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

by Shin Yeu Ong, Jing Quan Lim, Nicholas Grigoropoulos, Yurike Laurensia, Dachuan Huang, Burton Kuan Hui Chia, Daryl Cheah Ming Zhe, Sahil Ajit Saraf, Chee Leong Cheng, Wen-Yu Chuang, Ming-Chung Kuo, Yi-Jiun Su, Colin Phipps, Chandramouli Nagarajan, Yuh Shan Lee, Daryl Tan Chen Lung, Lee-Yung Shih, Yeow Tee Goh, Soon Thye Lim, and Choon Kiat Ong

Haematologica 2020 [Epub ahead of print]

Citation: Shin Yeu Ong, Jing Quan Lim, Nicholas Grigoropoulos, Yurike Laurensia, Dachuan Huang, Burton Kuan Hui Chia, Daryl Cheah Ming Zhe, Sahil Ajit Saraf, Chee Leong Cheng, Wen-Yu Chuang, Ming-Chung Kuo, Yi-Jiun Su, Colin Phipps, Chandramouli Nagarajan, Yuh Shan Lee, Daryl Tan Chen Lung, Lee-Yung Shih, Yeow Tee Goh, Soon Thye Lim, and Choon Kiat Ong. No association between ECSIT germline mutations and hemophagocytic lymphohistiocytosis in natural killer/T-cell lymphoma. Haematologica. 2020; 105:xxx

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Title: No association between ECSIT germline mutations and hemophagocytic lymphohistiocytosis in natural killer/T-cell lymphoma

Running title: ECSIT mutation not associated with HLH in NKTCL

Shin Yeu Ong1*, Jing Quan Lim2,3*, Nicholas Grigoropoulos1, Yurike Laurensia2, Dachuan Huang2,3, Burton Kuan Hui Chia2, Daryl Cheah Ming Zhe2, Sahil Ajit Saraf4, Chee Leong Cheng4, Wen-Yu Chuang5, Ming-Chung Kuo6, Yi-Jiun Su6, Colin Phipps1, Chandramouli Nagarajan1, Yuh Shan Lee1, Daryl Tan Chen Lung1, Lee-Yung Shih6, Yeow Tee Goh1, Soon Thye Lim2,3+, Choon Kiat Ong2,3,7+

1Department of Haematology, Singapore General Hospital, Singapore
2National Cancer Center, Singapore
3Duke-NUS Medical School, Singapore
4Department of Pathology, Singapore General Hospital
5Department of Pathology, Chang Gung Memorial Hospital at Linkou, and Chang Gung University, Taoyuan, Taiwan
6Division of Hematology-Oncology, Chang Gung Memorial Hospital at Linkou, and Chang Gung University, Taoyuan, Taiwan
7Genome Institute of Singapore, A*STAR

*These authors contributed equally to this work

Corresponding authors

Dr. Choon Kiat ONG
Principal Investigator
Lymphoma Genomic Translational Research Laboratory, Cellular and Molecular Research, National Cancer Centre Singapore, 11 Hospital Drive, 169610, Singapore
Email: cmrock@nccs.com.sg
Tel: +65 6436 8269

Prof. Soon Thye LIM
Deputy Director (Clinical)
National Cancer Centre Singapore, 11 Hospital Drive, 169610, Singapore
Email: lim.soon.thye@singhealth.com.sg
Tel: +65 6436 8173
Dear Editor,

Hemophagocytic lymphohistiocytosis (HLH) is a clinical syndrome of excessive immune activation with fever, cytopenia, and organ infiltration by activated macrophages. Secondary HLH associated with NK/T-cell lymphoma has extremely poor prognosis (1), and biomarkers that may predict patients who are more likely to develop HLH are lacking. Wen et al. recently showed an association between a somatic gene mutation in the Evolutionarily Conserved Signalling Intermediate In Toll Pathway (ECSIT) gene (c.T419C; p.V140A) and HLH in NK/T-cell lymphoma (NKTCL) (2). The variant ECSIT protein triggered NF-κB signalling pathway more potently, leading to aberrant secretion of proinflammatory cytokines by ECSIT-T419C-transfected NKTCL cell lines. They found that the ECSIT-T419C mutation was significantly enriched in individuals with NKTCL-associated HLH, which developed in 9/17 patients with and 5/36 patients without the mutation, respectively. Patients with ECSIT-T419C had elevated expression of proinflammatory cytokines and poorer prognosis. While intriguing, the prevalence of ECSIT-T419C and relation with HLH has not been assessed in independent cohorts. We therefore sought to examine whether the ECSIT-T419C mutation predisposes to HLH in multiple cohorts of patients with NKTCL and correlate 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-T419C mutation in 5 out of 25 subjects, but they were all germline mutations; heterozygously mutated in both matching tumour (variant allele frequency, VAF, mean=43.8%, 95% CI [38.8, 48.9]) and blood (VAF, mean=53.8%, 95% CI [51.5, 56.2]) samples from these 5 subjects (Figure 1A). The reported prevalence of somatic ECSIT-T419C mutation in Wen et
al.’s study was 19.3% (17/88), similar to the mutation frequency of Jiang et al’s cohort, but were all germline mutations.

