Infrequent occurrence of mutations in the PH domain of LNK in patients with JAK2 mutation-negative 'idiopathic' erythrocytosis

LNK (SH2B3) belongs to a family of adaptor proteins that contain a proline-rich N-terminal dimerization domain, a pleckstrin homology domain (PH), an Src homology-2 domain (SH2), and a conserved C-terminal tyrosine residue. By binding to cytokine receptors and JAK2 through the SH2 domain, LNK inhibits downstream signaling pathways.[1] Mice with *LNK* targeted deletion develop abnormal accumulation of erythroid, megakaryocytic and B cells.[2] *LNK* mutations were reported in myeloproliferative neoplasms (MPN),[3,4] also at leukemic transformation.[5] *LNK* exon 2 mutations were also found in patients with unexplained erythrocytosis,[6] subnormal EPO levels, no mutations in JAK2, MPL and EpoR, and in those not fulfilling the criteria for polycythemia vera (PV).[7] A non-synonymous polymorphism, pGlu400Lys, also located in the PH domain, was subsequently reported in 23 idiopathic erythrocytosis patients.[8] *LNK* mutations are found mainly in a "hot spot" encompassing exon 2 that, together with exons 3 and 4, encodes the PH domain.[3,6] Mutations in exon 7,[4] encoding the SH2 domain, and in exon 8,[4,9] encoding the C-terminal portion, have recently been reported in a few cases, also in association with *JAK2*V617F mutation.[4]

The aim of our study was to establish the occurrence of LNK mutations in a larger cohort of *JAK2* mutation-negative erythrocytosis. We studied 112 patients with erythrocytosis who tested negative for *JAK2* V617F and exon-12 mutations, could not be diagnosed with PV, had no familial history of unexplained erythrocytosis or MPN, and in whom no evidence of reactive erythrocytosis could be prgvided. All had EPO levels in the normal/subnormal range. We used the various combinations of 2008 WHO criteria to rule out a *JAK2* mutation-negative PV by performing bone marrow biopsy in 40%, growth of erythropoietin-independent colonies in 26%, or both in 34%. After a median follow up of 3.5 years, none of the patients developed an overt MPN. We genotyped *LNK* exons 2, 3 and 4 in DNA from granulocytes (n=94) or whole blood (n=18). Exon 2 was genotyped by direct bidirectional sequencing, exons 3 and 4 were screened by High Resolution Melting Analysis (HRMA) followed by confirmation of any abnormal curve by direct bidirectional sequencing. Since the

Table 1. Clinical features of LNK mutated patients.

Pt	LNK mutation	Age/Sex	WBC ($x10^9$/L)	Hb (g/dL)	Ht (%)	MCV (fL)	PLT ($x10^9$/L)	EPO (U/L)
# 1	E208Q	79 (F)	4.81	15.9	47.6	93	136	20.5
# 2	E208Q	52 (M)	9.57	20.9	63.3	101	210	15.1
# 3	E208Q	52 (M)	6.2	17.3	51.9	88	215	11.3
# 4	P155L	59 (M)	6.76	16.7	52.7	91	187	11.4
# 5	S213R	66 (M)	11.30	17	50.4	94	160	4
#6	T274A	74 (M)	5.30	18.1	53.9	89	181	18
N=106	Absent	56 (14-79) M 98 F 6	7.3 (2.7-11.8)	17.8 (15.9-22.9)	52.1 (51.3-62.9)	85.6 (81-98)*	221 (108-446)	(8.0 (0.3-29)

*All values are reported as median (range). *information available on 59 patients only.*

