Evaluation of Suspected Autosomal Alport Syndrome
Synonymous Variants

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Key Points
- Mutations registered in the database for autosomal Alport syndrome do not include synonymous variants.
- Certain synonymous variants can affect pre-mRNA splicing, and transcript analysis should be carried out to evaluate synonymous variants.
- Our in vivo and in vitro splicing assays showed that two of the four synonymous variants cause exon skipping.

Abstract
Background Alport syndrome is an inherited disorder characterized by progressive renal disease, variable sensorineural hearing loss, and ocular abnormalities. Although many pathogenic variants in COL4A3 and COL4A4 have been identified in patients with autosomal Alport syndrome, synonymous mutations in these genes have rarely been identified.

Methods We conducted in silico splicing analysis using Human Splicing Finder (HSF) and Alamut to predict splicing domain strength and disruption of the sites. Furthermore, we performed in vitro splicing assays using minigene constructs and mRNA analysis of patient samples to determine the pathogenicity of four synonymous variants detected in four patients with suspected autosomal dominant Alport syndrome (COL4A3 [c.693G>A (p.Val231=)] and COL4A4 [c.1353C>T (p.Gly451=), c.735G>A (p.Pro245=), and c.870G>A (p.Lys290=)]).

Results Both in vivo and in vitro splicing assays showed exon skipping in two out of the four synonymous variants identified (c.735G>A and c.870G>A in COL4A4). Prediction analysis of wild-type and mutated COL4A4 sequences using HSF and Alamut suggested these two variants may lead to the loss of binding sites for several splicing factors, e.g., in acceptor sites and exonic splicing enhancers. The other two variants did not induce aberrant splicing.

Conclusions This study highlights the pitfalls of classifying the functional consequences of variants by a simple approach. Certain synonymous variants, although they do not alter the amino acid sequence of the encoded protein, can dramatically affect pre-mRNA splicing, as shown in two of our patients. Our findings indicate that transcript analysis should be carried out to evaluate synonymous variants detected in patients with autosomal dominant Alport syndrome.

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Introduction
Alport syndrome is an inherited disorder characterized by progressive renal disease, variable sensorineural hearing loss, and ocular abnormalities (1). This disease arises from pathogenic variants in COL4A3, COL4A4, or COL4A5, the protein products of which are involved in the synthesis, assembly, deposition, and function of collagen IV α345, which is one of the most important components of the glomerular basement membrane (2).

A pathogenic variant in COL4A5 causes X-linked Alport syndrome (XLAS) because this gene is located on the X chromosome. In contrast, mutations in COL4A3 or COL4A4 are transmitted autosomally. Heterozygous variants in COL4A3 or COL4A4 are associated with autosomal dominant Alport syndrome (ADAS), and mutations in both the alleles of COL4A3 or COL4A4 cause autosomal recessive Alport syndrome (ARAS) (3,4).

Female patients with XLAS exhibit milder symptoms than male patients (5,6), whereas renal failure, hearing loss, and ocular abnormalities are equally common in men and women with ARAS (3,7). Kidney damage appears to progress more slowly in ADAS than in XLAS or ARAS and is associated with a wide range of phenotypes (3). The frequency of inheritance of XLAS, ADAS, and ARAS has been calculated to be 80%, 5%, and 15%, respectively (8,9). However, we have recently reported a prevalence of XLAS, ADAS, and ARAS of 74%, 17%, and 9%, respectively, in a Japanese cohort (10), indicating that the prevalence of ADAS is higher than previously reported. Furthermore, we have observed patients with suspected Alport syndrome, and pathogenic variants cannot be detected by next-generation sequencing (NGS) panel analysis in approximately 12% of these patients (10).

Numerous variants identified in COL4A3 (222 variants; NM_000091.4) and COL4A4 (163 variants; NM_000092.4; http://www.hgmd.cf.ac.uk/ac/index.php) in ARAS or ADAS, including splice site variants, have been registered in the Human Gene Mutation Database. However, none of these variants are synonymous variants.

