JAK2, *CALR*, and *MPL* mutation spectrum in Japanese patients with myeloproliferative neoplasms

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Supplemental data

Materials and Methods

Mutation analysis

The JAK2V617F mutation profile was first obtained in a previous study[1]. The patient specimens that exhibited a JAK2V617F mutant allele frequency below 10% in the previous study were re-evaluated via deep sequencing (see below). The MPLW515K/L mutation was assessed using a newly developed allele-specific polymerase chain reaction (PCR), called dual amplification refractory mutation system PCR (DARMS-PCR), and a subsequent capillary electrophoresis method using ABI3130xl (Life Technologies, Carlsbad, USA)[2]. The CALR mutation on exon 9 was examined using our in-house fragment analysis method. The *CALR* fragment including exon 9 was PCR-amplified from 20 ng of genomic DNA using Titanium Taq (TOYOBO, Osaka, Japan) with a FAM (5-carboxyfluorescein hydrate)-labeled forward primer (TGGTCCTGGTCCTGATGTC) and a reverse primer (GTGGATTTTGGTTTTGTTCC). The PCR conditions were as follows: an initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 68.5°C for 30 sec, extension at 72°C for 30 sec; and a final extension at 72°C for 2 min. The PCR products were diluted with formamide and then analyzed using an ABI3730 DNA Analyzer (Life Technologies) and Gene mapper 4.0 (Life Technologies).

Deep-sequencing analysis

The patient specimens that exhibited a *JAK2*V617F mutation rate of <10%, were positive for *MPL*W515K/L, or were positive for a *CALR* mutation were subjected to deep-sequencing analysis. According to the identified mutation, we PCR-amplified *JAK2* exons 12 and 14, *MPL* exon 10, or *CALR* exons 1-9 from genomic DNA using KOD plus Neo (TOYOBO); the primers are listed in supplemental Table 1. The specimens were negative for *JAK2*V617F, *MPL*W515K/L, and *CALR* exon 9 mutations based on the previously described PCR-based assays;

follow-up analyses were conducted by deep sequencing all exons of the three genes.

PCR-amplified fragments were purified using an Agencourt AMPure XP kit (Beckman Coulter), and the concentration of each aliquot was subsequently measured using a Quantus fluorometer (Promega, Madison, US). The purified amplicons were mixed together in an equal molecular ratio and were then fragmented to approximately 200 bp using a M220 Forced-ultrasonicator (Covaris, Woburn, USA). The sample library was prepared using TruSeq DNA LT Sample prep kits Sets A and B (Illumina, San Diego, USA) according to the manufacturer's instructions. The libraries were deep-sequenced using a MiSeq bench-top sequencer (Illumina). For the identification of the *JAK2*, *MPL, and CALR* mutations, the data were analyzed using CLC Genomics Workbench software version 6.5 (CLC Bio, Aarhus, Denmark). In addition, an alternative algorithm was used for the identification of *CALR* mutations. Sequence reads were mapped to the reference genome (hg19) using the BWA program[3]. Reads containing over 5 uncalled bases and unmapped reads were discarded from subsequent analyses. From the reads aligned to the *CALR* region, CIGAR strings from the reads in SAM/BAM format were parsed to call potential insertions or deletions using perl scripts.

Although mutation calls were not always consistent between two algorithms, at least one algorithm produced mutation calls that matched fragment analysis data. Conversely, deep-sequencing analysis revealed low-frequency *CALR* mutations in some specimens, whereas no mutations were detected using fragment analysis. In this case, we determined that no *CALR* mutations were present based on the fragment analysis data.

Statistical analysis

Adjusted p-values for multiple comparisons of laboratory parameters between patient groups with different mutations were determined by Tukey-Kramer test with R3.1.1 (Free Software Foundation, Boston, USA). *p*-values below 0.05 were considered significant.

References

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Supplemental Table 1: Primers used for exon amplification.

