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## Mutations in the RAS-BRAF-MAPK-ERK pathway define a specific subgroup of patients with adverse clinical features and provide new therapeutic options in chronic lymphocytic leukemia

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### ABSTRACT

Mutations in genes of the RAS-BRAF-MAPK-ERK pathway have not been fully explored in patients with chronic lymphocytic leukemia. We, therefore, analyzed the clinical and biological characteristics of chronic lymphocytic leukemia patients with mutations in this pathway and investigated the *in vitro* response of primary cells to BRAF and ERK inhibitors. Putative damaging mutations were found in 25 of 452 patients (5.5%). Among these, BRAF was mutated in nine patients (2.0%), genes upstream of BRAF (KITLG, KIT, PTPN11, GNB1, KRAS and NRAS) were mutated in 12 patients (2.6%), and genes downstream of BRAF (MAPK2K1, MAPK2K2, and MAPK1) were mutated in five patients (1.1%). The most frequent mutations were missense, subclonal and mutually exclusive. Patients with these mutations more frequently had increased lactate dehydrogenase levels, high expression of ZAP-70, CD49d, CD38, trisomy 12 and unmutated immunoglobulin heavy-chain variable region genes and had a worse 5-year time to first treatment (hazard ratio 1.8,  $P=0.025$ ). Gene expression analysis showed upregulation of genes of the MAPK pathway in the group carrying RAS-BRAF-MAPK-ERK pathway mutations. The BRAF inhibitors vemurafenib and dabrafenib were not able to inhibit phosphorylation of ERK, the downstream effector of the pathway, in primary cells. In contrast, ulixertinib, a pan-ERK inhibitor, decreased phospho-ERK levels. In conclusion, although larger series of patients are needed to corroborate these findings, our results suggest that the RAS-BRAF-MAPK-ERK pathway is one of the core cellular processes affected by novel mutations in chronic lymphocytic leukemia, is associated with adverse clinical features and could be pharmacologically inhibited.

## Introduction

The clinical course of patients with chronic lymphocytic leukemia (CLL) is highly heterogeneous.<sup>1,2</sup> The mutational status of the immunoglobulin heavy-chain variable-region genes (IGHV) and deletions/mutations of 11q/*ATM/BIRC3* and 17p/*TP53* are important determinants of the clinical outcome of patients with CLL.<sup>3-6</sup> Whole genome sequencing and whole exome sequencing have identified recurrent acquired mutations in the coding and non-coding regions of several genes. A few of them are mutated with moderate/low frequencies (11-15%), whereas the majority are mutated at much lower frequencies (2-5%).<sup>7-10</sup> This mutational landscape highlights the patients' heterogeneity. Several of the mutations, including some with a low incidence, have been reported to be associated with particular clinical features and disease evolution.<sup>9,11-13</sup>

BRAF is a member of the serine-threonine kinase RAF family, comprising RAF-1/CRAF, ARAF, and BRAF. In normal cells, BRAF functions as a mitotic signal transporter in the RAS/RAF/mitogen-extracellular signal-regulated kinase 1/2 (MEK1/2)/ extracellular signal-regulated kinase 1/2 (ERK1/2)/mitogen activated protein kinase (MAPK) pathway. This pathway plays a pivotal role in regulating embryogenesis, cell proliferation, differentiation, migration, and survival.<sup>14</sup> In the last decade, a high frequency of *BRAF* point mutations has been identified in melanoma and other human cancers.<sup>15,16</sup> *BRAF* mutations are also a characteristic of hairy cell leukemia (HCL), being detected in 95% to 100% of patients with this type of leukemia.<sup>17,18</sup> The most common *BRAF* mutation leads to the substitution of a valine for glutamic acid at amino acid 600 (V600E) in the kinase domain of the protein. This substitution mimics the phosphorylation of the activation loop, thereby leading to its constitutive activation and phosphorylation of MEK1 and MEK2, which in turn phosphorylate and activate the effector kinases ERK1 and ERK2.<sup>19</sup> ERK proteins target numerous substrates, such as protein kinases, transcription factors, and cytoskeletal or nuclear proteins. Moreover, they are able to affect protein functions either by phosphorylating proteins in the cytoplasm or by translocating them into the nucleus where they activate transcription factors that regulate proliferation- and cell survival-associated genes.<sup>20</sup>

*BRAF* mutations have been recurrently reported in CLL patients with a frequency of approximately 3%,<sup>21-24</sup> most of these mutations cluster within or near the activation loop. Recently, novel CLL drivers (*NRAS*, *KRAS*, *NRAS* and *MAP2K1*) of the RAS-BRAF-MAPK-ERK pathway have also been described.<sup>9,24</sup> However, the impact of *BRAF* mutations and other mutations in the RAS-BRAF-MAPK-ERK pathway in CLL is not well established.

We analyzed the clinical and biological characteristics and the impact of mutations in genes of the RAS-BRAF-MAPK-ERK pathway in CLL patients, the functional implications of these mutations and the *in vitro* response to different MAPK inhibitors.

## Methods

### Patients

Four hundred fifty-two patients (276 males/176 females) diagnosed with CLL according to the World Health Organization criteria<sup>25</sup> and included in the International Cancer Genome

Consortium for CLL (ICGC-CLL)<sup>7</sup> were analyzed. All patients gave informed consent to inclusion in this study, according to the guidelines of the ICGC-CLL project and the local ethics committees. The study was conducted in accordance with the Declaration of Helsinki.

### Primary chronic lymphocytic leukemia cells

CLL cells were isolated, cryopreserved and stored in the Hematopathology collection registered at the Biobank (Hospital Clínic-IDIBAPS; R121004-094) (*Online Supplementary Methods*). Functional studies were done in all patients with mutations in genes of the RAS-BRAF-MAPK-ERK pathway for whom cryopreserved material was available.

### Mutational analysis

Whole exome sequencing or whole genome sequencing was performed in 452 CLL patients. DNA from purified CLL cells (>95% tumor cells) was obtained before administration of any treatment, as described elsewhere.<sup>7</sup> The median interval between diagnosis and sample analysis was 36 months (range, 0-300 months). Mutations in genes of the RAS-BRAF-MAPK-ERK pathway according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (*KITLG*, *KIT*, *SOS2*, *PTPN11*, *GNB1*, *KRAS*, *NRAS*, *BRAF*, *MAP2K1*, *MAP2K2* and *MAPK1*) were selected for further analysis. Clonal mutations were considered when the variant allele frequency (VAF) was  $\geq 0.40$  and subclonal when the VAF was  $< 0.40$ . PolyPhen-2, SIFT and CADD algorithms were used for *in silico* prediction of the pathogenicity of the mutations. Coding mutations were considered pathogenic if they were reported as such by at least two algorithms (probably damaging by PolyPhen-2 and/or damaging by SIFT and/or with a phred-like score  $> 20$  by CADD).

### Gene expression analysis

The gene expression profile of 143 purified CLL samples with unmutated IGHV genes (U-IGHV) from the CLL-ICGC project<sup>7</sup> was analyzed using the Gene Set Enrichment Analysis (GSEA) package version 2.0. Enrichment of the MAPK gene signature was investigated using the C2 Biocarta and C2 KEGG collection version 6.1 as reported in the *Online Supplementary Methods*. Gene sets with a  $P \leq 0.05$ , a false discovery rate (FDR)  $q$ -value  $\leq 10\%$  and a normalized enrichment score (NES)  $\geq 1.5$  were considered to be significantly enriched in the group with mutations in the RAS-BRAF-MAPK-ERK pathway.

