No association between *ECSIT* germline mutations and hemophagocytic lymphohistiocytosis in natural killer/T-cell lymphoma

Hemophagocytic lymphohistiocytosis (HLH) is a clinical syndrome of excessive immune activation with fever, cytopenia, and organ infiltration by activated macrophages. Secondary HLH associated with natural killer (NK)/T-cell lymphoma (NKTCL) has extremely poor prognosis,[1] and biomarkers that may predict patients who are more likely to develop HLH are lacking. Wen *et al.*[2] recently showed an association between a somatic gene mutation in the evolutionarily conserved signaling intermediate in Toll pathway (*ECSIT*) gene (c.T419C; p.V140A) and HLH in NKTCL. The variant ECSIT protein triggered NF-κB signaling pathway more potently, leading to aberrant secretion of proinflammatory cytokines by *ECSIT*-T419C-transfected NKTCL cell lines. They found that the *ECSIT*-T419 mutation was significantly enriched in individuals with NKTCL-associated HLH, which developed in nine of 17 patients with and five of 36 patients without the mutation, respectively. Patients with *ECSIT*-T419 had elevated expression of proinflammatory cytokines and poorer prognosis. While intriguing, the prevalence of *ECSIT*-T419 and relation with HLH has not been assessed in independent cohorts. We therefore sought to examine whether the *ECSIT*-T419 mutation predisposes to HLH in multiple cohorts of patients with NKTCL and correlates its presence with clinical outcomes.

First, we studied the mutational profile of *ECSIT* in 25 subjects with sporadic NKTCL from China with available whole exome sequencing of paired tumour-blood samples.[3] Samples were sequenced with Illumina HiSeq X and NextSeq 6000, and variant-calling was performed by Strelka2 using default single-sample settings.[4] We found the *ECSIT*-T419 mutation in five of 25 subjects, but they were all germline mutations; heterozygously mutated in both matching tumour (variant allele frequency [VAF], mean, 43.8%, 95% Confidence Interval [CI]: 38.8-48.9) and blood (VAF mean, 53.8%, 95%CI: 51.5-56.2) samples from these five subjects (Figure 1A). The reported prevalence of somatic *ECSIT*-T419 mutation in Wen *et al.*'s study was 19.3% (17 of 88), similar to the mutation frequency of Jiang *et al.*'s cohort, but were all germline mutations.

In order to further verify whether *ECSIT*-T419 is a germline or somatic mutation, we studied 67 patients with NKTCL who provided written informed consent under respective institutions' Institutional Review Boards (IRB) from Singapore local hospitals and Sun Yat-Sen University Cancer Center in Guangzhou, China. We Sanger sequenced matched tumor- buccal swab (representative Sanger sequence in Figure 1B) or peripheral blood (representative Sanger sequence in Figure 1C) samples from NKTCL patients and *ECSIT*-T419 was validated in 7.5% (five of 67) of both the tumor and matching non-tumor samples. Targeted resequencing using next-generation sequencing method revealed the mean VAF of *ECSIT*-T419 to be 52.2%, 95%CI: 42.8-61.6 in the five tumors, 52.2%, 95%CI: 48.5-56.0 in four matched blood samples, and 53% in a matched buccal swab sample (P=0.90, 2-tailed Wilcoxon Rank-sum test, VAF of *ECSIT*-T419 between tumors and non-tumoral samples). The near 50% VAF, and the presence of *ECSIT*-T419 mutation observed in all matching tumor, blood and buccal swab DNA indicate that this is a germline heterozygous single-nucleotide polymorphism (SNP), with a report SNP ID of rs145036301. Among the five patients with *ECSIT*-T419 mutation, HLH information was available for three patients and none developed HLH, as defined by the HLH 2004 criteria.[5]

Given the discrepant findings, we re-analyzed the initial discovery cohort of paired tumor-normal exome data (n=5) from Wen *et al.*[2] In the sample where *ECSIT*-T419 mutation was reported as a somatic mutation, VAF was 52% in the tumor (150 of 288; alternate allele depth/reference allele depth) and 10% (5 of 51) in the matched normal sample (Figure 1D). Furthermore, the VAF exceeded the thresholds of 30% in tumor and 5% in matched normal sample as specified by Wen *et al.*[2] Thus, this variant should not be considered as a somatic mutation as based on the authors' analysis criteria.

