INTRODUCTION

Genetic information is coded on the genome, and transcription produces pre-mRNA as primary transcripts, which are further modified by several processes to generate mature mRNA, including 5′ cap, 3′ polyadenylation (poly[A] tail), and AS.1–4 Both the quantity and quality of the genetic information are influenced not only by transcriptional factors and chromatin loops but also by post-transcriptional regulation, including RNA splicing, editing, chemical modification (e.g., methylation), and polyadenylation.1–4 Recent studies have discovered cancer-associated changes in RNA editing, RNA modifications, and the expression of noncoding RNA species, including micro-RNAs and long noncoding RNAs.5–8 Especially, mutations in genes encoding core spliceosomal proteins and accessory regulatory splicing factors are now considered among the most recurrent genetic abnormalities in patients with cancer, particularly hematologic malignancies. These include mutations in the major (U2-type) and minor (U12-type) spliceosomes, which remove >99% and ~0.35% of introns, respectively. Growing evidence indicates that aberrant splicing of evolutionarily conserved U12-type minor introns plays a crucial role in cancer as the minor spliceosome component, ZRSR2, is subject to recurrent, leukemia-associated mutations, and intronic mutations have been shown to disrupt the splicing of minor introns. Here, we review the importance of minor intron regulation, the molecular effects of the minor (U12-type) spliceosomal mutations and cis-regulatory regions, and the development of minor intron studies for better understanding of cancer biology.

KEYWORDS
LZTR1, minor introns, splicing, U12 spliceosome, ZRSR2
detailed information on RNA splicing of normal and malignant cells on a genome-wide scale. For example, it is estimated that >90% of multiexon human genes undergo AS, an enzymatic process in which a single gene has the potential to produce multiple, potentially functionally distinct pre-mRNA and protein isoforms. Therefore, pre-mRNA splicing is considered a major mediator of proteome diversity due to its ability to generate various isoforms and transcripts with differing amino acid sequences from identical DNA sequences. Furthermore, due to the functional link between aberrant splicing and NMD, splicing serves as a step in regulating the expression of the entire gene. Indeed, specific splicing events occur at defined stages of tissue- or cell type-specific development, such as hematopoiesis, resulting in selective increases or decreases in the inclusion of individual exons to alter the function or stability of the encoded proteins to define a cell’s identity. In the new era of transcriptome exploration, recent studies have revealed that a small number of introns, called “minor” introns, spliced by distinct machinery, play pivotal roles in various diseases, such as congenital growth retardation, neuromuscular disease, and cancer. Here, we review our understanding of the regulatory mechanism of minor introns and the dysregulation involved in cancer predisposition and development.

2 | WHAT ARE MINOR (U12-TYPE) INTRONS?

Eukaryotic spliceosome consists of several snRNPs. Eukaryotic cells have two types of spliceosomes: U2 dependent (major) and U12 dependent (minor) (Figure 1A). Both spliceosomes existed in the last eukaryotic common ancestor, and most of the spliceosome-associated proteins are shared between the U2 and U12 spliceosomes. Nonetheless, the types of snRNAs in each spliceosome are unique. The U2 spliceosome contains the U1, U2, U4, U5, and U6 snRNAs, whereas the U12 spliceosome contains unique U11, U12, U4atac, and U6atac snRNAs; both the U2 and U12 spliceosomes contain U5 snRNA. Therefore, eukaryotic cells harbor two distinct pre-mRNA splicing machineries, referred to as the major (U2-dependent, >99% introns) and minor (U12-dependent, <0.35% introns) spliceosomes, which recognize and excise the major or minor class of introns, respectively. In line with the rarity of minor introns, the low abundance of their minor spliceosome (~100-fold compared with the major spliceosome), especially the catalytic component U6atac snRNP, has been reported. The major introns are characterized by GT–AG introns, BPS, and a PPT located immediately upstream of the 3′ ss (Figure 1A). In contrast with major introns, minor introns are characterized by AT–AC introns (although most minor introns have been shown to have a GT–AG dinucleotide), highly evolutionarily conserved 5′ ss, and BPS (Figure 1A). These introns also lacked the characteristic polypyrimidine tract present in U2-type introns. In human cells, splicing of U2-type introns is initiated with the U1 snRNP binding to the 5′ ss, SF1 binding to the BPS, and U2AF1/2 heterodimer binding to the 3′ ss and PPT, respectively. Subsequently, the U2 snRNP is recruited to the BPS and displaces SF1. SF3B1, a component of U2 snRNP, is involved in binding to the BPS. The preassembled U4/U6/U5 complex binds, and the U1 and U4 snRNPs are released to form a catalytically active spliceosome complex that mediates intron excision and proximal and distal exon ligation to synthesize mature mRNA. In contrast with U2-type introns, the 5′ ss and BPS of U12-type introns are cooperatively recognized by the U11 and U12 snRNAs, respectively, and the U4atac/U6atac/U5 tri-snRNP complex is used. These steps in splicing are similar to those in the U2- and U12-dependent pathways.