### Western blot analysis

Whole-cell protein extracts were obtained from CLL cells and peripheral blood mononuclear cells from healthy donors and western blot was performed with antibodies against phosphorylated-T202/Y204 ERK 1/2 and total ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (*Online Supplementary Methods*).

### Analysis of viability

Vemurafenib, dabrafenib, and ulixertinib (BVD-523) were purchased from Selleckchem (Houston, TX, USA). Primary CLL cells were incubated for 24 or 48 h with the indicated doses of the drugs and then stained and analyzed as reported in the *Online Supplementary Methods*.

### B-cell receptor stimulation and quantification of phosphorylated ERK by flow cytometry

B-cell receptors were stimulated by incubating CLL cells with 10  $\mu\text{g/mL}$  of anti-IgM (Southern Biotech, Birmingham, AL, USA) and cells were stained for phospho (T202 and Y204)-ERK1/2-phyco-

erythrin (Becton Dickinson, Franklin Lakes, NJ, USA) (*Online Supplementary Methods*).

### Statistical analysis

A Fisher test or non-parametric tests were used to correlate clinical and biological variables according to the presence of mutations in the RAS-BRAF-MAPK-ERK pathway. Time to first treatment (TTFT) was calculated from the date of sampling to the first treatment or last follow-up. Overall survival was calculated from the date of sampling to the date of death or last follow-up. All the analyses were conducted using SPSS 20 (www.ibm.com) software and are detailed in the *Online Supplementary Methods*. For primary cell cultures data are presented as the mean  $\pm$  standard error of the mean. Comparisons between groups were evaluated with a Wilcoxon paired test using GraphPad Prism 4.0 software. Results were considered statistically significant when the *P*-value was  $\leq 0.05$ .

## Results

### Clinical and biological impact of mutations in the RAS-BRAF-MAPK-ERK pathway

Four hundred fifty-two patients (276 males/176 females) with CLL were analyzed for the clinical and biological impact of mutations in genes of the RAS-BRAF-MAPK-ERK pathway (see *Online Supplementary Table S1* for the main characteristics of the series).

A total of 31 mutations affecting genes of the RAS-BRAF-MAPK-ERK pathway were observed in 30 of the 452 CLL patients (7%) (*Online Supplementary Figure S1* and Table 1). Mutations were missense (25/31; 81%) or non-coding mutations at the 3' or splice donor regions (6/31; 19%). The mean VAF for the 31 individual mutations was  $0.36 \pm 0.13$ . According to the results of the PolyPhen-2, SIFT and CADD algorithms used to predict the patho-

**Table 1.** Description of the mutations in genes of the RAS-BRAF-MAPK-ERK pathway in patients with chronic lymphocytic leukemia.

Case	Patient	Gene name	HGVS.p	Annotation	PolyPhen-2 prediction <sup>a</sup>	SIFT prediction <sup>b</sup>	CADD phred-like score <sup>c</sup>	VAF	IGHV	TP53	BIRC3	ATM
1	723	<i>KITLG</i>	n.a.	3' UTR	n.a.	n.a.	4.25	0.39	UM	UM	M	M
2	33	<i>KIT</i>	p.Val833Leu	missense	Probably damaging	Damaging	22.70	0.24	M	UM	UM	UM
3	1078	<i>KIT</i>	n.a.	3' UTR	n.a.	n.a.	5.91	0.55	UM	UM	UM	UM
4	850	<i>SOS2</i>	p.Pro7Ser	missense	Benign	Tolerated	10.21	0.50	M	UM	UM	UM
5	191	<i>PTPN11</i>	p.Ala72Val	missense	Probably damaging	Damaging	32.00	0.58	M	UM	UM	UM
6	677	<i>PTPN11</i>	p.Glu76Lys	missense	Probably damaging	Damaging	33.00	0.54	UM	M	UM	UM
7	1192	<i>PTPN11</i>	p.Asp61Val	missense	Probably damaging	Damaging	28.20	0.17	UM	UM	UM	UM
8	1226	<i>PTPN11</i>	p.Asp61Val	missense	Probably damaging	Damaging	28.20	0.50	UM	UM	UM	UM
9*	155	<i>PTPN11</i>	p.Ser502Pro	missense	Possibly damaging	Damaging	31.00	0.15	UM	UM	UM	UM
10	15	<i>GNB1</i>	p.Ile80Thr	missense	Probably damaging	Damaging	28.10	0.42	UM	UM	UM	UM
11	1564	<i>GNB1</i>	n.a.	3' UTR	n.a.	n.a.	1.21	0.31	UM	UM	UM	UM
12	398	<i>KRAS</i>	p.Gly12Val	missense	Probably damaging	Damaging	29.90	0.18	UM	UM	UM	UM
13	598	<i>KRAS</i>	p.Gln61His	missense	Benign	Damaging	23.50	0.42	UM	UM	UM	UM
9*	155	<i>KRAS</i>	p.Gly12Asp	missense	Possibly damaging	Damaging	25.30	0.30	UM	UM	UM	UM
14	1371	<i>NRAS</i>	p.Gln61Arg	missense	Benign	Damaging	23.10	0.22	UM	UM	UM	UM
15	27	<i>BRAF</i>	p.Glu501Lys	missense	Probably damaging	Damaging	34.00	0.15	UM	UM	UM	UM
16	100	<i>BRAF</i>	p.Lys601Glu	missense	Possibly damaging	Damaging	24.50	0.20	UM	UM	UM	UM
17	134	<i>BRAF</i>	p.Gly469Ala	missense	Probably damaging	Damaging	27.50	0.54	UM	UM	UM	UM
18	148	<i>BRAF</i>	p.Lys601Asn	missense	Possibly damaging	Damaging	24.30	0.38	UM	UM	UM	UM
19	279	<i>BRAF</i>	p.Asp594Gly	missense	Probably damaging	Damaging	29.70	0.49	UM	M	UM	UM
20	721	<i>BRAF</i>	p.Asn581Ser	missense	Probably damaging	Damaging	19.38	0.48	UM	UM	UM	UM
21	824	<i>BRAF</i>	p.Leu597Gln	missense	Probably damaging	Damaging	28.80	0.25	UM	UM	UM	UM
22	1079	<i>BRAF</i>	p.Val600Glu	missense	Probably damaging	Damaging	32.00	0.33	UM	UM	UM	UM
23	1431	<i>BRAF</i>	p.Gly534Arg	missense	Possibly damaging	Damaging	34.00	0.46	UM	UM	UM	UM
24	44	<i>MAP2K1</i>	p.Phe53Cys	missense	Probably damaging	Damaging	29.10	0.19	UM	UM	UM	UM
25	1365	<i>MAP2K1</i>	p.Gly128Asp	missense	Probably damaging	Damaging	32.00	0.29	UM	UM	UM	M
26	884	<i>MAP2K2</i>	n.a.	splice donor	n.a.	n.a.	23.30	0.39	M	UM	UM	UM
27	761	<i>MAP2K2</i>	p.Gln60Pro	missense	Probably damaging	Damaging	24.70	0.26	UM	UM	M	M
28	1477	<i>MAP2K2</i>	n.a.	3' UTR	n.a.	n.a.	11.64	0.43	M	UM	UM	UM
29	1568	<i>MAP2K2</i>	p.Tyr134Cys	missense	Probably damaging	Damaging	27.00	0.33	UM	M	UM	UM
30	442	<i>MAPK1</i>	n.a.	3' UTR	n.a.	n.a.	12.71	0.43	UM	UM	UM	UM