Notwithstanding the false somatic call, we wanted to examine whether the germline *ECSIT*-T419 mutation is associated with HLH in two independent cohorts of patients with NKTCL in Singapore and Taiwan. In Singapore, the cases were identified using local databases from two teaching hospitals and all samples and clinical information were collected after IRB approval. Cases were reviewed by a central pathologist and HLH was defined according to the HLH 2004 criteria. Sixty-four cases of NKTCL were identified between 2007-2017, and *ECSIT*-T419 mutations were found in 15.4% (two of 13) and 5.9% (two of 51) patients with and without HLH respectively. Out of the 13 patients with HLH, four were women. Median age was 43 (range, 18-60 years). At time of the last follow-up in December 2018, all patients had died. Seven of 12 patients received polychemotherapy, while one was treated with the HLH-2004 protocol (with dexamethasone, etoposide, cyclosporin), and two received steroids. Median survival was only 33 days (range, 1-389 days). Causes of death were lymphoma (n=6), HLH (n=6), and infection (n=1). The two individuals with *ECSIT* mutation succumbed at day 1 and day 89. Within these NKTCL patients with HLH in our Singapore cohort, there was no significant association of the *ECSIT* mutation with them (P=0.18, Fisher's exact test, *Online Supplementary Table S1*).

In the Taiwanese cohort of 85 NKTCL cases with clinical and sequencing data from the Chang Gung Memorial Hospital, *ECSIT*-T419 mutation frequency was observed at 11.8% (ten of 85). Nine cases developed HLH, and none of these samples harboured the *ECSIT*-T419C mutation. When both Singapore and Taiwan cohorts were combined for analysis, we did not find any statistical association between *ECSIT*-T419C mutation and HLH (OR=1.48, 95%CI: 0.38-5.76, $P\approx1.0$, Fisher's exact test, *Online Supplementary Table S2*). There were also no significant associations between the *ECSIT*-T419C mutation with clinical characteristics such as sex, stage, Eastern Cooperative Oncology Group (ECOG) performance status and international prognostic index (Figure 1E; *Online Supplementary Table S3*). Overall survival (*Online Supplementary Figure S1A*) and progression-free survival (*Online Supplementary Figure S1B*) were also not significantly associated with *ECSIT*-T419 mutation.

Given the rarity and fulminant nature of malignancy-associated HLH hindering the collection of biopsy specimens, we combined data from multiple cohorts to examine associations between *ECSIT*-T419 and HLH in NKTCL in the largest study to date. Strict diagnostic inclusion criteria were used for both HLH and NKTCL. Some possible explanations for the discordant results between Wen *et al.* and our study need consideration. Patients in Singapore and Taiwan developed HLH at around the time of diagnosis or relapse, as opposed to

Wen *et al.*'s cohort which developed HLH 3 to 6 months after diagnosis of NKTCL, during or after treatment. The onset of HLH might be triggered by the initiation of chemotherapy that leads to loss of immune homeostasis and further aggravates T-cell dysfunction which may further lower the threshold for triggering HLH in lymphoma patients.[6] It is possible that in the absence of chemotherapy in our patients, the activating effect of the *ECSIT*-T419 mutation on the NF-κB pathway is not strong enough to drive HLH. However, there were four *ECSIT* wild-type patients from Singapore who developed HLH again after chemotherapy initiation.

