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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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.1,2 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.3,4 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%).3,5 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.5,6,7

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.8 In the last decade, a high frequency of BRAF point mutations has been identified in melanoma and other human cancers.9,10 BRAF mutations are also a characteristic of hairy cell leukemia (HCL), being detected in 95% to 100% of patients with this type of leukemia.11,12 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 ERK2.13 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.14

BRAF mutations have been recurrently reported in CLL patients with a frequency of approximately 3%15–18 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.19 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 criteria20 and included in the International Cancer Genome Consortium for CLL (ICGC-CLL) 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 Clinic-IDIBAPS; P121004-094) (Online Supplementary Methods). Functional studies were done on 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.21 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 were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (KITLG, KIT, SOS2, PTPN11, CNB1, KRAS, NRAS, BRAF, MAP2K1, MAP2K2 and MAPK1) were selected for further analysis. Clonal mutations were considered when the variant allele frequency (VAF) was <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 project22 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 false discovery rate (FDR) q-value ≤10% and a normalized enrichment score (NES) ≥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 (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 µg/mL of anti-IgM (Southern Biotech, Birmingham, AL, USA) and cells were stained for phospho (T202 and Y204)-ERK1/2-phycotoxin-Fluorescein Isothiocyanate (Molecular Probes, Eugene, OR, USA).
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 ± 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 ≤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 nonsense mutations at the 3’ or splice donor regions (6/31; 19%). The mean VAF for the 31 individual mutations was 0.56 ± 0.13. According to the results of the PolyPhen-2, SIFT and CADD algorithms used to predict the patho-

| Case | Patient | Gene name | HGVS.p | Annotation | PolyPhen-2 prediction* | SIFT prediction* | CADD phred-like score* | VAF | IGHV | TPS3 | BIRC3 | ATM |
|------|---------|-----------|--------|------------|------------------------|-----------------|-----------------------|-----|------|------|-------|-----|
| 1    | 723     | KITLG     | n.a.   | 3' UTR     | n.a.                   | n.a.            | 4.25                  | 0.39 | UM   | UM   | M     | M   |
| 2    | 33      | KIT       | p.Val853Leu | missense       | Probably damaging          | Damaging        | 22.70                  | 0.24 | M    | UM   | UM   | UM  |
| 3    | 1078    | KIT       | n.a.   | 3' UTR     | n.a.                   | n.a.            | 5.91                  | 0.55 | UM   | UM   | UM   | UM  |
| 4    | 850     | SOG2      | p.Pro7Ser  | missense       | Benign                  | Tolerated       | 10.21                  | 0.50 | M    | UM   | UM   | UM  |
| 5    | 191     | PTPN11    | p.Ala72Val | missense       | Probably damaging          | Damaging        | 32.00                  | 0.58 | M    | UM   | UM   | UM  |
| 6    | 677     | PTPN11    | p.Glu76Lys | missense       | Probably damaging          | Damaging        | 33.00                  | 0.54 | M    | UM   | UM   | UM  |
| 7    | 1192    | PTPN11    | p.Asp61Val | missense       | Probably damaging          | Damaging        | 28.20                  | 0.17 | UM   | UM   | UM   | UM  |
| 8    | 1226    | PTPN11    | p.Asp61Val | missense       | Probably damaging          | Damaging        | 28.20                  | 0.50 | UM   | UM   | UM   | UM  |