\*CLL case with two mutations in genes of the RAS-BRAF-MAPK-ERK pathway; <sup>a</sup>Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet.* 2013; Chapter 7: Unit 7.20. <sup>b</sup>Ng CL, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res.* 2003 Jul 1;31(13):3812-4. <sup>c</sup>Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46(3):310-5. HGVS.p: Human Genome Variation Society protein sequence; PolyPhen-2: Polymorphism Phenotyping v2; SIFT: Sorting Intolerant From Tolerant; CADD: Combined Annotation-Dependent Depletion; VAF: variant allele frequency; IGHV: immunoglobulin variant heavy chain genes; 3'UTR: 3' untranslated region; n.a. not applicable; M: mutated, UM: unmutated.

genicity of the mutations, five mutations in the 3' untranslated region (cases 1, 3, 11, 28 and 30) and one missense mutation (case 4, *SOS2* gene) were discarded as not being pathogenic. We were able to demonstrate that the mutation in the 3' untranslated region of *KITLG* (case 1) was functional as we detected high levels of phosphorylated ERK, a surrogate marker of RAS-BRAF-MAPK-ERK pathway activation (Figure 3A). Due to the absence of cryopreserved material, we could not analyze the functionality of these mutations in the remaining cases. Therefore, considering only the putative functional mutations, a total of 26 functional mutations affecting genes of the RAS-BRAF-MAPK-ERK pathway were observed in 25 of 452 CLL patients (5.5%). In 11 of the 25 patients (44%) these mutations were clonal (VAF  $\geq$ 0.40) and in the other 14 patients (56%) they were subclonal (VAF <0.40). Mutations were detected in genes upstream of *BRAF* (*KITLG*, *KIT*, *PTPN11*, *GNB1*, *KRAS* and *NRAS*) in 12/452 patients (2.6%), in *BRAF* in 9/452 patients (2.0%), and in genes downstream of *BRAF* (*MAP2K1* alias *MEK1*, *MAP2K2* alias *MEK2*) in 5/452 patients (1.1%). The most frequent single mutated gene was *BRAF* (n=9/26, 34.6%) followed by *PTPN11* (n=5/26, 19.2%), *MAP2K2* (n=3/26, 11.5%), *KRAS* (n=3/26, 11.5%), and *MAP2K1* (2/26 cases, 7.7%); mutations of *GNB1*, *NRAS*, *KIT*, and *KITLG* were each found in one patient. One patient had concomitant mutations of *PTPN11* and *KRAS*. Interestingly, *BRAF* mutations were localized between exons 11 to 15 and most of them occurred in the activation loop (A-loop) near the V600 position or near the phosphate-binding loop (P-loop) at residues 464-469. Only in one case did the *BRAF* mutation correspond to V600E, the most common mutation described in a variety of human malignancies including HCL.<sup>17</sup>

#### Association of mutations in the RAS-BRAF-MAPK-ERK pathway with clinical and biological features

The main clinical and biological characteristics of the 25 patients with functional mutations in the RAS-BRAF-MAPK-ERK pathway are listed in Table 2.

The age, sex and clinical stage of the patients with mutations in the RAS-BRAF-MAPK-ERK pathway were similar to those of the patients without mutations. However, patients with mutations in RAS-BRAF-MAPK-ERK pathway genes more frequently had abnormal values of lactate dehydrogenase, high expression of ZAP-70, CD38 and CD49d, trisomy 12 and most of them had U-IGHV (21/24, 87%) ( $P \leq 0.05$  in all comparisons) (Table 2). Patients with mutations in the RAS-BRAF-MAPK-ERK pathway more frequently had three or more driver mutations than patients without mutations in the pathway, but no differences were observed in the genes most frequently mutated in CLL (*NOTCH1*, *SF3B1*, *BIRC3*, *TP53* or *ATM*) (Table 2). Six cases contemporaneously carried mutations in *TP53*, *ATM* or *BIRC3*. As most patients with mutations in the RAS-BRAF-MAPK-ERK pathway had U-IGHV, we conducted a similar analysis including only the subgroup of U-IGHV patients. As seen in Table 3, only lactate dehydrogenase and trisomy 12 maintained statistical significance. Figure 1 shows a brick-plot of concomitant gene mutations/cytogenetic aberrations for cases with RAS-BRAF-MAPK-ERK pathway mutations.

Patients with mutations in the RAS-BRAF-MAPK-ERK pathway required treatment more frequently, considering both the whole group (88% versus 43%;  $P < 0.001$ ) and

within the U-IGHV subgroup (95% versus 75%;  $P < 0.048$ ). There were no differences in the type of treatment received or the response achieved according to the presence or absence of mutations in the pathway (Table 2). Five-year TTFT of patients with Binet A or B disease was 82% [95% confidence interval (95% CI): 66-98%] in patients with mutations in the RAS-BRAF-MAPK-ERK pathway versus 50% (95% CI: 42-58%) in the unmutated group;  $P < 0.001$ . The comparison between clonal and subclonal mutated cases showed that the 5-year TTFT was

**Table 2.** Main clinical and biological characteristics of patients according to mutations in the RAS-BRAF-MAPK-ERK pathway.

Parameter	Category	Unmutated (n=427)	Mutated (n=25)	P-value
Gender	Male (%)	257 (60%)	19 (76%)	ns
Age (years), median (range)		61 (18-93)	61 (44-84)	ns
Binet stage	A	366 (87%)	21 (88%)	ns
	B	47 (11%)	1 (4%)	
	C	8 (2%)	2 (8%)	
Rai stage	0	278 (66%)	13 (54%)	ns
	I-II	130 (31%)	9 (38%)	
	III-IV	12 (3%)	2 (8%)	
Lymphocytes count (x10 <sup>9</sup> /L), median (range)		11 (1-203)	11 (1-75)	ns
Absolute CLL cell count (x10 <sup>9</sup> /L), median (range)		8 (0.4-192)	6 (0.7-83)	ns
Hemoglobin (g/L), median (range)		141 (45-177)	147 (125-159)	ns
Platelets (x10 <sup>9</sup> /L), median (range)		204 (49-791)	170 (99-315)	ns
B <sub>2</sub> microglobulin	UNV*	119/373 (32%)	7/18 (39%)	ns
Lactate dehydrogenase	UNV*	26/407 (6%)	6/19 (32%)	0.002
IGHV	Unmutated	145/421 (34%)	21/24 (87%)	<0.001
CD49d	>30%	92/290 (32%)	9/13 (69%)	0.012
CD38	>30%	96/403 (24%)	10/23 (43%)	0.046
ZAP-70	$\geq$ 20%	98/394 (25%)	14/21 (67%)	<0.001
Genetics	del(13q)(q14.3)	148/308 (48%)	3/13 (23%)	ns
	Trisomy 12	48/308 (16%)	6/13 (46%)	0.011
	del(11q)(q22.3)	26/307 (8%)	0/13 (0%)	ns
	del(17p)(p13.1)	11/308 (4%)	1/13 (8%)	ns
Driver mutations	$\geq$ 3	159/427 (37%)	17/25 (68%)	0.003
<i>NOTCH1</i>	Mutated	52/427 (12%)	5/25 (20%)	ns
<i>SF3B1</i>	Mutated	38/427 (9%)	1/25 (4%)	ns
<i>TP53</i>	Disrupted	21/397 (5%)	2/23 (9%)	ns
<i>BIRC3</i>	Disrupted	38/427 (9%)	2/25 (8%)	ns
<i>ATM</i>	Disrupted	47/427 (11%)	3/25 (12%)	ns
Treated		184/427 (43%)	22/25 (88%)	<0.001
Response to treatment*	CR	102 (55%)	12 (57%)	ns
	PR	48 (26%)	4 (19%)	
	Failure	13 (7%)	3 (14%)	
5-year TTFT (95% CI)*	A&B	50% (42-58)	82% (66-98)	<0.001
5-year OS (95% CI)	All	80% (74-86)	78% (60-96)	ns
5-year t-DLBCL	All	2% (1-3)	11% (0-25)	0.080

