

Mutational analysis of the DOK2 haploinsufficient tumor suppressor gene in chronic myelomonocytic leukemia (CMML).

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1 **Mutational analysis of the *DOK2* haploinsufficient tumor suppressor gene in**
2 **chronic myelomonocytic leukemia (CMML)**

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4 Letter to the Editor
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8 *Downstream of tyrosine kinases* (DOK) proteins are substrates of protein tyrosine
9 kinases, acting as negative regulators of cell signaling pathways. ¹ Loss of *DOK2*
10 gene expression has been detected in human lung adenocarcinomas, and mice with
11 *Dok2* haploinsufficiency develop lung cancers. ² Mice lacking both *Dok1* and *Dok2*
12 genes (the two first described *Dok* gene family members) present a
13 myeloproliferative chronic myelogenous leukemia (CML)-like syndrome. ^{3, 4}
14 Moreover, genetic ablation of *Dok* genes in a *BCR/ABL* transgenic background
15 accelerates the apparition of the blastic crisis and leukemia induced by the BCR-ABL
16 fusion oncoprotein, ^{3, 4} DOK1 and DOK2 adaptor proteins attenuate RAS/ERK and
17 PI3K/AKT dependent-signaling pathways involved in myeloid cell proliferation. ³⁻⁵
18 Based on these studies, animal models ²⁻⁴ and data from solid tumors, ^{2, 6} *DOK*
19 genes are now considered as tumor suppressors. However, the mutation status of
20 *DOK1* and *DOK2* genes in patients with a chronic myeloproliferative neoplasm
21 (MPN) remains to be defined.

22 Mutations in cell signaling genes have been reported in MPNs. ⁷ Chronic
23 myelomonocytic leukemia (CMML) belongs to the MPN class. ⁸ Upon white blood
24 cells (WBC) count CMML has been subdivided in myelodysplatic (MD-CMML) and

25 myeloproliferative (MP-CMML) subtypes. These two subtypes are associated with
26 different gene expression profiles.⁹

27 Under informed consent, we analyzed gene mutations in bone marrow samples from
28 30 MD-CMML and 36 MP-CMML patients (**Table S1**). Expectedly, mutations
29 previously reported in CMML patients (such as *NRAS*, *CBL*, *PTPN11*, *FLT3*, *JAK2*,
30 *NF1* genes)⁷ were present in our MP-CMML cohort. We analyzed somatic mutations
31 in *DOK1* and *DOK2* genes. Genomic DNA from bone marrow (BM) cells was
32 amplified with 6 primer pairs covering the entire coding region (exons 1-5) of each
33 *DOK* gene (**supplementary material**). We identified point mutations in the two *DOK*
34 genes. For *DOK1*, two variants were found; L60Q in exon 1 coding for a functional
35 protein-lipid interaction domain, a pleckstrin homology (PH) domain¹⁰ and D263E in
36 exon 5. For *DOK2*, four variants (2 x R201H, L238P, R215H) were found in 3/66
37 CMML and 1/2 unclassified-myeloproliferative myelodysplastic neoplasm (U-
38 MPN/MDS). These *DOK2* point mutations are located in exon 4 and the 5' end of
39 exon 5, which code for the phosphotyrosine-binding (PTB) domain of the DOK
40 protein.¹¹ Sorted CD3⁺ lymphocytes of peripheral blood from *DOK* variant patients
41 were only available for the MP-CMML patient with *DOK2* L238P mutation. The *DOK2*
42 L238P mutation was present in myeloid cells but not in lymphoid cells (**Figure 1a**).

43 A three-dimensional (3D) structure model revealed that the L238P substitution would
44 alter the structure of the DOK2 PTB domain, resulting in a loss of stable binding to
45 phosphotyrosyl peptides (**Figure 1b**).