Single-nucleotide variants (SNVs) are the most common variants in the human genome (11). Approximately 10,000 variants in the coding regions of the human genome that do not affect the resulting product protein sequence, termed synonymous SNVs (sSNVs), have been identified (12). It is generally believed that the effect of sSNVs on the molecular functioning of genes/proteins is minimal. Nonetheless, earlier studies have suggested that sSNVs can be as pathogenic as nonsynonymous variants and that they are associated with 1% of human diseases (13). sSNVs can disrupt transcription (14), splicing (15), cotranslational folding (16), mRNA stability (17), and cause various functionally relevant changes (18). mRNA splicing is the main predictive feature in some prediction tools (18).

sSNVs can affect exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSes), which are short DNA sequence motifs that promote or suppress the splicing of pre-mRNA by binding to SR proteins (proteins with long serine and arginine repeats) (19). In addition, sSNVs can alter the pre-mRNA affinity for spliceosomes and initiate false identification of exon-intron boundaries, resulting in the production of abnormal mRNAs and dysfunctional proteins (20). Among these mechanisms, pre-mRNA splicing is the most important, and mRNA expression analysis is necessary to assess pre-mRNA splicing in patients, but this is often difficult because of the fragility and/or low expression of pre-mRNA in accessible samples. Previously, we reported that splicing assessment using a minigene construct is useful in patients with XLAS harboring sSNVs (21), but its usefulness in those with ADAS has not yet been established. In this study, we evaluated four patients suspected of having Alport syndrome and harboring synonymous COL4A3 or COL4A4 variants via in vivo and in vitro splicing assays.

Materials and Methods

Patient Characteristics
Patient 1 was a 45-year-old female who was diagnosed with proteinuria (2 g/g creatinine [Cr]) and hearing loss. Because she had no hematuria, the possibility of Alport syndrome was considered to be low. However, a renal biopsy revealed a thin basement membrane. Moreover, family history assessment revealed that her mother had CKD along with proteinuria and hematuria. Therefore, we conducted a genetic analysis of COL4A3 and COL4A4. At the time of the genetic analysis, her kidney function was normal. Immunofluorescence staining for type IV collagen α5(α5[IV]) could not be conducted.

Patient 2 was a 35-year-old female who was diagnosed with proteinuria and hematuria at the age of 24 years (1.7 g/g Cr), but she had normal hearing and kidney functions. Pathologic findings indicated a thin basement membrane with normal α5(IV) expression in the glomerular basement membrane and Bowman’s capsule. In addition, deposition of IgA-predominant Ig and a mild increase in mesangial matrix were observed. Although these findings suggested a diagnosis of IgA nephropathy, genetic testing was performed to exclude Alport syndrome.

Patient 3 was a 66-year-old male who was diagnosed with proteinuria (0.5 g/g Cr) and hematuria at the age of 50 years. He had no hearing loss. His eGFR was 60 ml/min per 1.73 m². Pathologic findings revealed glomerulosclerosis. His father was on hemodialysis. Immunofluorescence staining for α5(IV) could not be conducted. Although it was very difficult to make the diagnosis of Alport syndrome on the basis of pathologic findings, his family history suggested a possibility of Alport syndrome; therefore, he was referred to our hospital for genetic testing.

Patient 4 was a 43-year-old female with (occasional gross) hematuria and proteinuria since childhood. Her family medical history revealed that her father had received hemodialysis at the age of 56 years. In addition, her brothers, sisters, and grandmother were undergoing hemodialysis. The pathologic findings revealed a thin basement membrane with lamellation and normal α5(IV) staining.

Compliance with Ethical Standards

Research Involving Human Participants
All procedures involving human participants in this study were performed in accordance with the ethical standards of the Institutional Review Board (IRB) of Kobe University Graduate School of Medicine (IRB approval number...
Table 1. Patient clinical characteristics and variant sites

