Transcriptome complexity in intravascular NK/T-cell lymphoma

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ABSTRACT

Aims Intravascular NK/T-cell lymphoma (IVNKTCL) is a rare disease, which is characterised by exclusive growth of large cells within the lumen of small vessels, Epstein–Barr virus infection and somatic mutations in epigenetic regulator genes. Here, we elucidate the transcriptomic complexity of IVNKTCL.

Methods IVNKTCL cases were retrieved from a single-centre cohort of 25 intravascular lymphomas. RNA-seq and whole exome sequencing (WES) were performed to analyse transcriptomic abnormalities and mutations in splicing factors.

Results Approximately 88% of the total reads from the RNA-seq were considered exonic, while the remaining reads (12%) were mapped to intronic or intergenic regions. We detected 28,941 alternative splicing events, some of which would produce abnormal proteins rarely found in normal cells. The detected events also included tumour-specific splicing alterations in oncogenes and tumour suppressors (e.g., *HRAS*, *MDM2* and *VEGFA*). WES identified premature termination mutations or copy number losses in a total of 15 splicing regulator genes, including *SF3B5*, *SRSF12* and *TNPO3*.

Conclusions This study raises the possibility that IVNKTCL may be driven by multiple complex regulatory loops, including non-exonic expression and aberrant splicing, in addition to defects in epigenetic regulation.

INTRODUCTION

Intravascular lymphoma (IVL) is a rare form of non-Hodgkin's lymphoma characterised by almost-exclusive growth of neoplastic cells within the lumina of small-sized or medium-sized blood vessels.[1] Most cases of IVL have a B-cell phenotype (intravascular large B-cell lymphoma, IVLBCL),[2–5] but extremely rare cases with an NK/T-cell phenotype (i.e., intravascular NK/T-cell lymphoma, IVNKTCL) have also been identified.[6–11] IVNKTCL has been first described in 2003, and only 20 cases have been reported in the literature. IVNKTCL is aggressive and has a poor overall prognosis, especially in patients with multisystem involvement (e.g., skin, liver and central nervous system). The pathobiology of IVNKTCL has been linked to infection with the Epstein–Barr virus (EBV), which is considered to be a cause of tumourigenesis and responsible for the aggressive features of this lymphoma.[6–11]

We have recently investigated the somatic mutations, mutation signatures and copy number alterations (CNAs) in IVNKTCL at whole-genome levels using exome sequencing.[11] Results obtained from this first genetic study not only identified frequent alterations in epigenetic regulators such as histone genes, histone deacetylase, DNA/RNA helicases and methylation-related enzymes, but it also revealed the intricate interwoven relationships that govern the biological behaviour of this aggressive lymphoma. More importantly, this result reinforced the concept that multiple genetic events are required to unleash the malignant progression of IVL, and that only a genome-wide approach could interpret the complicated scenarios in the biology of this hematopoietic neoplasm. Although our previous study contained promising results, the focus was on somatic alterations occurring at the DNA level. A systematic analysis of variation in transcript architecture has not yet been reported in IVLs.

While the human genome is estimated to contain about 20,000 protein-coding genes, their transcriptome is tremendously more complex, with 83,666 distinct mRNA sequences indexed on the current version (V.31) of GENCODE.[12] This remarkable discrepancy between the number of protein-coding genes can be explained by alternative splicing (AS), the process by which a single mRNA precursor can be spliced in different arrangements to produce functionally and structurally distinct protein variants.[13] AS has a profound effect on the biologic characteristics of the final protein, as AS can add or delete functional domains, change the stability of the protein and modify protein–protein interactions (PPI). Growing evidence has also demonstrated that AS is frequently modified in malignant neoplasms and is associated with tumour initiation and progression.[14–20]

Here, we comprehensively investigate the gene expression and AS patterns in IVNKTCL by running MISO (mixture of isoforms), a probabilistic framework that quantitates the expression level of alternatively spliced genes from high-throughput RNA-seq data.[21] Our study reveals the complicated regulatory loops driving this rare haematologic malignancy. To the best of our knowledge, this is the first study addressing the complexity of the IVL transcriptome.