46 DOK2 binds via its PTB domain to tyrosine phosphorylated DOK1 protein.¹² Two
47 arginine residues in positions 200 and 201 in the DOK2 PTB domain are critical for
48 the direct interaction with the DOK1 molecule. The double DOK2 RR200-201AA
49 mutant shows reduced PTB domain ligand binding.¹² We used this RR200-201AA

50 (RR) DOK2 mutant as a loss-of-function control. To test the ability of the L238P
51 DOK2 mutant to bind to DOK1, KG-1 myeloid cells were transfected with different
52 GFP-tagged DOK2 constructs. These cells were then treated with the potent tyrosine
53 phosphatase inhibitor sodium pervanadate (pV), which causes tyrosine
54 phosphorylation of the major cellular proteins (**Figure 1c**). Endogenous DOK1 was
55 immunoprecipitated from cell lysates and the GFP-DOK2 fusion proteins were
56 revealed by GFP Western-blots. A GFP signal in DOK1 immunoprecipitates was
57 detected only when a wild-type (WT) version of DOK2 but not when DOK2 L238P
58 mutant protein was transfected (**Figure 1c**).

59 The DOK2 PTB domain plays a role in the negative regulation of signaling events in
60 myeloid cells.¹³ PTB-deficient DOK2 molecules lose their inhibitory effects on
61 functional cell events in lymphoid cells.¹² Via its interaction with RAS GTPase
62 activating protein (RasGAP), a potent inhibitor of RAS, DOK2 acts as a negative
63 regulator of the RAS/ERK signaling pathway in myeloid cells.^{1, 5, 13} To test the
64 capacity of the L238P DOK2 mutant to attenuate extracellular signal-regulated
65 kinases-1/2 (ERK-1/2) activation, KG-1 cells were transfected with different GFP-
66 tagged DOK2 constructs. These cells were then treated or not with pV and ERK-1/2
67 activation was detected by phospho-ERK Western-blot (**Figure 1d**). DOK2 WT
68 overexpression reduced pV-induced ERK phosphorylation. In contrast, the DOK2
69 L238P mutant was unable to inhibit ERK activation, suggesting that it could be
70 considered as a loss-of-function mutant.

71 The major characteristic of MP-CMML is an abnormally high rate of cell proliferation.
72 ¹⁴ We designed a cell proliferation assay using WT and DOK1/DOK2-deficient mouse
73 embryonic fibroblasts (MEF) (**Figure 1e**). In DOK-deficient cells (**Figure 1e**, right
74 panel), DOK2 WT but not its PTB-deficient mutants reduced autonomous cell

75 proliferation. Moreover, expression of DOK2 L238P mutant in WT MEF induced a
76 hyperproliferation (**Figure 1e**, left panel). These results are in agreement with the MP
77 phenotype of the CMML patient harboring a *DOK2* L238P mutation.

78 The possibility that DOK2 gene expression is downregulated or mutated in MPN has
79 been previously suggested.¹⁵ Here, we have shown that point mutations in *DOK1*
80 and *DOK2* genes are detected with low frequency in CMML, but that they may have
81 consequences for the function of the DOK2 PTB domain.

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85 **Conflict of interest**

86 The authors declare no conflict of interest

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102 **Author contributions**

103 EC, VG-B, DB and JAN designed the experiments and wrote the manuscript. EC, XM
104 NC and AM performed and analyzed the data. P-PP provided important materials.

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124 Supplementary information is available at Leukemia's website

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196 **Figure legend**

197 **(a) Identification of an acquired and heterozygous *DOK2* L238P point mutation**
198 **in an MP-CMML patient.** Sequencing profile of *DOK2* MP-CMML patient (HD-1236).
199 DNA samples were extracted from whole bone marrow or sorted CD3⁺ T cells of
200 peripheral blood. The HGVS nomenclature of the identified *DOK2* gene variation is
201 c.713T>C (p.Leu238Pro) (Table S2).