| Patient No. (Identifier) | Age, yr | Sex | Variant Site | Allele Frequency (gnomAD) | Disease Onset, yr | Hearing Loss | Kidney Function (eGFR, ml/min per 1.73 m²) | Pathologic Findings |
|--------------------------|--------|-----|--------------|--------------------------|------------------|-------------|---------------------------------|---------------------|
| 1 (A419)                 | 45     | Female | COL4A4     | c.1353C>T, p.Gly451=A; COL4A3, c.693G>A, p.Val231= | Japanese = 0.28 | 45          | Yes                | 105 | Thin basement membrane, α5(IV) staining; N/A |
| 2 (A577)                 | 35     | Female | COL4A4     | c.876G>A, p.Lys290= | ND               | 24          | No                 | 110.3 | Thin basement membrane, normal α5(IV) staining; Glomerulosclerosis |
| 3 (A586)                 | 66     | Male   | COL4A4     | c.876G>A, p.Lys290= | All = 0.00071  | 50          | No                 | 60 | |
| 4 (A619)                 | 43     | Female | COL4A4     | c.876G>A, p.Lys290= | ND               | School age | N/A                | N/A | Thin basement membrane, normal α5(IV) staining |

gnomAD, Genome Aggregation Database; N/A, not available; α5(IV), type IV collagen α5; ND, no data.

301) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent
Informed consent was obtained from the parents of the patients or the patients who participated in the study.

Genomic DNA Analysis
Genomic DNA was isolated from patients’ peripheral blood leukocytes using the QuickGene-Mini80 System or QuickGene-Auto 12S (Kurabo Industries Ltd., Tokyo, Japan), according to the manufacturer’s instructions. NGS samples were prepared using the HaloPlex Target Enrichment System (Agilent Technologies, Santa Clara, CA), following the manufacturer’s instructions. COL4A3, COL4A4, COL4A5, and other podocyte-related genes were sequenced using the MiSeq NGS platform (Illumina, San Diego, CA). Variant calling was carried out using SureCall 4.0 software (Agilent Technologies).

mRNA Analysis
The total RNA was extracted from peripheral blood leukocytes using the RiboPure Kit (Thermo Fisher Scientific) and reverse transcribed into cDNA using ReverTra Ace-α (Toyobo, Osaka, Japan). The cDNA was amplified in 35 reaction cycles using primers designed to target variants in COL4A3 or COL4A4 (Supplemental Table 1). The PCR products were subjected to agarose gel analysis.

In Vitro Splicing Assay
An H492 vector, previously developed in our laboratory, was used to create hybrid minigene constructs (22,23). The vector is based on the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, OR) to mimic in vivo splicing. We amplified genomic DNA from peripheral leukocytes of patients and controls to create hybrid minigenes using primers for the target variants in COL4A3 and COL4A4, which were designed complementarily to the ends of the linearized vector using the primer design tool In-Fusion (HD Cloning Kit; Takara, Shiga, Japan). This enabled cloning of the PCR products into the multiple cloning site of the vector, located within an intron between exons A and B. We cloned introns 19–21 of COL4A4 from patient 1, introns 11–14 of COL4A3 from patient 2, introns 11 and 12 of COL4A4 from patient 3, and introns 12–15 of COL4A4 from patient 4.

We used an in-fusion cloning reaction, and the constructs were transfected into HEK293T and HeLa cells using Lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours later, the total RNA was extracted from the cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse transcribed using the RNA to cDNA EcoDry Premix (Double Primed; Takara) and PCRs were run using a forward primer complementary to a segment upstream of exon A (YH307, 5'-ATTACCTGCTCAGAAGCTGTGTTGC-3') and a reverse primer complementary to a segment downstream of exon B (Y308, 5'-CTGCCAGTTGCTAAGTGAGA-3') to amplify only transcripts from each minigene. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, followed by Sanger sequencing.

In Silico Splicing Assay
We predicted splicing domain strength using Human Splicing Finder (HSF; http://www.umd.be/HSF3/HSF.shtml) and a commercial software package Alamut (Interactive Biosoftware, Rouen, France).

Results
sSNVs Detected by NGS
Three heterozygous sSNVs in COL4A4 were found in three patients (patient 1, c.1353C>T; patient 3, c.735G>A; and patient 4, c.876G>A). One patient harbored heterozygous sSNVs in COL4A3 (patient 2, c.693 G>A, Table 1, Supplemental Figure 1). No other pathogenic variants causing Alport syndrome–like clinical or pathologic findings.
were detected. The classification of variants, based on that of the American College of Medical Genetics and Genomics (ACMG) (24), is listed in the Supplemental Table 3.