MATERIALS AND METHODS

Case selection

IVNKTCL cases were retrieved from the surgical pathology files (2000–2017) of the Kobe City Medical Center General Hospital. IVNKTCL was defined as an extranodal NK/T-cell lymphoma selectively growing within the lumina of vessels, particularly capillaries, while larger arteries and veins were spared. Patient background is shown in online supplementary table 1.

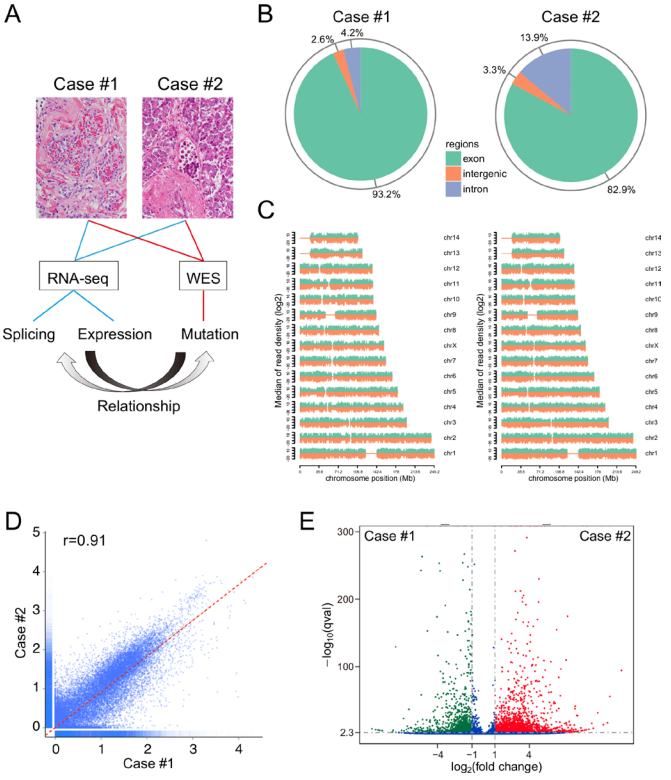

Figure 1 Gene expression analysis. (A) Study design and histology of intravascular NK/T-cell lymphoma (IVNKTCL). (B) Statistics for the distribution of reads on the genome. Reads are mapped to exons (green), introns (blue) or intergenic regions (orange). (C) Distribution of mapped reads on chromosomes. (D) Scatter plot representation of gene expression levels of all genes in two IVNKTCL cases. One blue dot corresponds to one gene. Pierson's r is indicated at the left upper corner. (E) Overview of differentially expressed genes displayed as a volcano diagram. The threshold for differential expression of genes was a $|\log_2(\text{foldchange})| > 1$ and a Q-value < 0.005.

RNA SEQUENCING

Five 10 µm sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue blocks, and areas with >60% tumour content were microdissected for RNA extraction. Total RNA was extracted from the tumour samples using the RNeasy FFPE Kit (Qiagen, Madison, WI). RNA-seq libraries were constructed using the NEBNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The quality-checked RNA-seq libraries were sequenced on a HiSeq X (Illumina, San Diego, CA). The raw image data file from the high-throughput sequencing was transformed to sequence data by CASAVA V.1.8. The sequence data were stored as FASTQ format files. TopHat2 was used to map quality-filtered reads onto the human genome build 19 (hg19), and sequencing reads were ultimately mapped to exonic, intronic or intergenic regions. Gene expression analysis was performed using the DESeq2 R package. Raw sequence data are available on request.

Quantification of alternative splicing events

The MISO Bayesian inference model was applied for the quantification of AS events. AS events were classified into the following five patterns: alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), mutually exclusive exon (MXE), intron retention (IR) and exon skipping (ES). 'Percent Spliced In (Ψ)' (PSI) was used to denote the fraction of mRNAs that represent the inclusion isoform, and the MISO model was used to calculate the posterior probability distributions of Ψ and ΔΨ for the two samples. The latter distribution was used to calculate the Bayes factor (BF), defined as the ratio of the posterior probability of the alternative hypothesis, $\Delta\Psi \neq 0$, to that of the null hypothesis, $\Delta\Psi = 0$; thus, higher values of the BF indicate increased confidence in differential regulation.