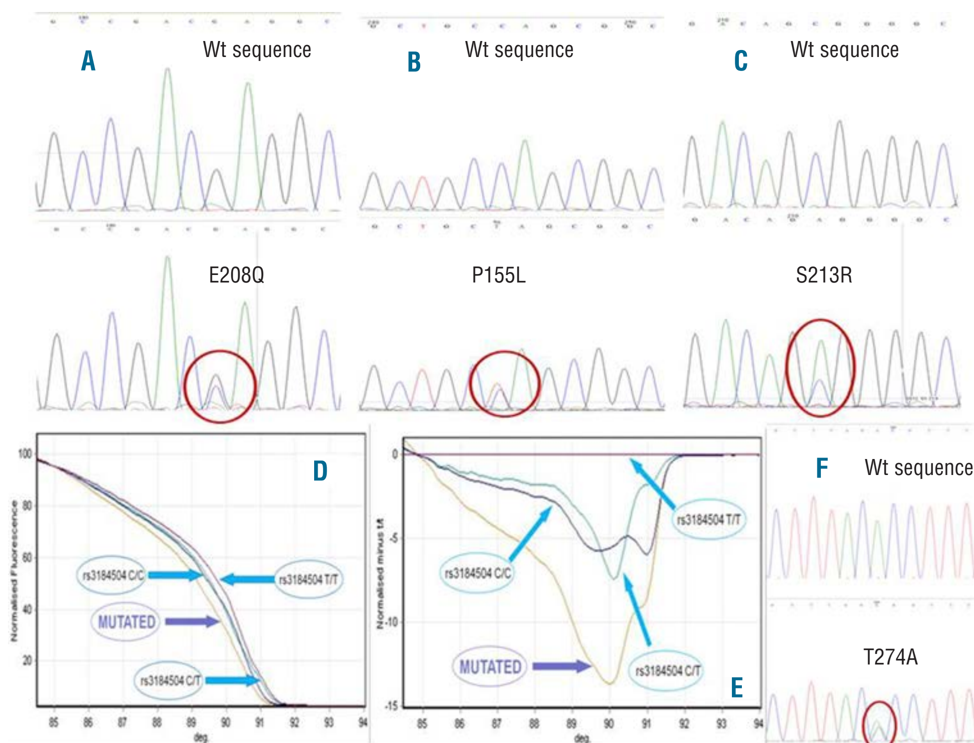

Figure 1. Top line shows mutated and corresponding wt sequence of exon 2 mutations E208Q (A), P155L (B) and S213R (C). Panels D and E represent the HRMA analysis of the patient with 820A>G substitution; shown are both the normalized graph of fluorescence *versus* temperature rising (D) and the difference graph *curves* normalized *versus* the rs3184504 T/T genotype (E); this exon 3 T274A mutation was confirmed by direct sequencing (F).

amplicon generated for exons 3 and 4 encompasses the rs3184504 T/C SNP, we included healthy donor DNA for the 3 different genotypes of the SNP in the HRMA. Samples that in the first HRMA reaction originated a curve overlapping that of the two rs3184504 homozygous donors were mixed with 50% of either the rs3184504 C/C or T/T donor DNA and re-analyzed. Statistical analysis was performed with the Kruskal-Wallis test and the χ^2 test using SPSS software.

Four different mutations were detected in 6 patients (5.3% of the series). Three patients exhibited the previously described heterozygous 622G>C substitution leading to E208Q mutation (Figure 1A). BaF3-MPL cells transduced with E208Q LNK mutant displayed augmented growth and downstream signaling activation in response to TPO.[3] Two patients presented other mutations in exon 2: i) a heterozygous 464C>T substitution leading to P155L variation (Figure 1B), indexed in 1000Genomes Project variation database as ESP_12_111856413, with an MAF<0.01, and predicted to be deleterious with SIFT software (available from: *sift.jcvi.org*) and probably damaging with PolyPhen (available from: *genetics.bwh.harvard.edu/pph/data/*); ii) a heterozygous 639C>A change causing S213R substitution (Figure 1C) that occurs at the same position of SNP rs111360561 (S213S); the A variant is predicted to be deleterious with SIFT and probably damaging with PolyPhen. The sixth patient's HRMA profile differed from the reference curves representing each genotype of the SNP included in the amplicon (Figure 1D and E). Direct sequencing revealed an 820A>G substitution in LNK exon 3 (Figure 1F). This mutation, not listed in the NCBI dbSNP or in the 1000Genomes Project database, is predicted damaging with SIFT and probably damaging with PolyPhen (score = 1.00). We did not find mutations in exon 4 in any of the patients evaluated. Due to the lack of constitutional material, we were unable to confirm the newly detected variants in paired germline DNA; however, they were not found in an extensive database search in the normal population. Finally, due to restrictions imposed by the Ethics Committee, we could not collect material from parents in order to exclude a germline mutation.

We evaluated whether *LNK* mutation could be associated with any clinical feature. In this patient cohort, males were predominant (92%), median age at diagnosis was 56 years (range 14-79), median leukocyte count 7.4x10^9/L (range 2.9-8.9), hemoglobin level 17.8 g/dL (range 16.3-22.9), platelet count 213x10^9/L (range 108-446), EPO level 9.0 U/L (range 2.9-29; reference range 5-30). Table 1 reports the main characteristics of *LNK* mutated patients compared with *LNK* wild type. We found no significant difference in values for splenomegaly, constitutional symptoms, thrombotic or hemorrhagic events or microvessel manifestations. However, the small size of the mutated patient cohort might be the reason why no meaningful difference was found.

We confirm and extend the findings of previous reports that *LNK* mutations occur in *JAK2* negative erythrocytosis previously defined as idiopathic erythrocytosis[10] although at low frequency. Therefore, detection of LNK mutations might represent a useful diagnostic test to reveal an underlying clonal disease in selected patients. However, due to its relative rarity, LNK mutation analysis should only be carried out in specialized laboratories.

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doi:10.3324/haematol.2013.090175

Funding: This study was supported by a special grant from Associazione Italiana per la Ricerca sul Cancro-"AIRC 5 per Mille"-to AGIMM, "AIRC-Gruppo Italiano Malattie Mieloproliferative" (#1005); for a description of the AGIMM project, see at www.progettoagimm.it) and by a FIRB grant (#RBAP11CZLK).

Key words: mutations, PH domain, Epo levels.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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