Minor introns constitute only ~0.35% of all human introns and have been reported to be present in 700–800 genes (https://midb.pnb.uconn.edu, https://genome.crg.es/cgi-bin/u12db/u12db.cgi). A recent study documented 770 minor introns in human MIGs, with each MIG typically carrying only a single U12-type minor intron and multiple U2-type introns in the other introns. Importantly, such introns are enriched in genes that represent a somewhat restricted set of functional classes and pathways rather than being randomly distributed throughout the genome. They are especially abundant in genes involved in information processing functions, such as DNA replication/repair, translation, RNA processing, transcription, cytoskeletal organization, voltage-gated ion channel activity, kinase, and vesicular transport. Gene ontology terms of MIGs are shown in Figure 1B. The identities of genes carrying minor introns and their positions in their host genes are highly conserved among humans, animals, and plants. One example of this is the first intron of the PTEN gene, which has been evolutionarily conserved even in European honey bees (Apis mellifera) (Figure 1C). However, the same intron 1 is a U2-type intron in C. elegans, indicating splice site mutations during evolution. In mammals, PTEN mRNA expression is positively regulated by the splicing of the minor intron.

3 | MINOR SPLICEOSOMAL ZRSR2 MUTATIONS AND HEMATOLOGIC MALIGNANCIES

Since 2011, several groups have reported a high frequency of splicing factor mutations in patients with MDS, characterized by clonal hematopoiesis, cytopenia, and abnormal cellular maturation/differentiation. This occurred most frequently in the U2 (SF3B1, SRSF2, and U2AF1) and U12 spliceosomes (ZRSR2). This striking finding was accompanied by the observation that these mutations were mutually exclusive, shared convergent functions, and that when co-mutated, they are synthetically lethal. Several studies have demonstrated that these four genes are recurrently mutated in various hematologic malignancies. The mutational frequency of each spliceosomal component is indicated in Figure 2A. Notably, in contrast with U1/U2 spliceosomal mutations detected in solid tumors, such as uveal/mucosal melanoma (SF3B1), breast cancer (SF3B1), and Sonic Hedgehog medulloblastoma (U1SnRNA), ZRSR2 mutations are
FIGURE 1 Characteristics of U12-dependent (minor) introns. (A) Main determinants for distinguishing U2-type and U12-type introns in terms of 5′/3′ splice sites (ss), branchpoint sequences (BPS), and polypyrimidine tract (PPT). Red-dotted rectangles indicate evolutionarily conserved sequences in U12-dependent (minor) introns. (B) Gene ontology analysis of minor intron-containing genes (MIGs). (C) Conservation of PTEN/Pten first intron as a minor intron across species.
restricted in hematologic malignancies, especially in MDS and BPDCN, SF3B1, SRSF2, and U2AF1 are subject to heterozygous, change-of-function missense mutations that affect specific residues. In contrast, the X chromosome-encoded ZRSR2 is enriched in nonsense and frameshift mutations across the open reading frame in male patients, consistent with loss of function. The mutational distribution across ZRSR2 coding sequence in MDS and AML is shown in Figure 2B (https://www.cbioportal.org ). For example, across >2000 sequentially sequenced myeloid neoplasm patients, 100% of these patients with somatic mutations in ZRSR2 were males, and no female had the ZRSR2 mutation. In line with the role of U12 spliceosome in minor intron splicing, Madan et al. first demonstrated that ZRSR2 loss results in impaired splicing of U12-type introns, and RNA-seq of MDS bone marrow (BM) revealed that loss of ZRSR2 activity causes increased missplicing. These splicing defects involved the retention of U12-type introns, while splicing of U2-type introns was mostly unaffected. Furthermore, global minor IR followed by NMD on a ZRSR2 null background was confirmed with the RNA-seq of another ZRSR2-mutated MDS cohort (Figure 2C) and the newly generated Zrsr2 conditional knockout murine model. A striking enrichment for U12-type IR in ZRSR2-mutant patients has been reported, but not in patients with SF3B1, SRSF2, or U2AF1 mutations (Figure 2D). Phenotypically, the analysis of the murine model enhanced self-renewal of Zrsr2-null HSCs in vivo, which stands in marked contrast with recent studies that evaluated the effects of hotspot mutations in SF3B1, SRSF2, and U2AF1, all of which identified a perplexing impairment in self-renewal when those mutations were induced in mice using similar transplantation methods. Although another Zrsr2-deficient model developed almost no hematologic phenotypes, both groups shared the same finding that Zrsr2-deficient myeloid progenitors exhibited missplicing of minor introns; however, the effects on splicing were less pronounced than those of the
ZRSR2-mutated human MDS cells. To this regard, Madan et al. reported a collective role of Zrsr1 (Zrsr2p1) and Zrsr2 in the murine U12-spliceosome.58