The significant differentially spliced events were determined by the following two parameters:

1. BF ≥ 10.
2. PSI values (percent-spliced-in, Ψ) ≥ 0.2.

Whole exome sequencing data

Information regarding somatic mutations and CNAs was retrieved from our previous whole exome sequencing (WES) data.[11] In brief, WES libraries were prepared from tumour and non-tumour tissues with a SureSelect Human Exon V6 enrichment system (Agilent, Santa Clara, CA), and paired-end sequencing was performed on a HiSeq X (Illumina) according

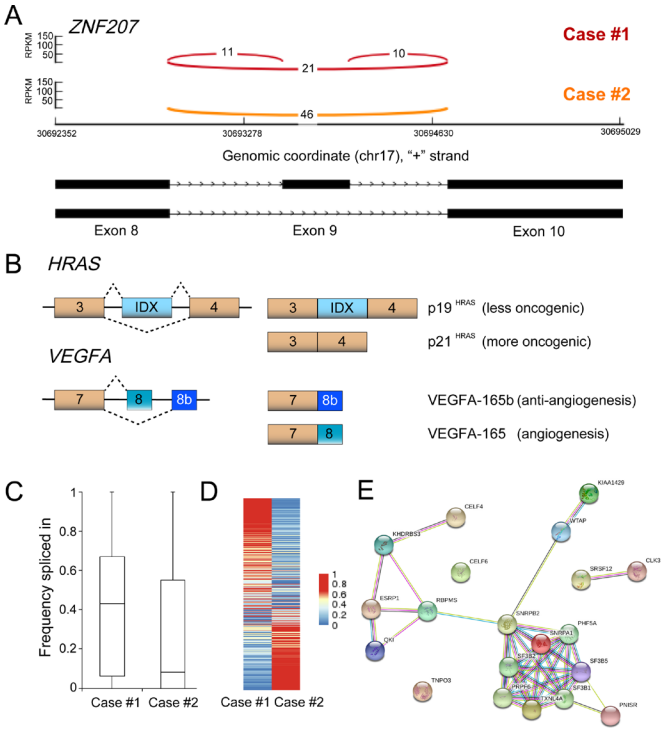

Figure 2 Alternative splicing analysis. (A) One representative example of the Sashimi plot of an alternative exon skipping event. Junction reads in the *ZNF207* gene are visualised as arcs connecting the pair of exons that the junction borders. The thickness of the arc is found to be in proportion to the number of junction reads present in the sample, but the actual number of junction reads is plotted. (B) Aberrant splice variants and oncogenic consequences for $p21^{HRAS}$ and VEGFA-165. *HRAS* pre-mRNA can be alternatively spliced to include the intron D exon (IDX) before exon 4, yielding the p19 and p21 proteins, respectively. Anti-angiogenic VEGFA-165b and pro-angiogenic VEGFA-165 are generated from the same transcript. (C) Box plot depicting the frequency of alternative exon skipping in cases #1 and #2. (D) Heatmap displaying the result of differential alternative splicing events between the two cases. Differently expressed AS events were identified based on PSI value. (E) The protein–protein interaction network analysis of somatically altered genes displayed using the STRING database. Broad defects in splicing factors were detected in this analysis. PSI, percent-spliced-in.

to the previous study.[22] BWA (http://bio-bwa.sourceforge.net/) and SAMtools (http://samtools.sourceforge.net/) were used for reading alignments and single nucleotide variant calling. Control-FREEC (http://boevalab.com/FREEC/) was applied to analyse somatic CNAs. Deleteriousness prediction scores were obtained using the SIFT, PolyPhen-2, MutationTaster and CADD algorithms. The functional interactome of the proteins was explored utilising STRING software V.11.0.

