c.844-5-6insT (NM_014588), c.*28G>T (DQ854811/DQ854812), and c.*50G>A (DQ854809/DQ854810). No other previously reported pathogenic or potentially sequence variants were detected in the study cohort.

Discussion

To date, *VSX1* remains the only major genetic factor to be identified in keratoconus pathogenesis. A number of protein coding changes were originally reported as being potentially pathogenic (p.L17P, p.D144E, p.L159M, p.G160D, p.R166W, p.H244R, and p.P247R),[23,35,46] and a functional impact of p.R166W on homeodomain binding has been shown.[23] However, there has been debate in literature with regard to the significance of *VSX1* in keratoconus development.[23,29,30,35,47] The frequency of *VSX1* mutations in keratoconus study cohorts is variable, with some authors questioning the role of *VSX1* in keratoconus.[29,30]

Furthermore, the *VSX1* knockout mouse did not have a corneal phenotype on light microscopy[48] and the initial report of *VSX1* expression in normal adult human cornea[32] has not been replicated in subsequent studies on normal and keratoconic corneas.[23,49,50] *VSX1* expression was detected at a low level in murine cornea[51–53] and was recently reported in human neonatal cornea.[33] There is experimental evidence that *VSX1* has a role in corneal wound healing, in which it participates in the differentiation of corneal keratocytes into myofibrobalsts,[54] explaining a potential biological role for its involvement in keratoconus pathogenesis.

VSX1 mutations have been reported in 4.7% (3 of 63)[23] and 8.75% (7 of 80) of unrelated keratoconus patients,[35] whereas other groups have failed to identify *VSX1* pathogenic sequence variants in keratoconus.[29,30,47] Mutations in *VSX1* have also been reported in PPCD1 (MIM#122000)[23,46] and in combination with keratoconus.[23] A p.A256S VSX1 mutation was reported in an African-American family with corneal endothelial abnormalities, craniofacial anomalies, and abnormalities in retinal and auditory bipolar cells.[55]

Recently, two novel exons downstream of the original *VSX1* gene sequence were identified and *VSX1* is now

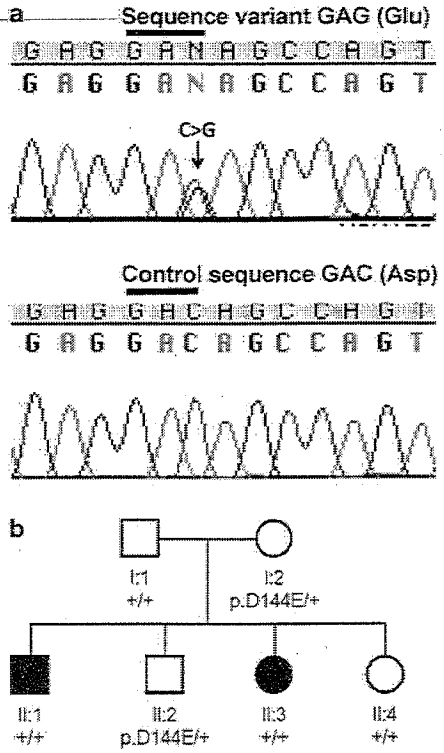

Figure 1 Sequence chromatogram of VSX1 c.432C>G (p.D144E) and segregation analysis. (a) DNA sequence chromatogram of the c.432C>G (p.D144E) sequence variant in *VSX1* exon 2 (NM_014588), resulting in the substitution of aspartic acid (GAC: Asp; D) with glutamic acid (GAG: Glu; E), both acidic-charged polar amino-acid residues. (b) Segregation of p.D144E in familial keratoconus. Blackened symbols (II:1 and II:3) refer to affected individuals, whereas unblackened symbols are unaffected relatives (I:1, I:2, II:2, and II:4) (drawn using Cyrillic 2.1). Two unaffected individuals are heterozygous for p.D144E (I:1 and II:2), whereas affected family members with keratoconus are wild type, showing that p.D144E does not segregate with disease status.