Blastic plasmacytoid dendritic cell neoplasm is a hematologic malignancy that is thought to develop from pDCs or their precursors.59 Male-predominant BPDCN can have leukemic involvement of the blood and BM, as well as tumor formation in the skin (in ~90% of cases), lymphoid organs, and other tissue.41,59 Togami et al.41 discovered ZRSR2 mutations in ~26% of BPDCN, most of which are nonsense or frameshift mutations. It is quite striking since BPDCN is the only hematologic malignancy in which ZRSR2 is the most prevalently mutated of the four spliceosomal proteins described above. Given that the minor (U12) spliceosome component, ZRSR2, is subject to recurrent, leukemia-associated mutations, minor intron splicing is essential in the hematopoietic system.

4 | DOWNSTREAM OF NMD TARGETS IN ZRSR2-MUTATED CELLS

With regard to the physiologic roles of the minor spliceosome, transcriptomic analyses of ZRSR2-mutated MDS cells revealed that only a subset of minor spliceosome-dependent genes is recurrently and robustly misspliced, implying that not all U12-type introns are equally crucial for disease pathogenesis.16 To identify the functionally important splicing target, we performed CRISPR enrichment screening using the custom sgRNA library for the protein-coding region of each of the 601 MIGs whose mRNAs were identified as differentially spliced in ZRSR2-mutant MDS patient samples versus spliceosomal wild-type MDS patient samples and predicted to result in NMD. Strikingly, in addition to PTEN, only one gene, LZTR1, was significantly enriched in all conditions (Figure 3A), and encodes a substrate-specific CUL3 adaptor regulating ubiquitination-mediated suppression of RAS-related GTPases,50–62 and is subject to loss-of-function mutations in glioblastoma,63 schwannomatosis,64 and the RASopathy known as Noonan syndrome.65 Indeed, the loss of LZTR1 resulted in a significant accumulation of RAS proteins, including RIT1, a RAS GTPase recently identified as an endogenous LZTR1 substrate,61 and a gene known to undergo activating mutations in RASopathies and various cancers.66,67 An inspection of the transcriptomic data from primary BM cells revealed that the retention of a U12-type intron in LZTR1 (intron 18) followed by NMD was specific to ZRSR2-mutant MDS and undetected in BM samples from ZRSR2 wild-type MDS and normal subjects.