| 9*   | 155     | PTPN11    | p.Ser502Pro | missense       | Possibly damaging          | Damaging        | 31.00                  | 0.15 | UM   | UM   | UM   | UM  |
| 10   | 15      | GNB1      | p.Ile80Thr  | missense       | Possibly damaging          | Damaging        | 28.10                  | 0.42 | UM   | UM   | UM   | UM  |
| 11   | 1564    | GNB1      | n.a.   | 3' UTR     | n.a.                   | n.a.            | 1.21                  | 0.31 | UM   | UM   | UM   | UM  |
| 12   | 398     | KRAS      | p.Gly12Val | missense       | Probably damaging          | Damaging        | 29.90                  | 0.18 | UM   | UM   | UM   | UM  |
| 13   | 598     | KRAS      | p.Gln12His | missense       | Benign                  | Damaging        | 23.50                  | 0.42 | UM   | UM   | UM   | UM  |
| 9**  | 155     | KRAS      | p.Gly12Asp | missense       | Possibly damaging          | Damaging        | 25.30                  | 0.30 | UM   | UM   | UM   | UM  |
| 14   | 1371    | Nras      | p.Gln12Arg | missense       | Benign                  | Damaging        | 23.10                  | 0.22 | UM   | UM   | UM   | UM  |
| 15   | 27      | BRAF      | p.Glu501Lys | missense       | Possibly damaging          | Damaging        | 34.00                  | 0.15 | UM   | UM   | UM   | UM  |
| 16   | 100     | BRAF      | p.Lys601Glu | missense       | Possibly damaging          | Damaging        | 24.50                  | 0.20 | UM   | UM   | UM   | UM  |
| 17   | 134     | BRAF      | p.Gly492Ala | missense       | Possibly damaging          | Damaging        | 27.50                  | 0.54 | UM   | UM   | UM   | UM  |
| 18   | 148     | BRAF      | p.Lys601Asn | missense       | Possibly damaging          | Damaging        | 24.30                  | 0.38 | UM   | UM   | UM   | UM  |
| 19   | 279     | BRAF      | p.Asp584Gly | missense       | Possibly damaging          | Damaging        | 29.70                  | 0.49 | UM   | UM   | UM   | UM  |
| 20   | 721     | BRAF      | p.Asn581Ser | missense       | Possibly damaging          | Damaging        | 19.38                  | 0.48 | UM   | UM   | UM   | UM  |
| 21   | 824     | BRAF      | p.Leu597Gln | missense       | Possibly damaging          | Damaging        | 28.80                  | 0.25 | UM   | UM   | UM   | UM  |
| 22   | 1079    | BRAF      | p.Val600Glu | missense       | Possibly damaging          | Damaging        | 32.00                  | 0.33 | UM   | UM   | UM   | UM  |
| 23   | 1431    | BRAF      | p.Gly534Arg | missense       | Possibly damaging          | Damaging        | 34.00                  | 0.46 | UM   | UM   | UM   | UM  |
| 24   | 44      | MAP2K1    | p.Phe535Cys | missense       | Possibly damaging          | Damaging        | 29.10                  | 0.19 | UM   | UM   | UM   | UM  |
| 25   | 1365    | MAP2K1    | p.Gly128Asp | missense       | Possibly damaging          | Damaging        | 32.00                  | 0.29 | UM   | UM   | UM   | UM  |
| 26   | 884     | MAP2K2    | n.a.   | splice donor | n.a.                   | n.a.            | 23.30                  | 0.39 | M    | UM   | UM   | UM  |
| 27   | 761     | MAP2K2    | p.Gln680Pro | missense       | Possibly damaging          | Damaging        | 24.70                  | 0.26 | UM   | UM   | M    | M   |
| 28   | 1477    | MAP2K2    | n.a.   | 3' UTR     | n.a.                   | n.a.            | 11.64                  | 0.43 | M    | UM   | UM   | UM  |
| 29   | 1568    | MAP2K2    | p.Try134Cys | missense       | Possibly damaging          | Damaging        | 27.00                  | 0.33 | UM   | UM   | UM   | UM  |
| 30   | 442     | MAP2K2    | 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: ‘Azizhuguei’ Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet. 2013; Chapter 7, Unit 7.20. *Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res. 2003 Jul 1;31(13):3812-4. kKircher 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.
Altered RAS-BRAF-MAPK-ERK pathway in CLL