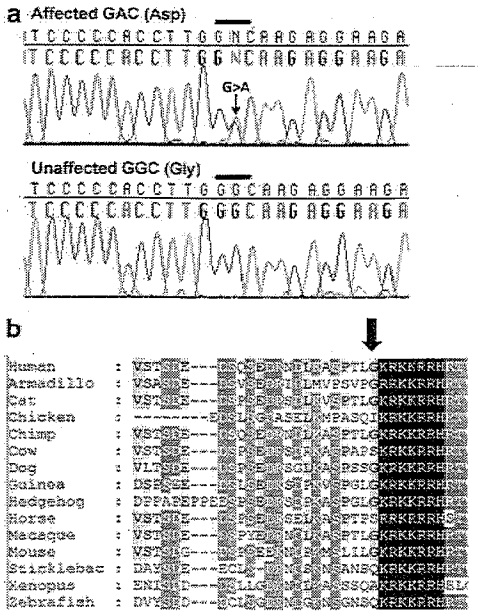

Figure 2 Sequence chromatogram of VSX1 c.479G>A (p.G160D) and conservation analysis. (a) DNA sequence chromatogram of the c.479G>A (p.G160D) sequence variant in *VSX1* exon 2 (NM_014588), resulting in the substitution of glycine (GGC: Gly; G) with aspartic acid (GAC: Asp; D), replacing a non-conservative substitution of a non-polar amino acid (glycine) with a charged polar residue (aspartic acid). (b) Multiple sequence alignment using ClustalX visualised using GeneDoc software for the glycine residue substituted in p.G160D showing 60% conservation across VSX1 from different species.

known to consist of seven exons with a complex splicing pattern producing a total of six transcripts.[33] The aim of this study was to screen the complete gene sequence of *VSX1* and to determine the contribution of the novel *VSX1* sequence to keratoconus pathogenesis.

The p.G160D variant was detected in two sporadic keratoconuses and was absent in 100 normal control individuals. This change was reported as being potentially pathogenic in posterior polymorphous dystrophy[23] on the basis of segregation and absence from 277 control individuals. Héon *et al*[23] reported that the G160 residue is not highly conserved in keeping with the multiple sequence alignment illustrated in Figure 2b. Bisceglia *et al*[35] detected p.G160D in two families with keratoconus. In one family, p.G160D was detected in a patient with keratoconus, in two relatives with topographically-suspected keratoconus, but was not seen in two further relatives with topographically suspected keratoconus. In the second family, p.G160D was present in a compound heterozygote with p.L17P and resulted in clinical keratoconus. Two other family members had p.L17P alone and had evidence of topographically suspected keratoconus. The change p.L17P was also reported as pathogenic in a sporadic case of keratoconus and was absent from 125 control individuals. Bisceglia *et al*[35] evaluated the segregation of p.G160D in family members with subclinical or 'forme fustre' keratoconus and it is difficult therefore to determine its true role in keratoconus pathogenesis. The pathogenicity of p.G160D can be debated from this evidence and it may represent a rare polymorphism or genetic modifier. Compound heterozygotes with p.G160D in association with p.L17P[35] and p.P247R[23] had clinically more severe corneal phenotypes.

The p.D144E variant was previously reported as disease causing in two affected family members with PPCD1 and keratoconus,[23] but has been detected in healthy controls[56] and glaucoma probands,[23] as well as in keratoconus patients.[29,35,47] In the cases with p.D144E reported by Bisceglia *et al*[35] from keratoconus families, one case was effectively a sporadic patient and in the

second family in which segregation was reported, three out of the four affected patients had a diagnosis of forme fustre or subclinical keratoconus. Recently, p.D144E was shown not to segregate with the disease phenotype in a family with clinical keratoconus.[47] Similarly, in this study, p.D144E was detected in a family with keratoconus, but segregation was not detected. Cumulative evidence suggests that p.D144E is a non-pathogenic polymorphism. Determining the pathogenicity of reported sequence variants has been assisted by screening familial keratoconus cohorts and assessing segregation.[35,47]

Two other sequence variants were detected in this study but were absent in controls. The intronic change c.844-13T>A, although predicted to have a significant impact on splice site strength, did not segregate in a family with keratoconus. The synonymous variant p.S263S was seen in a sporadic patient with keratoconus, although it was absent in controls. Synonymous or translationally silent sequence changes have the potential to alter the efficiency and specificity of alternative splicing.[57] The recent elucidation of the diverse transcript profile of *VSX1*[33] and the predicted effect of p.S263S on putative ESEs led us to evaluate the ability of this sequence variant to induce aberrant splicing. RNA analysis failed to detect aberrant *VSX1* transcript processing in the presence of p.S263S.

In summary, a comprehensive screening of the *VSX1* gene, including two newly identified exons in patients with sporadic and familial keratoconus, in a European population confirms that *VSX1* has a minor role in keratoconus pathogenesis and further study is required to determine the molecular genetic basis of this significant corneal disorder.

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Web Resources

NCBI dbSNP:
http://www.ncbi.nlm.nih.gov/projects/SNP/
Human Genome Variation Society:
http://www.hgvs.org/mutnomen/
ClustalX:
ftp://ftp.ebi.ac.uk/pub/software/clustalw2
GeneDoc:
http://www.nrbsc.org/gfx/genedoc/index.html
PolyPhen (*Poly*morphism *Phen*otyping):
http://genetics.bwh.harvard.edu/pph/
Consensus Sequence:
http://www.genet.sickkids.on.ca/~ali/splicesitescore.html
Neural Network:
http://www.fruitfly.org/seq_tools/splice.html
Information Theory:
https://splice.uwo.ca/
Maximum Entropy: http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html
ESE finder:
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