RESULTS

Study design

Two IVNKTCL cases were retrieved from a single-centre cohort of 25 IVLs. IVNKTCLs were characterised by aggregates of large atypical lymphoid cells occluding the lumina of small-sized blood vessels (figure 1A). RNA-seq and WES of these cases were performed to analyse gene expression, splicing abnormalities and somatic mutations in splicing factors. The data were finally integrated to uncover the pathogenesis of IVNKTCL.

Gene expression and alternative splicing in IVNKTCL

RNA-seq was successfully performed for both cases, and 7.8 Gb and 8.6 Gb of clean reads were produced for cases #1 and #2, respectively. Exonic reads provided 93.2% and 82.9% of the total reads from RNA-seq in cases #1 and #2, respectively, while the remaining reads (6.8% and 17.1%) were mapped to intron or intergenic regions, which highlighted the widespread occurrence of non-exonic transcription (figure 1B). Genomic location biases at the chromosome level were not detected in the mapping data (figure 1C). Gene expression analysis showed that two cases had a similar range of expression values (Pearson's r=0.91; figure 1D), although a small proportion of genes were differentially expressed (figure 1E).

RNA-seq was then analyzed by MISO software. We detected a total of 28,941 AS events in the form of five types of splicing events (A5SS, A3SS, MXE, IR and ES) (figure 2A; representative example of ES in *ZNF207*). Some AS events were tumour-specific alterations in oncogenes and tumour suppressors (e.g., *HRAS*,[23] *MDM2*,[24] *VEGFA*,[25] *FGFR2*,[26] *BCL2L1*,[27] *FAS*,[28] and *AIMP2*[29]), most of which had been previously reported in epithelial neoplasms (figure 2B and table 1). In addition, the ES patterns were largely different between the two cases (median frequency of alternative ES: case #1, 0.43; case #2, 0.08) (figure 2C). In total, 932 AS events were statistically designated as differentially expressed isoforms between the two cases (figure 2D). These results suggested that RNA splicing was highly perturbed at the whole genome level, while the gene expression levels remained relatively similar.

Somatic alterations in splicing factors

We retrieved our previous annotation of WES data[11] and constructed PPI maps of mutated or deleted genes with higher degrees of connectivity based on STRING networks. This PPI map revealed broad defects in splicing factors, comprising premature termination mutations in two splicing factors and copy number loss of 13 splicing factors (for a total of 15 affected splicing factors) (figure 2E and table 2). No somatic mutations were detected in the well-known cancer oncogenes and tumour suppressors described above (e.g., *HRAS*, *MDM2* and *VEGFA*) even though tumour-specific AS events were identified in these genes.

Table 1 Tumour-related alternatively spliced isoforms identified in IVNKTCL patients

Gene name	Alterations in cancer-related functions due to alternative splicing	Splicing type	ENSG_ID	Event	PSI (percentage-spliced-in) Case #1	Case #2
MDM2	Reduced p53 binding enhanced tumour progression	A3SS	ENSG00000135679	chr12:69207334:69207408:+@chr12:69233054|69233393:69233629:+	0.41	0.84
HRAS	Activates PKC pathway and induces the G1/S delay	ES	ENSG00000174775	chr11:533453:533612:-@chr11:533277:533358:-@chr11:532242:532755:-	0.27	0.01
FGFR2	Promotes EMT and metastasis	MXE	ENSG00000066468	chr10:123279493:123279683:-@chr10:123278196:123278343:-@ chr10:123276833:123276977:-@chr10:123274631:123274833:-	0.48	0.35
VEGFA	Enhanced proangiogenic function	A3SS	ENSG00000112715	chr6:43749693:43749824:+@chr6:43752278|43752344:43754223:+	0.60	0.36
BCL2L1	Apoptosis regulator	A5SS	ENSG00000171552	chr20:30310151:30309458|30309647:-@chr20:30252261:30253889:-	0.69	0.61
FAS	Loss of proapoptotic activity	ES	ENSG00000026103	chr10:90771756:90771838:+@chr10:90773100:90773124:+@chr10:90773876:90775542:+	0.51	0.87
AIMP2	Apoptosis regulation	ES	ENSG00000106305	chr7:6048882:6049129:+@chr7:6054777:6054983:+@chr7:6057445:6057676:+	0.86	0.66

A3SS, alternative 3′ splice site; A5SS, alternative 5′ splice site; ES, exon skipping; IVNKTCL, intravascular NK/T-cell lymphoma; MXE, mutually exclusive exon.