Togami et al.41 demonstrated that the ZRSR2 mutation in pDC causes abnormal pDC development and an inflammatory response via TLRs. Also, it impairs pDC activation by TLR stimulation with lipopolysaccharide and activation-induced apoptosis, all of which are associated with the retention of the minor intron in IRF7 and the inability to enhance IRF7 expression.41 Indeed, such events promote pDC expansion and signatures of decreased activation in vivo. Interestingly, the LZTR1 IR events observed in ZRSR2-mutated MDS cells were not reproduced in pDC-lineage cells with ZRSR2 depletion.41 Similarly, IRF7 IR was restricted in pDCs, indicating that
minor intron regulation by ZRSR2 is a highly context- and cellular-dependen event.\textsuperscript{16} Levesque et al. analyzed RNA-seq data from The Cancer Genome Atlas for 14 cohorts and reported that the differential splicing of minor introns is context dependent in tumorigenesis.\textsuperscript{68}

As described above, ZRSR2 dependency may vary across species, indicating a distinct vulnerability in specific human organs. Interestingly, in contrast with branchpoints (BPs) within U2-type introns, which are highly constrained in their location, BPs within U12-type introns exhibit a bimodal distribution, such that half of the U12-type introns have BPs similar in location to U2-type BPs, while half of the U12-type BPs occur in closer proximity (within 20 nucleotides) to 3′ ss.\textsuperscript{29} Interestingly, this bimodality is relevant to ZRSR2 responsiveness. Introns that respond to ZRSR2 loss had BPs that were significantly more proximal to the 3′ ss than nonresponsive introns. In contrast, nonresponsive U12-type introns exhibited no such spatially restricted enrichment, implying that BP location influences the U12-type intron susceptibility to retention in the absence of ZRSR2.\textsuperscript{29} These findings suggest that minor introns can be classified into two groups based on ZRSR2 dependency. Other than the ZRSR2 mutation, U12 snRNP components have not been reported to be recurrently mutated in cancer, although minor snRNP components, such as RNU4ATAc, RNPC3, and RNU12, have been shown to be mutated in congenital and degenerative diseases with neurological or developmental phenotypes.\textsuperscript{69-73}

5 | CANCER-ASSOCIATED MUTATIONS IN MINOR INTRONS

Despite the relative rarity of U12 spliceosomal mutations, there are other mechanisms for inhibiting minor intron splicing. An example is LZTR1 minor intronic mutation in a reported family with autosomal recessive Noonan syndrome in which a child died of acute leukemia,\textsuperscript{65} harboring mutations in LZTR1's minor intron (c.220-17C>A) and LZTR1's coding region (R210*). This same intronic sequence is also mutated in schwannomatosis.\textsuperscript{64} These mutations were detected in the U12 BPS-containing evolutionarily conserved region “TCCTTAAC.” Indeed, sgRNAs targeting either the BPS or coding regions promptly induced resistance to tyrosine kinase inhibitor in K562 cells endogenously harboring a BCR/ABL translocation (Figure 3B).\textsuperscript{36} Given that even a single base substitution or deletion disturbed the intron removal, the splicing fidelity of minor introns is highly dependent on the BPS, all of which were validated by the minigene reporter assay, in which the intronic sequences can be mutagenized in the plasmid, and minigene-derived splicing is evaluated by RT-PCR.\textsuperscript{56} As shown in Figure 4A,B, most mutations within “TCCTTAAC” induced transcripts with intron18 retention, followed by NMD.

 Conversely, splice site mutations do not simply cause IR. For example, in Peutz–Jeghers syndrome, an autosomal dominant disorder associated with gastrointestinal polyposis and increased cancer risk, it has been reported that the 5′ ss mutation (AT > GT) of STK11 (also known as LKB1) minor intron (Intron 2) resulted in aberrant splicing from the mutated 5′ ss to several cryptic, noncanonical 3′ ss immediately adjacent to the normal 3′ ss.\textsuperscript{74} Although only a few intronic mutations in the conserved BPS and splice sites have been phenotypically validated, recent efforts for aggregating and harmonizing both exome and genome sequencing data from various large-scale sequencing projects\textsuperscript{75} (gnomAD, https://gnomad.broadinstitute.org) have raised the possibility that various genomic variants affect the interaction with minor spliceosomes when they are located at the region essential for the canonical U12-type spliceosome. For example, PTEN and PARP1 are MIGs (in the first and last introns, respectively) and well studied tumor suppressors involved in genomic stability, metabolism, and DNA repair. Of note, both minor introns in PTEN and PARP1 have genomic variants that alter 3′ ss and BPS-containing evolutionarily conserved “TCCTTAAC” (Figure 4C). In fact, Oltshof et al. identified 51 pathogenic variants in a minor intron ss that reduce the ss strength and induce AS.\textsuperscript{33} Further studies are required to determine the extent to which similar variants or single-nucleotide polymorphisms are associated with cancer predisposition on a genome-wide scale.