**mRNA Analysis of Patients’ Peripheral Blood Leukocytes**

We found only normal transcripts in patients 1 and 2. Aberrant splicing in the form of exon skipping was detected in patients 3 and 4 (Figure 1, Table 2, Supplemental Figure 2).

**In Vitro Splicing Assay**

Aberrant minigene splicing in the form of exon skipping was revealed in two out of four patients (patients 3 and 4; Figure 1, Table 2, Supplemental Figure 3).

**In Silico Splicing Assay**

HSF predicted aberrant splicing in patients 1, 3, and 4, on the basis of a high variation score for the donor/acceptor site. ESE according to ESE finder matrices was predicted to be broken in all variants (Table 2, Supplemental Figure 4). In silico analysis by Alamut revealed all variants disrupt the donor/acceptor site with a possibility of exon skipping (Supplemental Figure 5).

**Discussion**

To the best of our knowledge, this is the first study to evaluate the pathogenicity of sSNVs in four patients with suspected ADAS by *in vivo*, *in vitro*, and *in silico* splicing analyses. All patients harbored sSNVs only in COL4A3 or COL4A4; we did not detect any variants that may cause
Table 2. Splicing assay results and variant effects on splicing motifs as assessed by Human Splicing Finder

| Patient No. | Variant | Exon | Gene | PSS/(Wild-Type versus Mutant Score)\(^a\) | Splicing Motifs | RT-PCR | Minigene Transcript |
|-------------|---------|------|------|------------------------------------------|-----------------|--------|---------------------|
| 1           | c.1353C>T | 20   | COL4A4 | Create new donor site (+68.2)/(39.34 versus 66.17) | Site broken via SC35 | ND | ND | r.═ | Wt: r.═,1370_1459del |
| 2           | c.693G>A  | 13   | COL4A3 | ND | ESE broken via SRp40 | ESS broken | ND | r.═ | Pt: r.═ |
| 3           | c.735G>A  | 12   | COL4A4 | Create new acceptor site (+62.8)/(46.62 versus 75.56); wild-type donor site broken (−11.38)/(92.92 versus 82.34) | ND | ESE broken via SC35 | ND | ND | r.[694_735del] | Wt: r.═ |
| 4           | c.870G>A  | 14   | COL4A4 | Create new acceptor site (+76.77)/(37.71 versus 66.66); wild-type donor and acceptor site broken (−13.5; −39.4) | ND | ESE broken via SF2/ASF | ND | ND | r.[817_870del] | Wt: r.═ |

PSS, potential splice site; WT, wild-type; MT, mutant; PBP, potential branch point; ESE, exonic splicing enhancer; ND, no disruption; Pt, patient; ESS, exonic splicing silencer.

\(^a\)PSS/(WT versus MT score) splicing motifs interpretation: The threshold is defined at 65. This means that every signal with a score above the threshold is considered to be a splice site (donor or acceptor). When a variant occurs, if the WT score is above the threshold, and the score variation (between wild type and mutant) is under −10\%, it is considered that the variant breaks the splice site. On the other hand, if the WT score is under the threshold and the score variation is above +10\%, it is considered that the variant creates a new splice site.
Alport syndrome—like clinical or pathologic findings, such as variants in LAMB2, LMX1B, PAX2, or MYH9. Several studies on Alport syndrome have investigated nonsynonymous variants, which cause changes in the amino acid sequence; however, it is important to study sSNVs because, although they do not affect the amino acid sequence, they may have several implications. Our results revealed that certain sSNVs can probably cause disease due to aberrant splicing.

Mature mRNA comprises only the coding sequence because introns are removed from the transcript during the splicing process (25). In most situations, adjacent pre-mRNA regulatory sequences, called ESEs or ESSs, influence splice site recognition and selection, which can have positive or negative effects on splice site utilization. ESEs largely mediate their effects as a binding site for trans-acting regulatory factors, which then recruit the splicing machinery small nuclear ribonucleoproteins subunits to the nearby splice site. ESEs are often bound by SR proteins (domain rich in alternating serine and arginine), whereas ESSs are typically bound by heterogeneous ribonucleoprotein (Supplemental Figure 6). Ultimately, whether a particular site is recognized by the spliceosome for inclusion of the adjacent exon in the mRNA product is determined by the sum of these multiple factors, which have positive and negative effects (Supplemental Figure 6) (26–28). Variants within the cis consensus sequences may produce aberrant transcripts due to improper exon-intron recognition in the mRNA, inclusion of cryptic exons, loss of an exon fragment, inclusion of an intronic fragment, or exon skipping, as seen in patients 3 and 4 in this study.