Table 2 Identification of somatic mutations and copy number alterations in mRNA splicing factors in IVNKTCL patients

	Gene name	*Gene ID*	*Exonic function*	*AA change*	*SIFT*	*Polyphen2*	*MutationTaster*	*CADD*
Case #1	*TNPO3*	NM_012470	Frameshift	p.Tyr843fs*	N/A	N/A	N/A	N/A
Case #2	*RBPMS*	NM_001008712	Stop-gain	p.Glu214*	N/A	N/A	Disease_causing	7.73
	Gene name		*Chromosome*	*Type*	*Copy number*	*Cytoband*	*Size (bp)*	
Case #2	*SRSF12, PNISR*		6	Loss	1	6q16.2-q16.3	22,895,517	
	SF3B5, WTAP, QKI		6	Loss	1	6q27-q25.2	31,197,821	
	ESRP1, KIAA1429		8	Loss	1	8q21.12-q21.2	21,422,296	
	KHDRBS3		8	Loss	1	8q24.23	3,236,255	
	CELF6, SNRPA1, CLK3		15	Loss	1	15q23-q24.1	33,151,892	
	CELF4		18	Loss	1	18q21.1-q12.3	16,064,221	
	TXNL4A		18	Loss	1	18q22.3-q21.32	26,113,240	

SIFT predictors: deleterious, tolerated.
Polyphen2 predictors: probably_damaging, possibly_damaging, benign.
MutationTaster predictors: disease_causing, polymorphism.
CADD: A CADD score of 10 corresponds to the top 10% deleterious variants in the genome. The score of 7.73 for case #2 is in the top 7.73% of deleterious variants.
IVNKTCL, intravascular NK/T-cell lymphoma.

DISCUSSION

The present study thoroughly investigated the transcriptomic landscape of two rare IVNKTCL cases. Twenty cases of IVNKTCL have been reported to date.[6–10] Our cases comprise the 21st and 22nd, and this is the first study focusing on transcriptomic modifications in IVNKTCL patients. The high-throughput RNA sequencing analysis allowed us to explore the tumour-related changes that occurred at the stage of transcription and splicing and to identify transcript isoforms that are specific to IVNKTCL.

We demonstrated that a subset of sequencing reads mapped to introns and intergenic regions. This subset was not a negligible portion, accounting for >10% of reads, on average. What are the possible causes of these intronic or intergenic mapped reads in RNA-seq? There are several possibilities for reads aligned to intergenic regions: (1) sequencing errors, (2) mapping errors, (3) unannotated genes or (4) true intergenic transcription. Intronic reads could be due to (1) immature transcripts with introns not yet spliced out, or (2) unannotated exons which might represent AS, if the sequence and mapping quality are reliable. Previous systematic studies on mammalian transcriptomes have suggested that more than 70% of the genome undergoes transcription, and that most of these transcripts are weakly expressed non-coding RNA.[30 31] In addition, although mRNA processing is a stringently regulated mechanism that enables the generation of multiple functional variants of an individual gene, many of these mechanisms are highly perturbed and can be seized to favour the malignant state of tumours.[14–20] As the RNA sequence quality was reliable in our study, it is reasonable to interpret the intronic and intergenic transcriptions as true reads.