\*It was not possible to assess the response to treatment in 21/184 (11%) of the unmutated patients and in 2/21 (9%) of the mutated patients. CLL: chronic lymphocytic leukemia; UNV: above normal value; CR: complete response, PR: partial response, TTFT: time to first treatment; OS: overall survival; 95% CI: 95% confidence interval; t-DLBCL: transformation into diffuse large B-cell lymphoma (Richter syndrome); ns: not significant.

92% (95 CI: 76-100%) for patients with subclonal mutations, 70% (95 CI: 42-98%) for patients with clonal mutations, and 51% (95% CI: 42-60%;  $P \leq 0.001$ ) for those without mutations. The adverse effect of mutations in genes of the RAS-BRAF-MAPK-ERK pathway was observed independently of the mutated gene (*Online Supplementary Figure S2*). Overall, patients with mutations in the RAS-BRAF-MAPK-ERK pathway had a worse TTFT than that of patients without mutations ( $P < 0.001$ ) (Figure 2A). However, when other adverse mutations (*TP53*, *ATM* or *BIRC3*)<sup>26,27</sup> were taken into account, patients with mutations in both the RAS-BRAF-MAPK-ERK pathway and in *TP53*, *ATM* or *BIRC3* (n=6, 1%) had the shortest 5-year TTFT (100%) followed by patients with mutations in *TP53*, *ATM* or *BIRC3* [n=64, 15%; 5-year TTFT of 83% (CI 95%: 71-95%)], patients with mutations only in the RAS-BRAF-MAPK-ERK pathway [n=16, 4%; 5-year TTFT of 75% (CI 95%: 54-96%)], and patients without mutations [n=337, 79%; 5-year TTFT of 44% (CI 95%: 34-54%)]

( $P \leq 0.001$ ) (Figure 2B). In the subgroup of patients with Binet A or B CLL with U-IGHV, those patients with adverse gene mutations concomitantly with mutations in RAS-BRAF-MAPK-ERK pathway genes (n=6, 4%) again had a worse 5-year TTFT (all treated) than patients with only mutations in *TP53*, *ATM* or *BIRC3* (n=45, 30%; 5-year TTFT: 87%, CI 95%: 77-97%), patients with only mutations in RAS-BRAF-MAPK-ERK pathway genes (n=13, 8%; 5-year TTFT: 85%, CI 95%: 65-100%), and patients without mutations in these genes (n=88, 56%; 5-year TTFT: 71%, CI 95%: 60-82%) ( $P = 0.001$ ) (Figure 2C). A multivariate analysis including IGHV status, mutations in RAS-BRAF-MAPK-ERK pathway genes, and mutations in *TP53*, *ATM* or *BIRC3* in a final model with 418 patients showed an independent impact on TTFT for IGHV status [hazard risk (HR) 3.4 (95% CI: 2.5-4.8),  $P < 0.001$ ], mutations in the RAS-BRAF-MAPK-ERK pathway [HR 1.8 (95% CI: 1.1- 3),  $P = 0.016$ ] and adverse mutations [HR 2.0 (95% CI: 1.5-2.8),  $P < 0.001$ ].

**Table 3.** Main clinical and biological characteristics of patients according to the presence or absence of mutations in genes of the RAS-BRAF-MAPK-ERK pathway in the subgroup with unmutated IGHV chronic lymphocytic leukemia.

Parameter	Category	Unmutated (n=145)	Mutated (n=21)	P-value
Gender	Male (%)	94 (65%)	16 (76%)	ns
Age (years), median (range)		61 (18-93)	61 (44-78)	ns
Binet stage	A	105/142 (74%)	18/20 (90%)	ns
	B	32/142 (22%)	1/20 (5%)	
	C	5/142 (4%)	1/20 (5%)	
Rai stage	0	67/141 (47%)	11/20 (55%)	ns
	I-II	66/141 (47%)	8/20 (40%)	
	III-IV	8/141 (6%)	1/20 (5%)	
Lymphocytes count (x10 <sup>9</sup> /L), median (range)		10.7 (1-106)	12 (1-26)	ns
Absolute CLL cells count (x10 <sup>9</sup> /L), median (range)		8 (0.8-114)	7 (0.7-83)	ns
Hemoglobin (g/L), median (range)		140 (45-166)	149 (125-159)	ns
Platelets (x10 <sup>9</sup> /L), median (range)		210 (49-470)	163 (99-315)	ns
B <sub>2</sub> microglobulin	UNV	57/128 (45%)	7/15 (47%)	ns
Lactate dehydrogenase	UNV	15/137 (11%)	6/16 (37%)	0.011
CD49d	>30%	42/89 (47%)	8/11 (73%)	ns
CD38	>30%	61/136 (45%)	10/19 (53%)	ns
ZAP-70	≥20%	77/131 (59%)	13/18 (72%)	ns
Genetics	del(13q)(q14.3)	38/102 (37%)	1/10 (10%)	ns
	Trisomy 12	22/102 (22%)	6/10 (60%)	0.015
	del(11q)(q22.3)	21/102 (20%)	0/10 (9%)	ns
	del(17p)(p13.1)	5/102 (5%)	1/10 (10%)	ns
Driver mutations	≥3	96/145 (66%)	14/21 (67%)	ns
<i>NOTCH1</i>	Mutated	43/145 (30%)	5/21 (24%)	ns
<i>SF3B1</i>	Mutated	21/145 (15%)	1/21 (5%)	ns
<i>TP53</i>	Disrupted	9/134 (7%)	2/20 (10%)	ns
<i>BIRC3</i>	Disrupted	30/145 (21%)	2/21 (12%)	ns
<i>ATM</i>	Disrupted	38/145 (26%)	3/21 (14%)	ns
Treated		108/145 (75%)	20/21 (95%)	0.048
5-year TTFT (95% CI)	A&B	78% (68-88)	90% (76-100)	0.025
5-year OS (95% CI)	U-IGHV	70% (60-80)	84% (64-100)	0.020
5-year t-DLBCL	All	9% (5-13)	12% (0-26)	ns

TTFT: time to first treatment; OS: overall survival; 95% CI: 95% confidence interval; t-DLBCL: transformation into diffuse large B-cell lymphoma (Richter syndrome); ns: not significant; UNV: above normal value; 95% CI: 95% confidence interval; U-IGHV: unmutated IGHV genes