6 | PROSPECTS OF A MINOR INTRON STUDY IN THE CANCER FIELD

The exploration of genetic variants or somatic mutations at 5′/3′ ss and BPS is underway by several groups. Given that the involvement in both alleles is considered stochastically rare, such studies should focus on mutations in regions prone to deletion or loss of heterozygosity. A recent study by Oltshof et al. proposed that AS of minor introns occurs more frequently than previously considered and occurs in a tissue-specific manner.\textsuperscript{33} Other than simple loss of function of MIGs due to NMD, such AS or somatic/germline mutations may result in translation into a truncated protein, exerting dominant-negative or gain-of-function effects, as most minor introns are expected to have in-frame stop codons. Additionally, Oltshof et al. discovered that AS events were more prevalent in long minor introns, whereas retention was favored in shorter introns.\textsuperscript{33} Quantitative proteomics or ribosome profiling combined with RNA/whole genome-seq will shed light on previously unrecognized variants.

Minor introns are less efficiently excised from pre-mRNA than major introns. Therefore, it has been postulated that minor introns serve as “molecular switches” to regulate the expression of their host genes, with the rate of removal of a single minor intron within a gene regulating the expression of the entire host mRNA.\textsuperscript{76,77} This may enable each MIG to be fine tuned in context-specific time-controlled regulation. Another significance of minor introns might be solved with the analysis of animal models depleted with specific U12-type introns or with a dCas13d-based RNA tracking system.\textsuperscript{78} These ideas are indirectly supported by the evidence of the high instability of U6atac snRNP, a crucial component of the minor spliceosome catalytic core,\textsuperscript{77} resulting in inefficient minor intron splicing. Alternatively, rapid MIG expression is achieved when U6atac is stabilized by the stress-activated protein kinase, MAPK14 (p38MAPK),
which in turn activates these molecular switches to allow the expression of genes required to deal with cellular stress, such as PTEN.77 Whether specific IR events in tumor suppressors are shared among cancers and contribute to tumorigenesis remains unelucidated. Our study demonstrated that LZTR1’s minor intron was efficiently excised in all normal samples.16 However, a notable subset of tumors without ZRSR2 or LZTR1 mutation in almost all profiled cancer types exhibited IR that were specific to LZTR1’s minor intron.16 Furthermore, the extent of LZTR1 IR varied across samples and cancer types, with 11.1% of all profiled cancer samples exhibiting LZTR1 minor IR exceeding that observed in any peritumoral control normal tissue.16 Considering tissue-specific MIG expression and minor IR events, shared retention events among patients with cancer may enable us to identify unnoticed tumor suppressors among MIGs.

Finally, ZRSR2 mutations in the X chromosome exhibit marked male predominance,16,41 indicating a sex bias in the vulnerability to aberrant splicing and dysregulated MIGs. ZRSR2 is considered an example of “EXITS,” which stands for “escape from X inactivation tumor suppressor.”79 At the same time, we separated minor introns into ZRSR2-responsive and ZRSR2-unresponsive groups, implying a ZRSR2-independent regulatory mechanism.16 Future studies should examine the sex bias in relation to ZRSR2 sensitivity. The ZRSR2-independent splicingosome complex could be revealed by dCas13-APEX2 tools that catalyze biotin-labeling to neighboring biomolecules at U12 BPS.80 Additionally, the development of nanopore sequencing will allow us to investigate not only full-length MIG mRNA but also its dynamics, modification, and further speculation.81

FIGURE 4 Conserved branchpoint sequences (BPS) of minor introns is essential for canonical splicing. (A) LZTR1 exon18–minor intron–exon19 minigene reporter plasmids with indicated mutations were generated. WT, wild-type. (B) RT-PCR of LZTR1 minigene with specific primers for the minigene reporter. The mutants in red induced significant intron retention. (C) Examples of genetic variants at BPS and 3′ splice sites (ss) in representative minor intron-containing genes (MIGs) (PTEN, PARP1, and LZTR1)

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D.I., W.Z., K.N., and H.Y. conceived the project and wrote the paper.

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CONFLICT OF INTEREST
The authors have no conflict of interest.

ETHICS STATEMENT
The review article contains published or publicly available datasets.

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