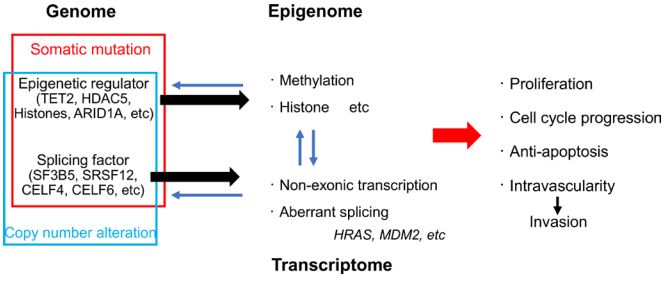

Figure 3 Schematic diagram of the pathogenesis of intravascular NK/T-cell lymphoma.

AS perturbations have been recently recognised as important signatures for tumour progression and therapy.[14–20] Some AS changes recapitulate tumour-associated phenotypes by regulating cell proliferation, DNA damage, invasion and apoptosis. In addition, tumour-specific splice variants of genes that control cell proliferation and apoptosis have been reported in the last decade.[23–29 32 33] These processes are influenced both by genetic factors and environmental factors, for example, immune responses,[34 35] age,[36] heat stress[37] and DNA damage.[38] CNAs have also been described as having a particular association with AS.[16 19] In this study, we identified previously known tumour-specific AS in cancer driver genes involved in disease pathogenesis (e.g., *HRAS*, *MDM2*, *FGFR2* and *VEGFA*). AS changes could partly originate from somatic mutations in splicing factors, as well as through mutations that disrupt splicing regulatory motifs in exon–intron boundaries, both of which could impact the splicing of cancer-related genes. Future functional studies will be necessary to determine the consequences of the genetic alterations and AS changes identified in this study.

The pathogenesis of IVNKTCL is summarised in figure 3. In our latest research, we identified frequent alterations in genes encoding epigenetic regulators, such as histone clusters, *TET2*, *HDAC5*, *ARID1A*, *ARID1B*, *DDX3X* and *WRN*,[11] which suggest that IVNKTCL is an epigenetic disease with EBV infection-associated etiopathogenesis. Along with theses previous data, the current whole transcriptome analysis further raises the possibility that IVNKTCL may be driven by multiple complex regulatory loops, including defects in epigenetic regulators and AS. This self-amplifying malignant loop is genome-wide, and could induce ever-worsening abnormalities during tumourigenesis of IVNKTCL.

Targeting the tumour-associated AS events or non-exonic transcriptions could be possible therapeutic approaches for treating IVL patients. Several strategies using small-molecule compounds and antisense oligonucleotides have already been proposed for modulating the core spliceosome machinery or eliminating tumour-specific transcripts.[15 32 33 39] These drugs have been developed for other solid tumours and are already shown to potentially inhibit excessive aberrant AS activities in limited types of cancer cells. They may be applicable to IVNKTCL in the future.

We are currently investigating the cause of the intravascular growth pattern in IVL, but it is proving difficult to elucidate. The

intravascular feature has been hypothesised to be secondary to defects in homing receptors present on the neoplastic cells, such as a lack of ICAM-1 (CD54) or ITGB1 (CD29).[40 41] However, in our genetic studies, we could not detect clear evidence of genetic and/or transcriptomic alterations in these adhesion molecules. Further genetic and functional characterisation of molecular defects will contribute to our understanding of the cause of the intravascular growth pattern in IVL.

This study has revealed the widespread transcriptomic abnormalities in IVNKTCL. This rare and aggressive disease could be driven by multiple complex regulatory loops; including non-exonic transcription, AS and defects in epigenetic regulators. Our observations may provide new insights into tumourigenesis and management of this aggressive entity.

Take home messages

- RNA-seq analysis revealed that non-exonic transcription and aberrant alternative splicing are prevalent in intravascular NK/T-cell lymphoma (IVNKTCL).
- Tumour-specific alternative splicing in oncogenes and tumour suppressors was detected in IVNKTCL.
- Premature termination mutations or copy number losses of splicing factors were identified in IVNKTCL.

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Contributors KF designed the study, performed the experiments and wrote the manuscript. MY collected clinical data. KU assisted bioinformatic analysis. All authors read and approved the submitted version of manuscript.

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