The overall survival of patients with mutations in RAS-BRAF-MAPK-ERK pathway genes was similar to that of patients without mutations in this pathway (Table 2). When mutations in *TP53*, *ATM* or *BIRC3* were taken into account, the overall survival of patients with mutations in genes of the RAS-BRAF-MAPK-ERK pathway alone was similar to that of patients without adverse mutations (Figure 2D) [5-year overall survival of patients without mutations, 84% (95% CI: 78-92%); with mutations only in the RAS-BRAF-MAPK-ERK pathway, 80% (95% CI: 64-99%); with adverse mutations only, 66% (95% CI: 53-79%); and with both abnormalities in RAS-BRAF-MAPK-ERK pathway genes and adverse mutations, 66% (95% CI: 45-100%),  $P=0.003$ ]. Multivariate analysis including IGHV status, mutations in genes of the RAS-BRAF-MAPK-ERK pathway, and adverse mutations in a final model with 439 patients showed an independent impact on overall survival for IGHV status [HR 3.3 (95% CI: 1.9-5.9),  $P<0.001$ ] and adverse mutations [HR 1.7 (95% CI: 1.1-2.8),  $P=0.02$ ].

### Functional and gene expression analysis

To assess the functional impact of these genomic alterations on the RAS-BRAF-MAPK-ERK pathway, we analyzed the phosphorylation status of ERK as a surrogate marker of activation of the pathway. Western blotting with an antibody that specifically recognizes the dually phosphorylated and active forms of ERK1 and ERK2 showed higher levels of endogenous ERK phosphorylation (3.3- to 4.4-fold induction) in CLL cases with mutations in *KITLG*, *BRAF*, *MAP2K2* and *MAP2K1* genes compared to U-IGHV CLL cases with no alterations in the MAPK/ERK pathway (Figure 3A). The same results were obtained when analyzing the phosphorylated forms of ERK by flow cytometry, labeling cells with phospho (T202/Y204)-ERK1/2-phycoerythrin. Figure 3B shows that cases with mutations in genes of the RAS-BRAF-MAPK-

ERK pathway (*PTPN11*, *BRAF*, and *MAP2K1* mutations) had higher basal levels of phosphorylated ERK than cases of U-IGHV CLL (5- to 10-fold).

To identify the differential biological characteristics of cells carrying mutations in the RAS-BRAF-MAPK-ERK pathway, we conducted a gene expression profiling study in CD19<sup>+</sup> tumor CLL cells from 143 CLL cases, 17 of which carrying functional mutations according to PolyPhen-2, SIFT and CADD phred-like predictions. With the C2 Biocarta analysis, we detected 126 of 149 gene sets upregulated in the group carrying mutations in genes of the RAS-BRAF-MAPK-ERK pathway, including the Biocarta MAPK pathway (NES=1.90;  $P<0.001$ ; FDR=0.013) (Online Supplementary Table S2 and Figure 3C). Similar results were obtained when carrying out a C2 KEGG analysis. We detected 104 of 178 gene sets upregulated in the group carrying mutations in genes of the RAS-BRAF-MAPK-ERK pathway, including the KEGG MAPK signaling pathway (NES=1.85;  $P<0.001$ ; FDR=0.013) (Online Supplementary Table S3 and Figure 3D). Genes belonging to the Biocarta and KEGG MAPK pathways are listed in Online Supplementary Tables S4 and S5, respectively.

### Response to MAPK pathway inhibitors

We next evaluated the effect of BRAF inhibitors (vemurafenib, a specific inhibitor of the BRAF V600E mutation, and dabrafenib, specific for BRAF V600E and V600K variants) in cells from 17 CLL cases, nine containing mutations in genes of the RAS-BRAF-MAPK-ERK pathway (*KITLG*, *PTPN11*, *KRAS*, *BRAF*, *MAPK1*, *MAP2K1* and *MAP2K2*) and eight U-IGHV CLL cases with no alterations in this pathway. Vemurafenib, at a dose of 2.5  $\mu$ M, was not able to inhibit basal ERK phosphorylation or after anti-IgM stimulation in mutated cases, while a slight effect was observed after treatment with 2.5  $\mu$ M of dabrafenib. Furthermore, upregulation of

