

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

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► **To cite this version:**

Emilie Coppin, Véronique Gelsi-Boyer, Xavier Morelli, Nathalie Cervera, Anne Murati, et al.. Mutational analysis of the DOK2 haploinsufficient tumor suppressor gene in chronic myelomonocytic leukemia (CMML).. *Leukemia*, Nature Publishing Group: Open Access Hybrid Model Option B, 2014, 29 (2), pp.500-2. <10.1038/leu.2014.288>. <inserm-01069773>

**HAL Id: inserm-01069773**

**<http://www.hal.inserm.fr/inserm-01069773>**

Submitted on 30 Sep 2014

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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. <sup>1</sup> Loss of *DOK2*  
10 gene expression has been detected in human lung adenocarcinomas, and mice with  
11 *Dok2* haploinsufficiency develop lung cancers. <sup>2</sup> 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. <sup>3, 4</sup>  
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, <sup>3, 4</sup> DOK1 and DOK2 adaptor proteins attenuate RAS/ERK and  
17 PI3K/AKT dependent-signaling pathways involved in myeloid cell proliferation. <sup>3-5</sup>  
18 Based on these studies, animal models <sup>2-4</sup> and data from solid tumors, <sup>2, 6</sup> *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. <sup>7</sup> Chronic  
23 myelomonocytic leukemia (CMML) belongs to the MPN class. <sup>8</sup> 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.<sup>9</sup>

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)<sup>7</sup> 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) domain<sup>10</sup> 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.<sup>11</sup> Sorted CD3<sup>+</sup> 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.<sup>12</sup> 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.<sup>12</sup> 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-blot. 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.<sup>13</sup> PTB-deficient DOK2 molecules lose their inhibitory effects on  
61 functional cell events in lymphoid cells.<sup>12</sup> 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.<sup>1, 5, 13</sup> 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<sup>14</sup> 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.<sup>15</sup> 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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88 **Acknowledgements**

89 The authors are thankful to the research facilities of the Centre de Recherche en  
90 Cancérologie de Marseille for technical support (oncogenomics, animal facility, and  
91 cytometry). We thank Julie Nunès and Lucy Foster for thoughtful reading the first  
92 draft of the manuscript. This work was supported by institutional grants from the  
93 Institut National de la Santé et de la Recherche Médicale, Centre National de la  
94 Recherche Scientifique and Aix-Marseille Université to CRCM, and by specific grants  
95 from the Fondation ARC Recherche contre le Cancer and the Groupement des  
96 Entreprises Françaises dans la Lutte contre le Cancer (GEFLUC Marseille-Provence  
97 - Projet LMMC) (JAN). EC was supported by a fellowship from the Région Provence  
98 Alpes Côte d'Azur (PACA) – Innate Pharma, then by the Fondation ARC Recherche  
99 contre le Cancer. JAN was a recipient of a Contrat d'Interface Clinique with the  
100 Department of Hematology (Institut Paoli Calmettes).

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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<sup>+</sup> 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  $\mu$ 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  $\mu$ 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 reprobbed for  $\beta$ -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.