To further verify whether ECSIT-T419C is a germline or somatic mutation, we studied 67 patients with NKTCL who have 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-T419C was validated in 7.5% (5/67) of both the tumor and matching non-tumor samples. Targeted resequencing using next-generation sequencing method revealed the mean VAFs of ECSIT-T419C to be 52.2%, 95% CI [42.8, 61.6] in the 5 tumors, 52.2%, 95% CI [48.5, 56.0] in 4 matched blood samples, and 53% in a matched buccal swab sample (P=0.90, 2-tailed Wilcoxon Rank-sum test, VAFs of ECSIT-T419C between tumors and non-tumoral samples). The near 50% VAFs, and the presence of ECSIT-T419C mutation observed in all matching tumor, blood and buccal swab DNA indicate that this is a germline heterozygous single-nucleotide polymorphism, with a report SNP ID of rs145036301. Among the five patients with ECSIT-T419C 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 reanalysed the initial discovery cohort of paired tumor-normal exome data (n=5) from Wen et al (2). In the sample where ECSIT-T419C mutation was reported as a somatic mutation, VAF was 52% in the tumor (150/288; alternate allele depth/reference allele depth) and 10% (5/51) in the matched normal sample (Figure 1D). Furthermore, the VAFs exceeded the thresholds of 30% in tumor and 5% in matched normal sample as specified by Wen et al. Thus, this variant should not be considered as somatic mutation as based on the authors’ analysis criteria.
Notwithstanding the false somatic call, we wanted to examine whether the germline \textit{ECSIT}-T419C 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 \textit{ECSIT}-T419C mutations were found in 15.4\% (2/13) and 5.9\% (2/51) patients with and without HLH respectively. Out of the 13 patients with HLH, 4 were women. Median age was 43 (range 18-60 years). At time of last follow-up in December 2018, all patients have died. 7 out 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 day to 389 days). Causes of death were lymphoma (n=6), HLH (n=6), and infection (n=1). The two individuals with \textit{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 \textit{ECSIT} mutation with them (P=0.18, Fisher’s exact test, Supplementary Table 1).

In the Taiwanese cohort of 85 NKTCL cases with clinical and sequencing data from Chang Gung Memorial Hospital, \textit{ECSIT}-T419C mutation frequency was observed at 11.8\% (10/85). Nine cases developed HLH, and none of these samples harboured the \textit{ECSIT}-T419C mutation. When both Singapore and Taiwan cohorts were combined for analysis, we did not find any statistical association between \textit{ECSIT}-T419C mutation and HLH (OR=1.48, 95\% CI [0.38, 5.76], P≈1.0, Fisher’s exact test, Supplementary Table 2). There were also no significant associations between the \textit{ECSIT}-T419C mutation with clinical characteristics such as sex, stage, ECOG performance status and international prognostic index (Figure 1E and Supplementary Table 3). Overall survival (Supplementary Figure 1A) and progression-free
survival (Supplementary Figure 1B) were also not significantly associated with \textit{ECSIT-T419C} 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 \textit{ECSIT-T419C} 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 \textit{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 \textit{et al}’s cohort which developed HLH three to six 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 \textit{ECSIT-T419C} mutation on the NF-κB pathway is not strong enough to drive HLH. However, there were four \textit{ECSIT}^{WT} patients from Singapore who developed HLH again after chemotherapy initiation. 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 \textit{et al}’s cohort had early stage disease).

In summary, our data from multiple cohorts do not support the risk effect of \textit{ECSIT-T419C} mutation (SNP rs145036301) on HLH in NKTCL. Furthermore, this is a germline rather than somatic mutation that appears in dbSNP(v153) and has now been flagged as a common polymorphic variant by COSMIC(v90) databases (7, 8). Additionally, there were no differences in clinical characteristics or prognosis between NKTCL patients with and without \textit{ECSIT-T419C} 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.
Acknowledgements

The study was supported by grants from the Singapore Ministry of Health’s National Medical Research Council (NMRC-OFLCG-18May0028 and NMRC-ORIRG16nov090), Tanoto Foundation Professorship in Medical Oncology, New Century International Pte Ltd, Ling Foundation, and Chang Gung Memorial Hospital (OMRPG3C0021), Taiwan.

Contributions

CKO conceived the project and designed the study, SYO drafted the initial manuscript, CCL, SAS, and WYC performed pathological studies, YL performed sequencing studies, JQL and BKHC performed the bioinformatics analysis. SYO, JQL, DCH, DCMZ, CP, CN, YSL, DTCL, STL, MCK, YJS, and LYS recruited participants, managed subject information and tissue samples, and contributed to data analysis. CKO, JQL, NG and YTG participated in critical revision of the manuscript.