target	forward	reverse
JAK2 exon 1	GAAGTGGGAGTGGTGTGGG	AAGCCAGGCAGAGATAACACC
JAK2 exon 2	CCCCTGGATTTATGTGGTAGTAG	CAGCGATAAAGGACAGCACAC
JAK2 exon 3	CCAGCCCATTTGTAACTTTATTG	AAAACAATCCATGCATAAAAAGG
JAK2 exon 4	CGACTGCTATTACATTTTGTTCC	TTTGCCTTTTAGCATTAAGTGAG
JAK2 exon 5	CGTTTGTATTTGAACTATTTGGAAGC	CAAGACAGAACTGCAATTTTCC
JAK2 exon 6	TGGCCAATTTGTATCTTGTAAATG	TCACACTTATGTGTAAGGATTTGC
JAK2 exon 7	TGTATGTGCTTTTTTATCCCTAGC	CTTTGTTTTTATTTCCATTGTACTTA
JAK2 exon 8	AATGGCTCTGTAAATTCTACCCG	TTTAAAACTACAATCAAAATTCCTACC
JAK2 exon 9	TAATTCATATTGAGTACTGAGCCA	CAGCATTTACTTTTCTAAAAATTTACTT
JAK2 exon 10	TGATTGTTTTAGATGACACTTGGTC	TTTGTACAGAATCTCTGAGGAAATTAAG
JAK2 exon 11	GATGTCCATTGTGACTATCCCTC	TACTTCACGCCACATAAACAATC
JAK2 exon 12	TGGAGCAATTCATACTTTCAGTG	AACACAAGGTTGGCATATTTTTC
JAK2 exon 13	TCCTACTTCGTTCTCCATCTTTAC	TCTTTAAACAGCATAAACTACATGAAC
JAK2 exon 14	GAGAAAGTGCATCTTTATTATGGC	TACACTGACACCTAGCTGTGATCC
JAK2 exon 15	TTGTTTAGACTCCTACTCTTGCTG	TTTGTTTCCAGGTAAAGATAATTTG
JAK2 exon 16	TTCTTCTTTAAATCTGTTTTGGGG	TTTACACCACTGCCCAAGTAAAG
JAK2 exon 17	TTGGTTTACTTGTGAATTATTTAACCC	CCTTCTTTTTAAAATTAGATGGGC
JAK2 exon 18	AAGAAGGTTGGTGTGGCATTAC	CCCAAATGACATCAAGAAAATG
JAK2 exon 19	TTTTGGTCAACTTGAATGTATATCAG	CAAGCACTCCTTAAAATGTTGTAG

JAK2 exon 20	CTTGAAAAACTTGGTATTTCCATCC	TTTAAAATAGGTTTCAATGGGCAG
JAK2 exon 21	GCAGAGTAAAACATTATTTCCACC	TTCCTAATGTCTACTTCAACACGG
JAK2 exon 22	AATAAAGGGAATATATAGGGTTAAGACC	CAATAAAGTTTGAAGTCTGTGCTC
JAK2 exon 23	TTTGCAGGTAAAAATCAAGAGTCC	GGTGGATACCCTAAAAGCTCTG
JAK2 exon 24	TTGACTGGAGGAAAATTGAGAAAG	CAGGAAGTGAACTGAAGTCCTTG
JAK2 exon 25	GCCCTTAGTGTTCATTTAATTTTG	TTTTCAGGTCTCAAAAAGCTGAG
	TTCTGAGACCAAAGTAGATTTTACAGAAC	GCGATATAAATGAACAAGGACAC
	TTTCAACTCAGCTTTTTGAGACC	CCACAAATAGATACAAGGGAAACC
	TTTCACATAAAGGGAACAAATGTC	TTTATGGCTTAAGCTTAAATTTAGT
	TGGTGAATGTGTTTTTTAAATGG	CAAAACCCACCATATTTTATTTTTAGC
	TGCAGCAGGTTAAGAATTTTTTC	CCTTCAACCCAAAATCAGAAGTC
MPL exon 1–3	CTGTATCTGACAGGAACCTGAGG	GTGACAGGAGGATGGCTCT
MPL exon 4	GTGGTACTCAGAGTTCTGATGTG	CAAGATTGAAGGTAGGAGATAAGA
MPL exon 5–6	GTTGGAGGCTCTCTCAGCTG	AACCTCAATCAGCAGTTCAG
MPL exon 7–8	AGGCCTGATTCAATGACTCT	TGAGGTCTGTGGGCATTTGTTG
MPL exon 9	TGCAGGATTTGGGTCAAACAG	GGCTTGCCTCACCGGTCT
MPL exon 10	CGAAGTCTGACCCTTTTTGTCT	GGTCACAGAGGCGAACCAAGA
MPL exon 11–12	CCTGCCAATCCACTGCCA	TTACCTTAATCCCATGCCAGC
CALR exon 9	TGGTCCTGGTCCTGATGTC	GGAACAAAACCAAAATCCAC

Supplemental Table 2: List of *CALR* mutations identified in the present study

Type	Nucleotide	Amino Acid	Pts. (ET/PMF)
–	wild type	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL-	
1	c.1092_1143del	AAEKQMKDKQDEEQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	11 (5/6)
2	c.1154_1155insTTGTC	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDNCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	8 (7/1)
4	c.1102_1135del	AAEKQMKDKQDEEQRLRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
22	c.1120_1123del	AAEKQMKDKQDEEQRLKEEEEDNAKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
28	c.1131_1152del	AAEKQMKDKQDEEQRLKEEEEDKKRKRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
33	c.1154_1155insATGTC	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	2 (2/0)
34	c.1154delinsCTTGTC	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDTCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
42	c.1100_1133del	AAEKQMKDKQDEEQRRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
43	c.1126_1144del	AAEKQMKDKQDEEQRLKEEEEDKKQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
44	c.1153_1154insTCTGT	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAEDICRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
45	c.1148_1154>GAC	AAEKQMKDKQDEEQRLKEEEEDKKRKEEEEAGRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA-	1 (1/0)
Total			29 (22/7)

* types 1, 2, 4, 22, 28, 33, 34[4]