Patient 3 displayed severe symptoms of decreased renal function and glomerulosclerosis at the age of 66 years. Patient 4 had had proteinuria since childhood, and pathologic findings revealed a thin basement membrane. Both patients harbored an sSNV at the last nucleotide of an exon (patient 3, c.735G>A in COL4A4 exon 12; patient 4, c.870G>A in COL4A4 exon 14). On the basis of in silico analysis, we found that these nucleotide substitutions caused disruption of the wild-type donor site and ESEs. ESEs have been reported to be under strong selection constraints up to 50–100 bp from the exon ends (29). It has been estimated that at least 4% of sSNVs are deleterious due to an effect on enhancer function (30). Although patients 3 and 4 harbored normal alleles in addition to heterozygous variants, in vivo mRNA analysis of patient 4 showed only a faint normal transcript band (Figure 1, Supplemental Figure 7), and we detected no normal transcript in patient 3. This finding can be explained by the tendency of PCR to easily amplify short sequences. Moreover, in-frame transcript stability and persistence may be another contributing factor, because only the (42-bp) exon 12–skipping fragment in patient 3 was amplified. The minigene transcript analysis results were concordant with the results of mRNA analysis of peripheral blood leukocytes, indicating the minigene system can be used as an alternative to in vivo splicing assays.

Pathogenicity could not be confirmed for the remaining two variants by in vivo or in vitro transcript analysis. Patient 1 had an sSNV, but in vivo and in vitro analyses showed no transcriptional abnormalities. This finding was consistent with the low in silico prediction score (66.17) for variants that may interfere with the 5′ splice site. This patient had hearing loss, asymptomatic proteinuria, but no hematuria. We believed these clinical signs were not indicative of Alport syndrome; however, review of a renal biopsy specimen revealed a thin basement membrane and her family history revealed CKD with hematuria and proteinuria, which compelled us to conduct genetic analysis. Because no abnormalities were found, Alport syndrome was excluded. Aberrant splicing was also not found in patient 2. The variant in patient 2 was predicted to interrupt the ESEs (31). Because this variant did not interfere with the potential splice site strength, the earliest assembly phase of the spliceosome, binding of the SFI/BP protein or U2 auxiliary factor (U2AF) to the branch point site, and polypyrimidine tract would be well maintained and the 5′ and 3′ splice sites would be effectively recognized. The relative strength of a given splice site plays a crucial role in determining the final mRNA sequence (26). As for patient 2, IgA-predominant Ig deposition and mesangial matrix were observed and, therefore, a diagnosis of IgA nephropathy seemed likely. The in vivo and in vitro analyses of patients 1 and 2 did not suggest that the synonymous variant is the causative variant for Alport syndrome, but this confirms the certainty of our minigene analysis. Additional exon skipping was observed in the minigene transcript in the patient and the wild type (exon 21 skipping in patient 1) due to minigene overexpression, which is a limitation of this assay.

The importance of variants influencing splicing is being uncovered, and their possible role in genetic disease is gaining the attention of medical geneticists in clinical practice. Currently, analysis of the genomic DNA is the most common method used in laboratories. Yet, examining genomic DNA alone is not adequate to determine the pathogenicity in some situations. We can refer to the ACMG criteria and the results of in silico analyses using HSF and Alamut, but it is currently difficult to make a definitive diagnosis on the basis of these results alone. Because evidence of a damaging effect on the gene product is necessary, the easiest and most reliable approach is to analyze the patient’s RNA to detect splicing abnormalities. Nevertheless, owing to limited RNA sample availability (particularly specific tissue samples) and limitations in the use of these laboratory techniques, our present knowledge of splicing has not been routinely used in clinical practice (32). Therefore, the propagation of in vitro tools, such as those involving minigenes, is desirable.