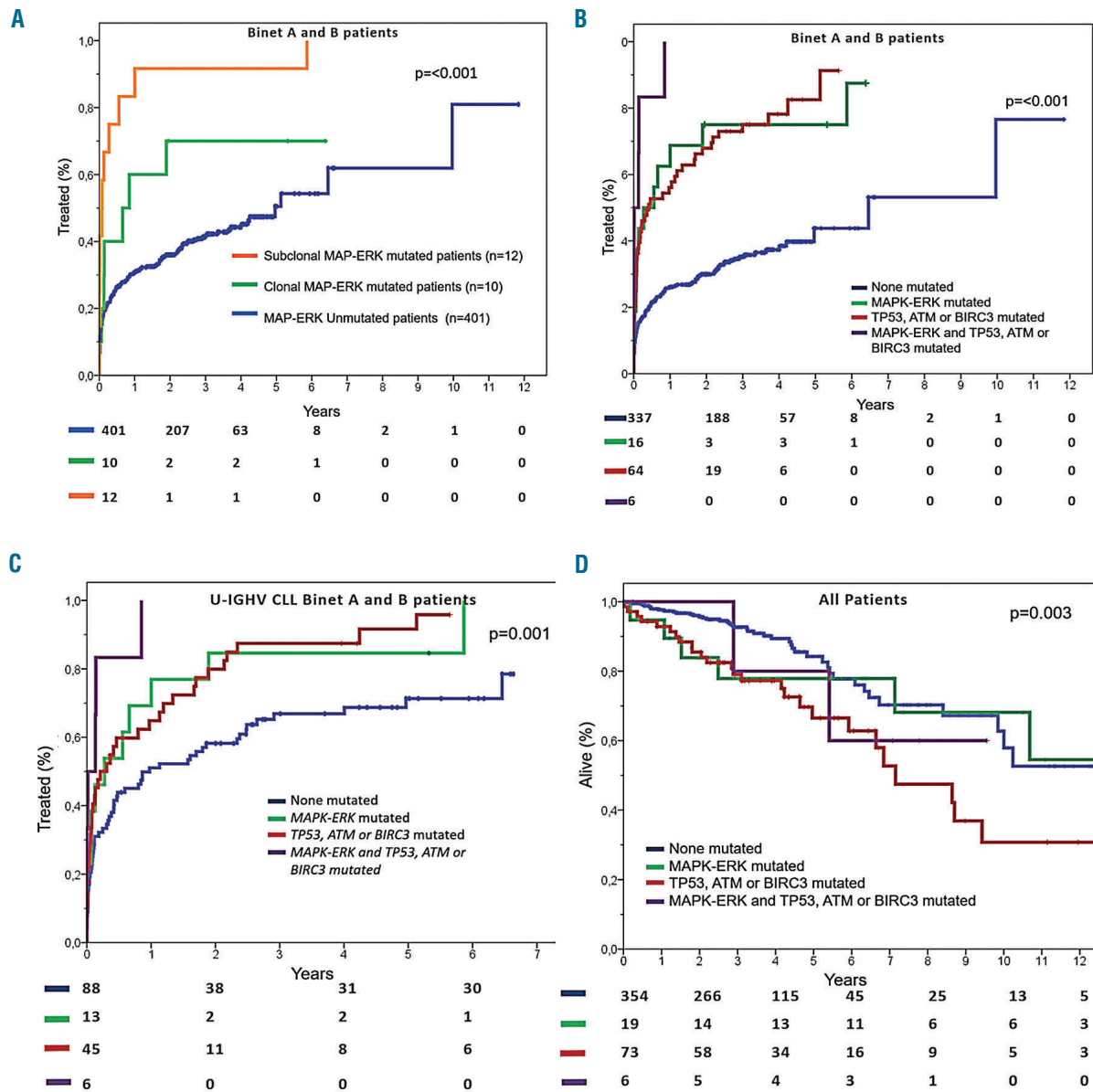


Figure 1. Brick-plot showing gene mutations, cytogenetic abnormalities and the type of RAS-BRAF-MAPK-ERK pathway mutations. Clonal mutations are labeled in dark blue, subclonal mutations in light blue, normal genes or chromosomal regions in light gray, and mutated/deleted genes or chromosomal regions in dark gray. Adverse alterations: *TP53*, *ATM* or *BIRC3*.

phosphorylated ERK, was observed in the U-IGHV CLL cases with no mutations in the RAS-BRAF-MAPK-ERK pathway after incubation with 2.5 μM of dabrafenib ( $P<0.05$ ) (Figure 4A).

We next analyzed the cytotoxic effect of these drugs at different doses (0.5 to 5 μM) and times (24 h and 48 h): vemurafenib did not have any cytotoxic effect, while dabrafenib exerted some degree of cytotoxicity at the higher doses in both mutated RAS-BRAF-MAPK-ERK cases and U-IGHV CLL cases after 24 h of incubation ( $P<0.05$ ) and at all doses after 48 h of incubation ( $P<0.05$  at 0.5 μM and  $P<0.01$  at 1-5 μM) (Figure 4B).

Finally, we compared the effect of the pan-ERK inhibitor ulixertinib (BVD-523) in six patients carrying mutations in the RAS-BRAF-MAPK-ERK pathway (*KITLG*, *PTPN11*, *BRAF*, *MAP2K1*, *MAP2K2* and *MAPK1*) and six U-IGHV CLL cases without mutations. In contrast to the lack of effect of vemurafenib and dabrafenib at 2.5 μM, ulixertinib was able to inhibit basal ERK phosphorylation (by 60%) in all cases with mutations in the RAS-BRAF-MAPK-ERK pathway at doses of 2.5 μM, and after stimulation with anti-IgM at much lower doses (100 nM) (Figure 4C). This effect was not observed in RAS-BRAF-MAPK-ERK pathway unmutated, U-IGHV cells.



**Figure 2. Outcome of patients according to mutations in genes of the RAS-BRAF-MAPK-ERK pathway.** (A) Time to first treatment (TTFT) in Binet stage A and B patients according to mutations in the RAS-BRAF-MAPK-ERK pathway (the green line represents patients with clonal mutations, the orange line represents patients with subclonal mutations and the blue line represents patients with no mutations in the RAS-BRAF-MAPK-ERK pathway). (B) TTFT in Binet stage A and B patients according to the presence or absence of mutations in the RAS-BRAF-MAPK-ERK pathway and/or adverse mutations (*TP53*, *ATM* or *BIRC3*). (C) TTFT in U-IGHV CLL Binet A and B patients according to the presence or absence of mutations in the RAS-BRAF-MAPK-ERK pathway and/or adverse mutations (*TP53*, *ATM* or *BIRC3*). (D) Overall survival of all CLL patients according to the presence or absence of mutations in the RAS-BRAF-MAPK-ERK pathway and/or adverse mutations (*TP53*, *ATM* or *BIRC3*).

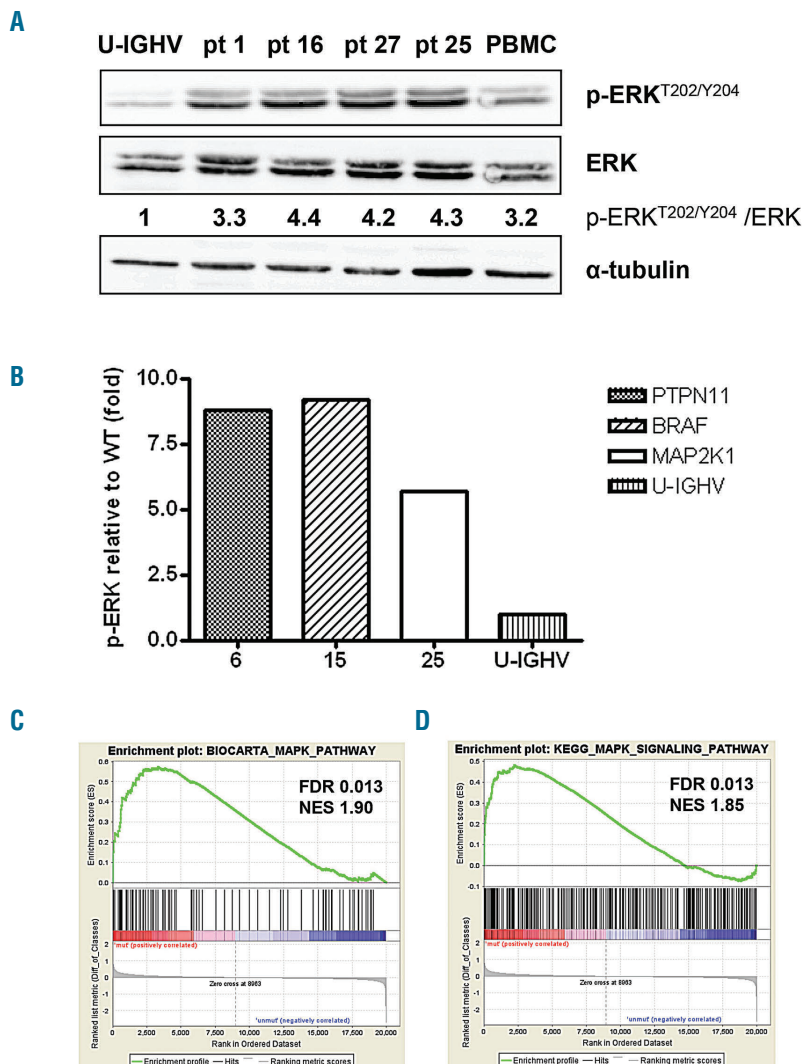
## Discussion

CLL is characterized by a heterogeneous mutational landscape, with the presence of certain mutations being associated with progression of the disease and refractoriness to immuno-chemotherapy, which lead to a poor outcome.<sup>6,13,28</sup> Recently, it has been proposed that the MAPK-ERK pathway could be one of the cellular processes affected in CLL through mutations in novel CLL drivers such as *NRAS*, *KRAS*, *BRAF*, *PTPN11* and *MAP2K1*.<sup>9,24</sup> The RAS-BRAF-MAPK-ERK pathway plays a central role not only in regulating normal cellular processes involved in proliferation, growth, and differentiation, but also in oncogenesis,<sup>29</sup> and it is an important key dysregulated pathway in cancer.<sup>30</sup>

