To the editor:

The \textit{BRAF-V600E} mutation in circulating cell-free DNA is a promising biomarker of high-risk adult Langerhans cell histiocytosis

We read with great interest the recent review article on Langerhans cell histiocytosis (LCH) by Delprat and Aricò.\textsuperscript{1} As they mentioned, LCH is a rare disorder characterized by local accumulation of dysplastic Langerhans cells and a wide range of organ involvement. Although the precise pathophysiology remains unknown, recent findings suggest that LCH is likely to be a clonally expanding myeloid neoplasm. One of the strongest lines of evidence is a report by Badalian-Very et al that the oncogenic \textit{BRAF-V600E} mutation was detected in LCH lesions from a majority of patients.\textsuperscript{2} Furthermore, Berres et al found that patients with active, high-risk LCH carried the \textit{BRAF-V600E} mutation in circulating CD11c\textsuperscript{+}/CD14\textsuperscript{+} cell fractions as well as in bone marrow CD34\textsuperscript{+} progenitor cells.\textsuperscript{3} In patients with various solid tumors, circulating cell-free DNA (cfDNA) in peripheral blood contains cancer-derived genomic DNA and has been used in a noninvasive diagnostic procedure, the so-called “liquid biopsy.” In a recent report, \textit{BRAF-V600E} was detected successfully in cfDNA from patients with colorectal cancer, with 100% sensitivity and specificity.\textsuperscript{4} LCH can involve organs and tissues not readily accessible for biopsy, and the specimens are sometimes not available for genetic analyses after pathologic procedures. Thus, we evaluated the \textit{BRAF} mutation in cfDNA as a potential biomarker of LCH using an allele-specific quantitative polymerase chain reaction (ASQ-PCR).

We cloned normal and mutant \textit{BRAF} alleles that included exon 15 and its neighboring sequences into pCR2.1 to prepare a standard curve. cfDNA was prepared from the plasma of adult LCH patients by using the QIAamp DNA Blood Mini Kit (Qiagen) and was subjected to genotyping for the \textit{BRAF} alleles by ASQ-PCR that was specifically designed to detect \textit{BRAF-V600E} by using a 3’-phosphate-modified oligonucleotide blocker, according to Thierry et al.\textsuperscript{4} Each assay reaction was performed in triplicate. The mutant \textit{BRAF} load was estimated from the standard curve in each assay and was expressed as the mean percentage of mutant alleles relative to the total number of alleles by using the StepOnePlus Real-Time PCR System (Life Technologies).

Plasma cfDNA was prepared from 8 adult patients with LCH (listed in Table 1) as well as 8 normal participants. DNA from lesion tissues was not available for all patients. The mean quantity of cfDNA recovered from patients with LCH vs normal participants was 316.5 pg/mL (median, 290.4 pg/mL) vs 92.0 pg/mL (median, 91.8 pg/mL). Three high-risk patients with active multiple lesions were positive for \textit{BRAF-V600E} but 8 normal participants were not. In these patients, the mean ratio of mutant \textit{BRAF} alleles to total alleles was 3.25% (median, 2.59%). Immunohistochemical analyses that used a \textit{BRAF-V600E}–specific antibody (Spring Bioscience) in biopsy specimens from 2 patients revealed that patient 3 (unique patient number 3 [UPN 3]) was positive for \textit{BRAF-V600E} but UPN 7 was negative, which may be explained by the lower sensitivity of the detection method and/or the possibility that some but not all lesions are positive for \textit{BRAF-V600E} in patients with multisystem LCH. Next, we compared the sensitivity of ASQ-PCR for \textit{BRAF-V600E} between cfDNA and cellular DNA in the same blood sample. Naturally, much more DNA was recovered from mononuclear cells than from the same blood volume of plasma, but the ratio of mutant to total alleles was more than 10-fold higher in the cfDNA, suggesting that LCH-derived genomes are significantly enriched in cfDNA compared with cellular DNA and that cfDNA is adequate for liquid biopsies in LCH with \textit{BRAF-V600E}.