Ethics declarations

Conflict of interest

The authors declare that they have no conflict of interest.
References


Figure Legends

**Figure 1.** ECSIT-T419C is a germline mutation not associated with HLH in NKTCL patients. (A) Sanger sequencing electropherogram profile for tumor-normal paired samples with heterozygous ECSIT\textsuperscript{V140A} mutation, identified as L12, L14, L20, L21, and L24 in Jiang et al., *Nature Genetics*, 2015. (B and C) Representative Sanger sequencing electropherogram profile for two tumor- peripheral blood (B) and buccal swab (C) samples for the ECSIT\textsuperscript{V140A} mutation from Singapore local hospitals and Sun Yat-Sen University Cancer Center in Guangzhou, China. (D) IGV snapshot centered around heterozygous germline ECSIT-T419C mutation of the paired tumor-normal exome sequencing data of sample NKT1 from Wen et al. VAFs were calculated from 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 NKTCL patients in Singapore and Taiwan. VAF: variant allele frequency, IPI: international prognostic index, ECOG: eastern cooperative oncology group, HLH: hemophagocytic lymphohistiocytosis, Mut: mutant, WT, wild-type.
Supplementary information

Supplementary Figure 1. Kaplan-Meier survival curves for all 149 patients from Singapore and Taiwan showing (A) Overall Survival (OS) and (B) Progression-Free Survival (PFS) according to ECSIT$^{V140A}$ mutation status. ECSIT$^{V140A}$ was not associated with survival (PFS, \(P=0.91\); OS, \(P=0.96\), log-rank)

Supplementary Table 1. Hemophagocytic lymphohistiocytosis (HLH) was not associated with ECSIT$^{V140A}$ in the 64 patients from Singapore (OR=4.45, 95% CI [0.56, 35.17], \(P=0.18\), Fisher’s exact test).

Supplementary Table 2. Hemophagocytic lymphohistiocytosis (HLH) was not associated with ECSIT$^{V140A}$ in the 149 patients from Singapore and Taiwan (OR=1.48, 95% CI [0.38, 5.76], \(P\approx1.0\), Fisher’s exact test).

Supplementary Table 3. Association between ECSIT mutation and clinical characteristics of NKTCL patients in Singapore and Taiwan
Supplementary Figure 1. Kaplan-Meier survival curves for all 149 patients from Singapore and Taiwan showing (A) Overall Survival (OS) and (B) Progression-Free Survival (PFS) according to ECSITV140A mutation status. ECSITV140A was not associated with survival (PFS, $P=0.91$; OS, $P=0.96$, log-rank)
**Supplementary Table 1.** Hemophagocytic lymphohistiocytosis (HLH) was not associated with ECSIT<sup>V140A</sup> in the 64 patients from Singapore (OR=4.45, 95% CI [0.56, 35.17], \( P=0.18 \), Fisher’s exact test).

\[
\begin{array}{c|cc}
 & \text{ECSIT}^\text{MUT} & \text{ECSIT}^\text{WT} \\
\hline
\text{HLH} & 2 & 11 \\
\text{No HLH} & 2 & 49 \\
\end{array}
\]

**Supplementary Table 2.** Hemophagocytic lymphohistiocytosis (HLH) was not associated with ECSIT<sup>V140A</sup> in the 149 patients from Singapore and Taiwan (OR=1.48, 95% CI [0.38, 5.76], \( P\approx1.0 \), Fisher’s exact test).

\[
\begin{array}{c|cc}
 & \text{ECSIT}^\text{MUT} & \text{ECSIT}^\text{WT} \\
\hline
\text{HLH} & 3 & 21 \\
\text{No HLH} & 11 & 114 \\
\end{array}
\]
**Supplementary Table 3.** Association between *ECSIT* mutation and clinical characteristics of NKTCL patients in Singapore and Taiwan

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>ECSIT</em>&lt;sup&gt;WT&lt;/sup&gt;</th>
<th><em>ECSIT</em>&lt;sup&gt;MUT&lt;/sup&gt;</th>
<th>P value</th>
<th>Univariate OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLH, Y/N</td>
<td>21/114</td>
<td>3/11</td>
<td>1.0</td>
<td>1.48 [0.38, 5.76]</td>
</tr>
<tr>
<td>&lt; 60 years, Y/N</td>
<td>10/4</td>
<td>42/49</td>
<td>0.0921</td>
<td>2.92 [0.85, 9.98]</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>7/7</td>
<td>93/42</td>
<td>0.230</td>
<td>0.45 [0.15, 1.37]</td>
</tr>
<tr>
<td>Stage I+II, Y/N</td>
<td>8/5</td>
<td>71/55</td>
<td>0.777</td>
<td>1.24 [0.38, 4.0]</td>
</tr>
<tr>
<td>ECOG 0-1, Y/N</td>
<td>9/3</td>
<td>102/15</td>
<td>1.0</td>
<td>0.44 [0.11, 1.82]</td>
</tr>
<tr>
<td>IPI 0-2, Y/N</td>
<td>6/6</td>
<td>80/38</td>
<td>0.220</td>
<td>0.48 [0.14, 1.57]</td>
</tr>
</tbody>
</table>

* OR, odds ratio; HLH, hemophagocytic lymphohistiocytosis; IPI, international prognostic index