In our series, we observed mutations in genes belonging to the RAS-BRAF-MAPK-ERK pathway in 5% of CLL patients, a frequency similar to that already described.<sup>13</sup> When we evaluated each mutation specifically, *BRAF* mutations were detected in 2% of our CLL series, as previously reported.<sup>9,21</sup> *BRAF* mutations did not involve the canonical hotspot (V600E) seen in other malignancies,<sup>17</sup> which leads to constitutive activation of BRAF, but rather were clustered around the activation segment of the

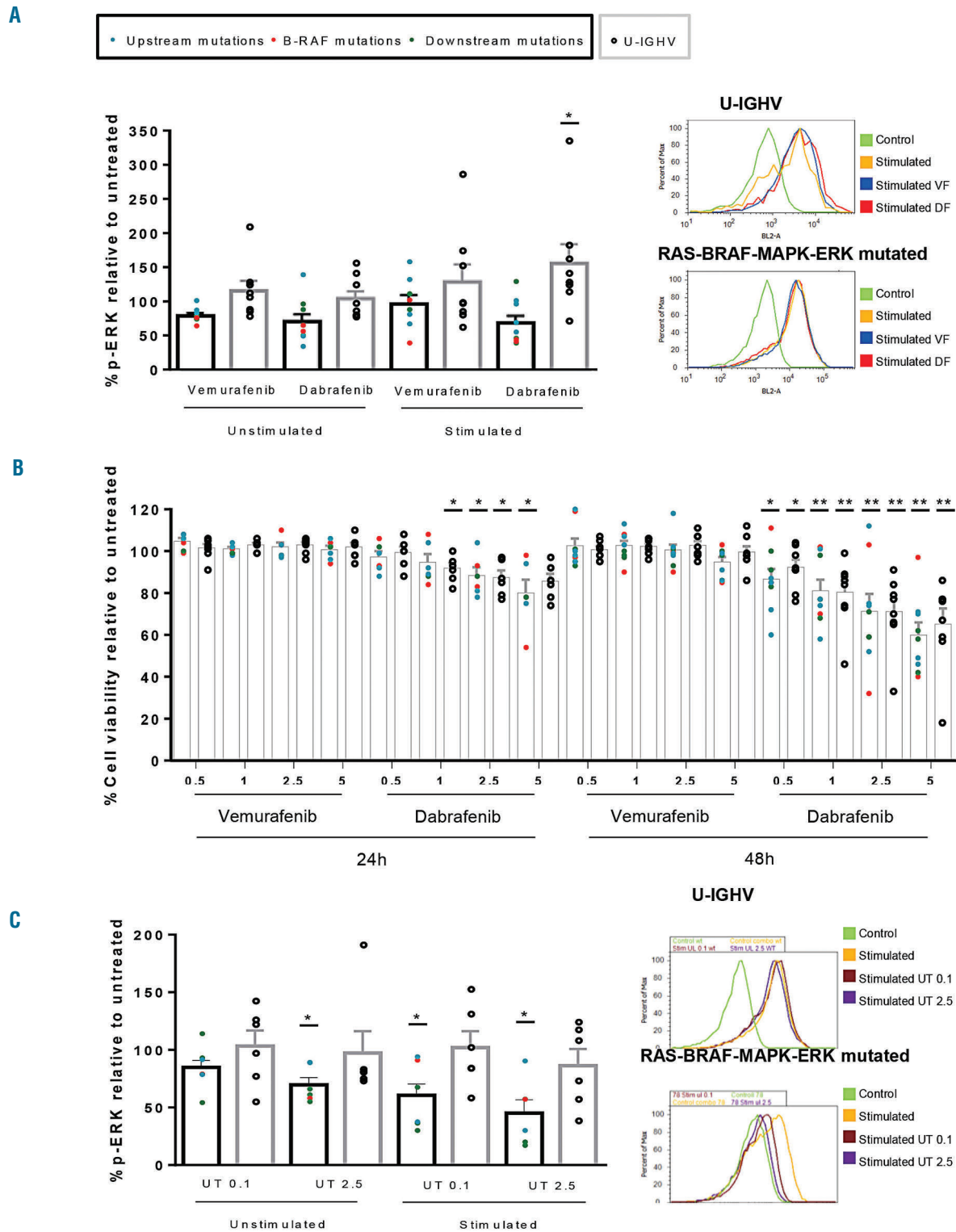
kinase domain.<sup>9,23</sup> Mutations in these positions confer variable but increased signaling and have oncogenic capacity.<sup>31</sup> Mutations in exon 15 of *BRAF* have been associated with refractoriness to fludarabine<sup>22</sup> although they do not seem to be selected during progression to refractory CLL.<sup>21</sup> Furthermore, the frequency of *BRAF* V600E mutations is higher in Richter syndrome than in untransformed CLL<sup>32</sup>, and this mutation could be acquired during the evolution of CLL. Recently, our group reported that the mere detection of a *BRAF* mutation, even at a very low frequency, had a prognostic impact on TTFT.<sup>33</sup> However, given the low frequency of mutations observed in CLL patients, larger series of patients are needed to corroborate these observations.

Mutations in genes upstream and downstream of *BRAF* were observed in 64% (16/25) of cases. *MAP2K1* mutations have already been described in HCL-variant and conventional HCL with rearranged IGHV4-34,<sup>34</sup> Langerhans cell histiocytosis,<sup>35</sup> and pediatric-type follicular lymphoma.<sup>36</sup> This mutation, similar to those of *BRAF*, leads to activation of the downstream target, ERK.<sup>36</sup> Moreover, we found mutations in additional genes of this pathway, such as *MAP2K2*, which encodes MEK2, and *PTPN11*, which encodes SHP-2. Both these proteins participate in the reg-



**Figure 3. Activation of the RAS-BRAF-MAPK-ERK pathway.** (A) Basal phosphorylated (p)-ERK and ERK levels analyzed by western blot in cases of chronic lymphocytic leukemia (CLL) with mutations in genes of the RAS-BRAF-MAPK-ERK pathway (case 1: *KITLG* mutation, case 16: *BRAF* mutation, case 27: *MAP2K2* mutation and case 25: *MAP2K1* mutation), in unmutated IGHV (U-IGHV) CLL and in peripheral blood mononuclear cells (PBMC).  $\alpha$ -tubulin was used as a loading control. p-ERK/ERK levels were quantified relative to the U-IGHV case. (B) Basal p-ERK levels were analyzed by flow cytometry in CLL cases with mutations in genes of the RAS-BRAF-MAPK-ERK pathway (case 6: *PTPN11* mutation, case 15: *BRAF* mutation and case 25: *MAP2K1* mutation). Expression levels are relative to those in U-IGHV CLL. (C) Gene set enrichment analysis (GSEA) plots of the Biocarta-MAPK and KEGG MAPK signaling pathway gene sets regarding mutational status in genes of the RAS-BRAF-MAPK-ERK pathway in U-IGHV cases. The enrichment plot contains profiles of the running enrichment scores (ES) and positions of gene set members on the rank ordered list in GSEA (126 unmutated and 17 mutated CLL cases). NES, normalized enrichment score. FDR, false discovery rate.