Next, in UPN 7, we observed the mutant \textit{BRAF} load during the course of initial chemotherapy. The ratio of mutant to total alleles was estimated as 1.00% prior to chemotherapy and was unmeasurable after chemotherapy. These data were compatible with the improved findings of computed tomography and positron emission tomography performed at the same time. Based on these results, ASQ-PCR for \textit{BRAF-V600E} in cfDNA may contribute to planning risk-based treatment as well as monitoring treatment efficacy in LCH, especially in a group with active, high-risk LCH. Several \textit{BRAF}-targeted inhibitors have been approved or are in clinical trials for various cancers with \textit{BRAF} mutations, and one of those inhibitors, vemurafenib, is also active against LCH with \textit{BRAF-V600E}.\textsuperscript{5}

Despite an obviously very small cohort, we demonstrated the feasibility of \textit{BRAF-V600E} in cfDNA as a biomarker of active, high-risk LCH. The utility of \textit{BRAF-V600E} in cfDNA should be validated in a larger cohort of LCH patients.

Masayuki Kobayashi
Division of Molecular Therapy, Advanced Clinical Research Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

\begin{table}[h]
\centering
\caption{Characteristics of patients with adult LCH}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
UPN & Age, years & Gender & Organ involvement & Risk & Activity & Treatment & \textit{BRAF-V600E} immunohistostaining & \textit{BRAF-V600E} (%) \\
\hline
1 & 56 & F & Multi & High & Inactive & Completed & N/A & 0 \\
2 & 38 & F & Single & High & Inactive & Completed & N/A & 0 \\
3 & 65 & F & Multi & High & Active & Interrupted & Positive & 2.59 ± 0.21 \\
4 & 48 & M & Single & High & Inactive & Completed & N/A & 0 \\
5 & 41 & F & Single & High & Inactive & During & N/A & 0 \\
6 & 28 & M & Multi & High & Inactive & During & N/A & 0 \\
7 & 29 & M & Multi & High & Active & During & N/A & 0 \\
8 & 47 & F & Multi & High & Active & Interrupted & Negative & 1.00 ± 0.28 \\
9 & 27 & M & Multi & High & Inactive & Completed & N/A & 6.16 ± 0.33 \\
\hline
\end{tabular}
\end{table}

F, female; M, male; N/A, not available; UPN, unique patient number.
\textsuperscript{a}Mean ± standard error.
Calreticulin mutation does not modify the IPSET score for predicting the risk of thrombosis among 1150 patients with essential thrombocythemia

An international prognostic score for the risk of thrombosis (IPSET-thrombosis) in essential thrombocythemia (ET) was developed. Risk factors included the following: age >60 years (1 point), cardiovascular (CV) risk factors (1 point), previous thrombosis (2 points), and the presence of JAK2 V617F mutation (2 points). Low-, intermediate-, and high-risk categories were identified by scores 0 to 1, 2, and ≥3, respectively. Mutations in the exon 9 of calreticulin (CALR) gene were recently identified in a large proportion of patients with JAK2 V617F-negative ET and associated with a reduced thrombotic risk as compared with JAK2 V617F-positive patients. However, the utility of incorporating CALR mutation status into current risk stratification for thrombosis in ET is not yet tested. Answering this question was the purpose of the present study.

Under the auspices of the Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative, 4 Italian centers convened to create a database of 1150 patients previously diagnosed and treated for ET. The study was approved by each Institutional Review Board. Patients’ eligibility criteria included diagnosis.