One option is to use in silico prediction tools to filter out variants with a low possibility of being deleterious, reducing the number of candidate variants for further experimental validation (33). Our group previously reported that only about half of splicing patterns can be predicted using HSF (34). In this study, although HSF accurately predicted the splicing abnormality in our two patients, the results of patient 1 show that in silico analysis alone cannot be used an alternative to transcript analysis to determine pathogenicity. Other in silico tools are listed in Supplemental Table 2. Furthermore, it has not been determined whether the synonymous variant in this study can be reliably predicted by using those tools, which is one of the limitations of our study.

Patient 1 exhibited differential splicing patterns in HEK293T and HeLa cells, although the same minigene construct was used. The rules governing the inclusion of alternative exons in different cell types to generate protein diversity are complex and manifold. Several experiments have shown that additional information exists in short degenerate sequence motifs that lie both within and outside the exonic “splicing codes,” which interact with specific RNA-binding proteins to enhance or silence splicing. This splicing code can be interpreted differently in various cellular environments (35). In previous studies using a minigene assay of XLAS (34,36,37), HEK293T cells were used and yielded transcripts that were concordant with the in vivo analysis results. In this study, the results of the HEK293T cell-based minigene assay for patient 1 were similar to those of RNA sequencing. Despite being epithelial in origin, the biochemical machinery of HEK293T cells is capable of most of the post-translational folding and processing required to produce a functional and mature protein from a wide range of mammalian and nonmammalian nucleic acids (38), which explains why it is a popular choice among other cell lines for molecular studies.

In this study, we identified two novel sSNVs of COL4A4 that are pathogenic due to aberrant splicing in the form of exon skipping in four patients suspected of having Alport syndrome. To avoid missing disease variants, more patients with sSNVs should be examined, especially those that have variants that are predicted to have a high chance of splicing abnormalities.

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Author Contributions
Y. Aoto, T. Horinouchi, K. Iijima, S. Ishiko, S. Ishimori, A. Kondo, S. Matsu, M. Nagahama, S. Nagai, C. Nagano, H. Nagase, K. Nakanishi, T. Ninno, K. Nozu, E. Okada, N. Sakakibara, Y. Shima, K. Tamagaki, Y. Ubara, and T. Yamamura were responsible for validation; Y. Aoto, S. Ishiko, S. Ishimori, A. Kondo, S. Nagai, C. Nagano, E. Okada, N. Sakakibara, and T. Yamamura were responsible for investigation; T. Horinouchi reviewed and edited the manuscript; T. Horinouchi, K. Iijima, and K. Nozu were responsible for funding acquisition; T. Horinouchi, M. Matsu, K. Nozu, and R. Rossanti were responsible for methodology; T. Horinouchi, K. Nozu, and R. Rossanti conceptualized the study; K. Iijima, M. Matsu, H. Nagase, K. Nakanishi, T. Ninno, K. Nozu, and Y. Shima provided supervision; S. Matsu, M. Nagahama, R. Rossanti, K. Tamagaki, and Y. Ubara were responsible for data curation; and R. Rossanti was responsible for formal analysis and wrote the original draft.

Supplemental Material
This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0005252021/-/DCSupplemental.

Supplemental Figure 1. Sanger sequence of Alport synonymous cases.
Supplemental Figure 2. In vivo transcript sequence of 4 variants suspected ADAS.
Supplemental Figure 3. Minigene transcript sequence of 4 variants suspected ADAS.
Supplemental Figure 4. Mutations’ effect prediction on splicing motifs by Human Splicing Finder (HSF).
Supplemental Figure 5. In silico prediction analysis by the Alamut.
Supplemental Figure 6. Molecular interactions in the early phase of spliceosome assembly.
Supplemental Figure 7. In vivo mRNA analysis form patient 4 using high-sensitivity DNA assay.

Supplemental Table 1. Primer Set for in vivo transcript PCR analysis.

Supplemental Table 2. ACMG* classification for synonymous COL4A3 and COL4A5 variants.

Supplemental Table 3. Summary of input, output, and interpretation of prediction scores for 5' and 3' splice site prediction.

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