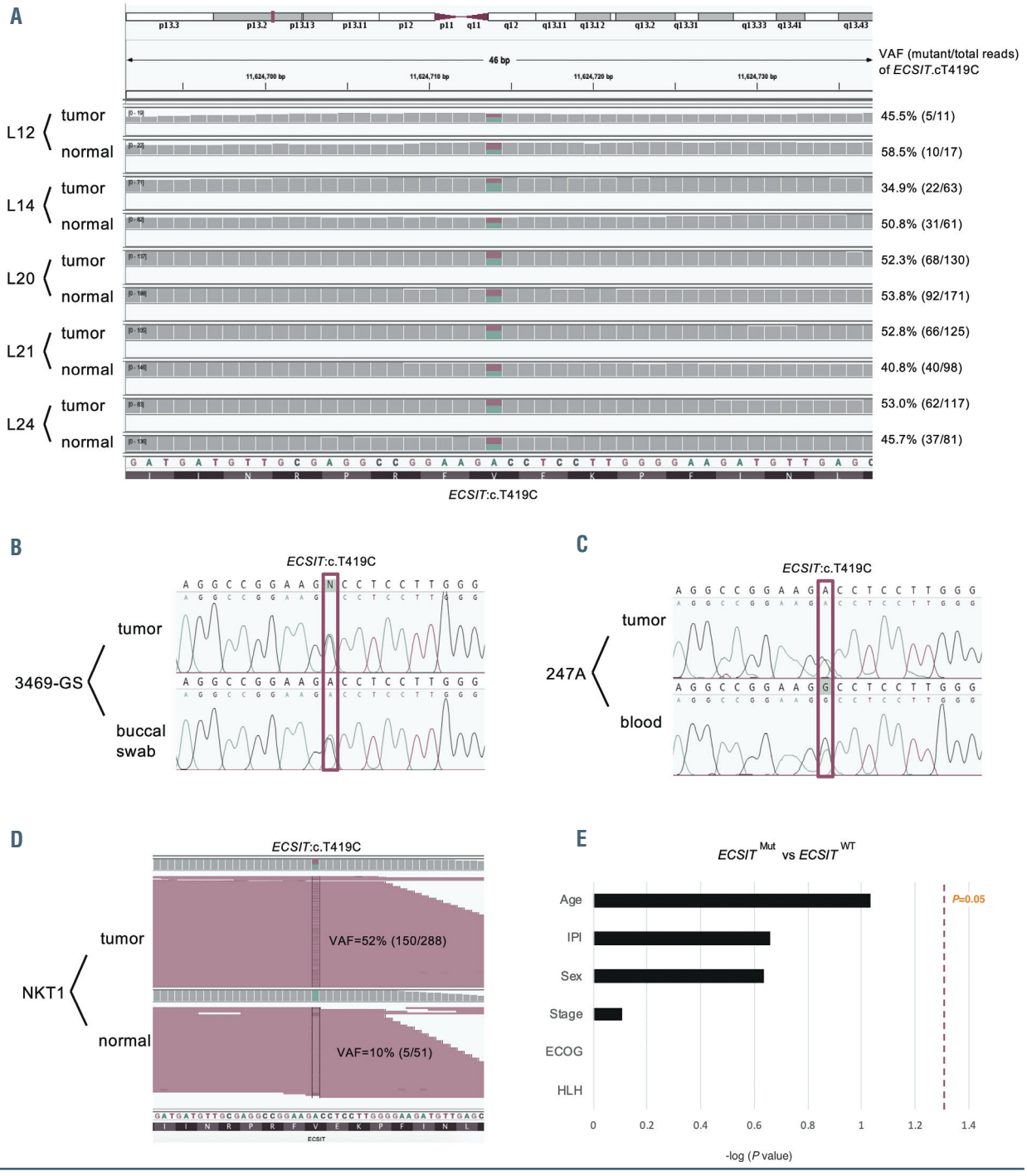

Figure 1. *ECSIT*-T419C is a germline mutation not associated with hemophagocytic lymphohistiocytosis in natural killer/T-cell lymphoma patients. (A) Sanger sequencing electropherogram profile for tumor-normal paired samples with heterozygous *ECSIT*-V140A mutation, identified as L12, L14, L20, L21, and L24 in Jiang *et al.*[3] (B and C) Representative Sanger sequencing electropherogram profile for two tumor- peripheral blood (B) and buccal swab (C) samples for the *ECSIT*-V140A mutation from Singapore local hospitals and the Sun Yat-Sen University Cancer Center in Guangzhou, China. (D) Integrative Genomics Viewer (IGV) snapshot centered around heterozygous germline *ECSIT*-T419C mutation of the paired tumor-normal exome sequencing data of sample NKT1 from Wen *et al.*[2] Variant allele frequencies (VAF) were calculated from the number of variant-supporting/total read-counts at *ECSIT*-T419C. Aligned reads were colored pink according to the read-strand that they were aligned with onto the human reference genome. (E) No association between ECSIT mutation and clinical characteristics of natural killer/T-cell lymphoma patients in Singapore and Taiwan. *ECSIT*: evolutionarily conserved signaling intermediate in Toll pathway; IPI: international prognostic index, ECOG: Eastern Cooperative Oncology Group, HLH: hemophagocytic lymphohistiocytosis, Mut: mutant; WT: wild-type.

Differences in other patient characteristics may also explain the discordance (e.g., patients with HLH in Singapore had stage III or IV disease, while most patients with HLH in Wen *et al.*'s cohort had early stage disease).

In summary, our data from multiple cohorts do not support the risk effect of *ECSIT*-T419 mutation (SNP rs145036301) on HLH in NKTCL. Furthermore, this is a germline rather than somatic mutation that appears in SNP database (dbSNP v153) and has now been flagged as a common polymorphic variant by Catalogue of Somatic Mutations in Cancer (COSMIC v90) databases.[7,8] Additionally, there were no differences in clinical characteristics or prognosis between NKTCL patients with and without *ECSIT*-T419 mutation. One limitation of our study is not being able to examine whether germline variants in genes associated with familial HLH are enriched in patients with NKTCL-associated HLH. However, recent studies have not shown an association of biallelic pathogenic variants in HLH-associated genes with adult HLH, albeit in cohorts that comprise a mixture of lymphoma and non-lymphoma subtypes.[9,10] Ultimately, additional efforts to define disruptive variants in a larger number of genes, in expanded cohorts of adults with lymphoma associated HLH, may further refine our understanding and treatment of this devastating condition.

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