Table 1. Patients’ characteristics at diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>CALR+ (A)</th>
<th>JAK2V617F+ (B)</th>
<th>MPLWS15+ (C)</th>
<th>CALR, JAK2, MPL wild type (D)</th>
<th>P A vs B</th>
<th>P A vs C</th>
<th>P A vs D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients, (%)</td>
<td>1150*</td>
<td>164 (14)</td>
<td>736 (64)</td>
<td>44 (4)</td>
<td>198 (17)</td>
<td>0.001</td>
<td>0.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>Gender M/F, n (%)</td>
<td>403/739 (35/65)</td>
<td>84/80 (51/49)</td>
<td>37/47 (22/23)</td>
<td>21/25 (10/13)</td>
<td>84/158 (20/80)</td>
<td>&lt;0.001</td>
<td>0.33</td>
<td>0.001</td>
</tr>
<tr>
<td>Age, years, median (5th-95th percentile)</td>
<td>57.6 (27-82)</td>
<td>53.5 (27-81)</td>
<td>60.8 (28-83)</td>
<td>59.7 (27-87)</td>
<td>47.8 (21-78)</td>
<td>0.01</td>
<td>0.396</td>
<td>0.245</td>
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<td>Hemoglobin, g/dL, median (5th-95th percentile)</td>
<td>14.1 (11.6-16.3)</td>
<td>13.7 (11.6-16.1)</td>
<td>14.5 (11.9-16.4)</td>
<td>13.4 (11.6-16.0)</td>
<td>13.6 (11.7-15.8)</td>
<td>&lt;0.001</td>
<td>0.681</td>
<td>0.099</td>
</tr>
<tr>
<td>Hematocrit, %, median (5th-95th percentile)</td>
<td>43.0 (36.0-48.8)</td>
<td>42.1 (35.6-47.6)</td>
<td>43.7 (37.2-49.3)</td>
<td>41.8 (35.0-48.5)</td>
<td>41.0 (35.1-47.0)</td>
<td>0.002</td>
<td>0.880</td>
<td>0.133</td>
</tr>
<tr>
<td>White blood cell count, ×10^3/L, median (5th-95th percentile)</td>
<td>8.7 (5.4-14.7)</td>
<td>7.8 (5.2-12.0)</td>
<td>9.0 (5.7-15.1)</td>
<td>7.9 (4.8-14.0)</td>
<td>8.4 (5.3-14.0)</td>
<td>&lt;0.001</td>
<td>0.725</td>
<td>0.034</td>
</tr>
<tr>
<td>Platelet count, ×10^12/L, median (5th-95th percentile)</td>
<td>718 (486-1313)</td>
<td>842 (551-1769)</td>
<td>704 (490-1234)</td>
<td>834 (544-1700)</td>
<td>647 (464-1318)</td>
<td>&lt;0.001</td>
<td>0.971</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CV risk factors, n (%)</td>
<td>568 (50)</td>
<td>71 (43)</td>
<td>366 (52)</td>
<td>27 (61)</td>
<td>84 (42)</td>
<td>0.034</td>
<td>0.033</td>
<td>0.868</td>
</tr>
<tr>
<td>Smoke, n (%)</td>
<td>98 (9)</td>
<td>7 (4)</td>
<td>66 (9)</td>
<td>5 (11)</td>
<td>20 (10)</td>
<td>0.046</td>
<td>0.073</td>
<td>0.035</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>107 (9)</td>
<td>11 (7)</td>
<td>77 (10)</td>
<td>5 (11)</td>
<td>14 (7)</td>
<td>0.143</td>
<td>0.303</td>
<td>0.892</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>459 (40)</td>
<td>59 (36)</td>
<td>314 (43)</td>
<td>21 (3)</td>
<td>65 (6)</td>
<td>0.116</td>
<td>0.497</td>
<td>0.175</td>
</tr>
<tr>
<td>Previous major thrombosis, n (%)</td>
<td>167 (15)</td>
<td>13 (8)</td>
<td>122 (17)</td>
<td>9 (20)</td>
<td>23 (12)</td>
<td>0.005</td>
<td>0.016</td>
<td>0.243</td>
</tr>
<tr>
<td>IPSET score, n (%)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.012</td>
<td>0.144</td>
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</table>

*Eight patients with double positivity for JAK2 V617F and MPLWS15 were excluded from further analysis.
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Masayuki Kobayashi and Arinobu Tojo