**Figure 4. Effect of RAS-BRAF-MAPK-ERK inhibitors in cases of RAS-BRAF-MAPK-ERK-mutated and unmutated IGHV chronic lymphocytic leukemia.** (A) Cells from 17 cases of chronic lymphocytic leukemia (CLL), nine containing mutations in the RAS-BRAF-MAPK-ERK pathway (*KITLG*, *PTPN11*, *KRAS*, *BRAF*, *MAPK1*, *MAP2K1*, *MAP2K2*) and eight with unmutated IGHV genes (U-IGHV) with no alterations in genes of the RAS-BRAF-MAPK-ERK pathway were treated with vemurafenib 2.5  $\mu$ M or dabrafenib 2.5  $\mu$ M. p-ERK levels were analyzed by flow cytometry after 1.5 h of treatment and expressed relative to untreated cells (Ct) at basal levels (unstimulated) or after stimulation with anti-IgM (stimulated) (\* $P$ <0.05). Histograms showing anti-IgM stimulation of ERK (T202/Y204) phosphorylation with and without vemurafenib or dabrafenib (2.5  $\mu$ M) treatment in representative CLL cases (U-IGHV CLL and case 17 with a *BRAF* mutation). (B) Cell viability after treatment for 24 and 48 h with vemurafenib or dabrafenib at doses of 0.5-5  $\mu$ M. Bars represent the mean  $\pm$  standard error of the mean (SEM) of all samples analyzed (n=9 in the group of CLL cases with mutations in genes of the RAS-BRAF-MAPK-ERK pathway and n=8 in the unmutated CLL group) (\* $P$ <0.05; \*\* $P$ <0.01). (C) p-ERK levels after treatment with 0.1 or 2.5  $\mu$ M ulixertinib (UT) relative to untreated (Ct) samples analyzed by flow cytometry at basal levels (unstimulated) or after stimulation with anti-IgM (stimulated). Bars represent the mean  $\pm$  SEM of six samples analyzed in each group, six with mutations in genes of the RAS-BRAF-MAPK-ERK pathway (*KITLG*, *PTPN11*, *BRAF*, *MAP2K1*, *MAP2K2*, and *MAPK1*) and six U-IGHV CLL cases. Histograms showing anti-IgM stimulation of ERK (T202/Y204) phosphorylation and its inhibition by 100 nM and 2.5  $\mu$ M ulixertinib (UT) in representative CLL cases (U-IGHV: CLL and case 15: *BRAF* mutation). Each patient is represented by a different color depending on the RAS-BRAF-MAPK-ERK mutational status and the mutation position relative to *BRAF*.

ulation of the RAS-BRAF-MAPK-ERK signaling pathway.<sup>37</sup> Mutations in this pathway seem to be mutually exclusive as only in one case were two different mutations observed simultaneously in the pathway. In this way, oncogene mutations that activate common downstream pathways often occur in a mutually exclusive fashion,<sup>38</sup> as has been reported for *BRAF* and *MAP2K1* in HCL-variant.<sup>34</sup>

The upregulation of genes of the MAPK pathway observed in the gene expression profiling analysis as well as the higher levels of phosphorylated ERK, a surrogate marker of MAPK pathway activation,<sup>39</sup> in cases with mutations in genes of the RAS-BRAF-MAPK-ERK pathway suggested the activation of this pathway in this subgroup of patients. Importantly, no ERK phosphorylation was observed in unmutated cases. Overall, these results agree with those found in other cancers, in which it has been postulated that the activation of RAS-RAF-MEK-ERK signaling can occur through mutations in several genes in the pathway.<sup>40</sup>

Our data suggest that mutations in the RAS-BRAF-MAPK-ERK pathway are associated with adverse biological features such as U-IGHV, high expression of ZAP-70, CD38 and CD49d, abnormal values of lactate dehydrogenase, and accumulation of three or more driver mutations. Importantly, mutated CLL cases had a 5-year TTFT similar to that of patients with adverse mutations (*TP53*, *ATM* or *BIRC3*), whereas patients carrying both types of mutations simultaneously had the worst 5-year TTFT, as reported by our group and others.<sup>7,9,22,33</sup> In our series of patients, the impact of mutations in genes of the RAS-BRAF-MAPK-ERK pathway on TTFT was independent of that of IGHV status and mutations in *TP53*, *ATM* or *BIRC3*. However, mutations in genes of the RAS-BRAF-MAPK-ERK pathway did not affect overall survival. Recently it was reported that *BRAF* mutations were associated with adverse overall survival, whereas *KRAS* and *NRAS* mutations were not.<sup>24</sup>

Vemurafenib (in 2011) and dabrafenib (in 2013) were the first selective BRAF inhibitors clinically approved for the treatment of melanoma with *BRAF* mutations.<sup>30</sup> MEK inhibitors have also shown efficacy in *BRAF*-mutant melanoma and in 2014 and 2015 the Food and Drug Administration approved the use of MEK inhibitors in combination with BRAF inhibitors as standard-of-care for *BRAF*-mutant advanced melanoma.<sup>41</sup> With these compounds, clinical response rates of around 50% and increased survival have been reported in *BRAF*-mutant melanoma<sup>42</sup> as well as in cases of HCL refractory to conventional therapy.<sup>43,44</sup> However, the majority of responses are transient and resistance is often associated with a plethora of different mechanisms that allow tumor cells to bypass BRAF/MEK inhibition and restore ERK-dependent signaling.<sup>45</sup> Our results showed that vemurafenib and dabrafenib were not able to decrease levels of ERK phosphorylation significantly in mutated cases, although a slight effect was observed after dabrafenib treatment which could be an off-target effect. Accordingly, a different spectrum of efficacy against non-V600 BRAF mutants has been described for vemurafenib and dabrafenib.<sup>46</sup> In contrast, activation of ERK was detected in unmutated CLL cases, potentially due to ERK activation by the B-cell receptor signaling complex as it has been described that

*BRAF* inhibitor-related ERK phosphorylation can be partially abrogated by blocking B-cell receptor signaling with SYK inhibitors.<sup>47</sup>

It has been postulated that cancer cells can dynamically rewire their signaling networks to restore ERK activity and override the actions of inhibitors that act upstream of ERK.<sup>48</sup> We, therefore, consider ERK itself as one of the "best" nodes for effective disruption of ERK signaling. Our results demonstrated that ulixertinib (BVD-523), a potent and highly selective inhibitor of ERK1/2, was able to inhibit ERK phosphorylation *in vitro* in all CLL cases with mutations in genes of the RAS-BRAF-MAPK-ERK pathway. Ulixertinib has shown activity in *BRAF*- and *RAS*-mutant cell lines. Results of phase I studies in solid tumors have documented a safe and well-tolerated effect in patients who harbored *BRAF*-, *NRAS*- and *MEK*-mutant solid tumors, supporting the ongoing development of ulixertinib for patients with MAPK-activating alterations.<sup>49</sup> Recently it was reported that CLL cells with trisomy 12 showed increased sensitivity to MEK and ERK inhibitors, pointing to an essential role for MEK/ERK signaling in CLL with trisomy 12.<sup>50</sup>

In conclusion, we showed that the RAS-BRAF-MAPK-ERK pathway is one of the cellular processes affected in CLL and identified novel CLL drivers. Patients with mutations in genes of the RAS-BRAF-MAPK-ERK pathway had adverse biological features and most of them required treatment. Furthermore, our results suggest that inhibition of ERK phosphorylation in this subgroup of mutated CLL patients can be achieved using new, specific ERK inhibitors that have recently entered clinical trials. Pharmacological inhibition of the RAS-BRAF-MAPK-ERK pathway may represent a therapeutic approach to improve responses in this subgroup of CLL patients.

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