202 **(b) *DOK2* L238P mutation alters the 3D structure of the *DOK2* PTB domain.** *Top:*
203 Linear structure of the *DOK2* molecule, containing an N-terminal PH domain, a
204 central PTB domain and several phosphorylable tyrosine (Y) residues in the C-
205 terminal part. The L238P point mutation (red asterisk) is located in the PTB functional
206 domain. *Bottom:* 3D structural model of the *DOK2* PTB domain (green ribbon)
207 interacting with *DOK1* EMLENLpY phospho-peptide (red) complex. The L238P single
208 mutation (grey) would affect the 3D conformation of the protein, bending the helix $\alpha 2$
209 (represented by light green arrows that illustrate the consequences of such mutation)
210 thus preventing the clamp between the C-terminal part of the protein and the
211 phospho-peptide.

212 **(c) *DOK2* L238P mutation induces a loss of *DOK2* heterodimerization with the**
213 ***DOK1* protein.** KG-1 myeloid cells were transfected by nucleofection with plasmids
214 coding for GFP-tagged wild-type (wt) *DOK2*, a control *DOK2* mutant with a loss-of-
215 function in the PTB domain (RR200-201AA, RR), the L238P point mutant (L238P) or
216 GFP alone (mock). KG-1 cells were treated with sodium pervanadate (pV) at 50 μ M
217 for 5 min at 37°C. Cells were lysed and lysates were immunoprecipitated using an
218 anti-Dok1 antibody followed by SDS-PAGE and anti-GFP western-blot (WB) allowing
219 the identification of *DOK2* GFP-fusion proteins with the endogenous *DOK1* molecule.
220 *DOK1* immunoprecipitates were controlled by *DOK1* WB. In parallel, whole cell

221 lysates (WCL) were separated by SDS-PAGE and blotted for GFP expression.
222 Molecular weight markers were reported in the left size of the blots. This panel shows
223 representative blots of 2 independent experiments.

224 **(d) The L238P mutant DOK2 molecule is unable to reduce pervanadate-induced**
225 **ERK-1/2 phosphorylation in KG-1 myeloid cells.** KG-1 myeloid cells were
226 transfected by nucleofection with plasmids encoding for GFP-tagged wild-type (WT)
227 DOK2, a control DOK2 mutant with a loss-of-function in the PTB domain (RR200-
228 201AA, RR), the L238P point mutant (L238P) or GFP alone (mock). KG-1 cells were
229 treated or not with sodium pervanadate (pV) at 1 μ M for 5 min at 37°C. Whole cell
230 lysates (WCL) were separated by SDS-PAGE and subsequently immunoblotted for
231 phospho-ERK-1/2 (p-ERK1/2 WB). The blots were reprobed for β -tubulin expression
232 as a loading control and GFP expression to detect the presence of GFP-DOK2 fusion
233 proteins. Molecular weight markers were reported in the left size of the blots. This
234 panel shows representative blots of 2 independent experiments.

235 **(e) Expression of the L238P mutant DOK2 molecule increases cell proliferation.**
236 Mouse embryonic fibroblasts (MEFs) from wild-type (left: MEFs *wt*) or *Dok1-Dok2*
237 double KO (right: MEFs *Dok DKO*) mice were infected by retroviruses expressing
238 DOK2 expression constructs (pMIG alone, pMIG *Dok2 wt*; pMIG *Dok2 RR* for
239 RR200-201AA and pMIG *Dok2 L238P*) and sorted for positive GFP expression.
240 Absolute cell counts were performed by flow cytometry. Only DOK2 PTB loss-of-
241 function mutants (L238P and RR) induce an increase of cell proliferation in MEFs *wt*
242 compared to mock-infected cells. DOK2 *wt* is acting as a cell proliferation attenuator
243 in the absence of endogenous DOK proteins (MEFs *Dok DKO*). * $P < 0.05$; ***
244 $P < 0.005$ (two-way ANOVA test). The data are representative of 3 independent
245 experiments.