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 ≥ 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, GNBT1, 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=9/452, 34.6%) followed by PTPN11 (n=5/452, 12.2%), MAP2K2 (n=3/452, 11.5%), KRAS (n=3/452, 11.5%), and MAP2K1 (2/452 cases, 0.4%). Among the mutations of RAS-BRAF-MAPK-ERK pathway, 50% (95% CI: 42.5-58%) of the cases maintained 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 generally similar to the whole group (88% versus 78%; P=0.048).

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) | A | 61 (18-93) | 61 (44-84) | ns |
| Binet stage | A | 368 (87%) | 21 (88%) | ns |
| Rai stage | A | 278 (69%) | 13 (54%) | ns |
| Lymphocytes count (x109/L), median (range) | A | 11 (1-203) | 11 (1-75) | ns |
| Platelets (x109/L), median (range) | A | 8 (0.4-192) | 6 (0.7-83) | ns |
| RAS-BRAF-MAPK-ERK pathway | A | 8 (0.7-32) | 145 (17-159) | ns |
| B, microglobulin | A | 119 (32%) | 7 (32%) | ns |
| Lactate dehydrogenase | A | 264 (6%) | 6 (6%) | 0.002 |
| Hemoglobin (g/L), median (range) | A | 11 (1-203) | 11 (1-75) | ns |
| B2 microglobulin | A | 119 (32%) | 7 (32%) | ns |
| Genetics | A | 264 (6%) | 6 (6%) | 0.002 |
| Response to treatment* | A | 159 (37%) | 17 (25%) | 0.003 |
| NOTCH1 | A | 52 (12%) | 5 (20%) | ns |
| SF3B1 | A | 38 (9%) | 3 (12%) | ns |
| TP53 | A | 21 (5%) | 2 (5%) | ns |
| Driver mutations | A | 159 (37%) | 17 (25%) | 0.003 |
| NOTCH1 | A | 52 (12%) | 5 (20%) | ns |
| SF3B1 | A | 38 (9%) | 3 (12%) | ns |
| TP53 | A | 21 (5%) | 2 (5%) | ns |
| ATM | A | 47 (11%) | 3 (12%) | ns |
| Treated | A | 184 (42%) | 32 (88%) | <0.001 |
| Response to treatment* | A & B | 30 (42%) | 82 (66-98%) | <0.001 |
| 5-year OS (95% CI) | A & B | 80% (74-86%) | 78% (60-96%) | ns |
| 5-year t-TFCL | A & B | 4% (0.2) | 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; CI: 95% confidence interval; t-TFCL: 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≤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)26,27 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=557, 79%; 5-year TTFT of 44% (CI 95%; 34-54%)] (P≤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 1.3 (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                         |              | 52/142 (22%)      | 1/20 (5%)      | ns      |
| C                         |              | 5/142 (4%)        | 1/20 (5%)      | ns      |
| Rai stage                  |              |                   |                |         |
| 0                         |              | 67/141 (47%)      | 11/20 (55%)    | ns      |
| I-II                      |              | 66/141 (47%)      | 8/20 (40%)     | ns      |
| III-IV                    |              | 8/141 (6%)        | 1/20 (5%)      | ns      |
| Lymphocytes count (x10^9/L), median (range) | | 10.7 (1-106) | 12 (1-26) | ns |
| Absolute CLL cells count (x10^9/L), median (range) | | 140 (45-166) | 149 (125-159) | ns |
| Hemoglobin (g/L), median (range) | | 8 (8.8-114) | 7 (8.7-83) | ns |
| Platelets (x10^9/L), median (range) | | 21 (49.470) | 163 (39-315) | ns |
| B, 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%         | 7/1131 (59%)      | 9/138 (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 (0%)      | ns      |
| del(17p)(p13.1)           |              | 5/102 (5%)        | 1/10 (10%)     | ns      |
| Driver mutations           | ≥3           | 96/145 (66%)      | 14/21 (67%)    | ns      |
| NOTCHI                    | Mutated      | 43/145 (30%)      | 5/21 (24%)     | ns      |
| SFBPI                     | Mutated      | 21/145 (15%)      | 1/21 (5%)      | ns      |
| TP53                      | Disrupted    | 9/134 (7%)        | 2/20 (10%)     | ns      |
| BIRC3                     | Disrupted    | 30/145 (21%)      | 2/21 (12%)     | ns      |
| ATM                       | 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% (9-26)     | 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+ 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
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.
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. 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. 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, and it is an important key dysregulated pathway in cancer.

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. When we evaluated each mutation specifically, BRAF mutations were detected in 2% of our CLL series, as previously reported. BRAF mutations did not involve the canonical hotspot (V600E) seen in other malignancies, which leads to constitutive activation of BRAF, but rather were clustered around the activation segment of the kinase domain. Mutations in these positions confer variable but increased signaling and have oncogenic capacity. Mutations in exon 15 of BRAF have been associated with refractoriness to fludarabine although they do not seem to be selected during progression to refractory CLL. Furthermore, the frequency of BRAF V600E mutations is higher in Richter syndrome than in untransformed CLL, 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. 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, Langerhans cell histiocytosis,31 and pediatric-type follicular lymphoma. This mutation, similar to those of BRAF, leads to activation of the downstream target, ERK. 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](image_url)
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 μM or dabrafenib 2.5 μM. 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 μM) 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 μM. Bars represent the mean ± 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 μM 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 ± 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 μM ulixertinib (UT) in representative CLL cases (U-IGHV: CLL and case 17; 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.\textsuperscript{27} 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,\textsuperscript{31} as has been reported for \textit{BRAF} and \textit{MAP2K1} in HCL-variant.\textsuperscript{34}

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,\textsuperscript{39} 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.\textsuperscript{40}

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 (\textit{TP53, ATM} or \textit{BIRC3}), whereas patients carrying both types of mutations simultaneously had the worst 5-year TTFT, as reported by our group and others.\textsuperscript{7,9,22,30} 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 \textit{TP53, ATM} or \textit{BIRC3}. However, mutations in genes of the RAS-BRAF-MAPK-ERK pathway did not affect overall survival. Recently it was reported that \textit{BRAF} mutations were associated with adverse overall survival, whereas \textit{KRAS} and \textit{NRAS} mutations were not.\textsuperscript{44}

Vemurafenib (in 2011) and dabrafenib (in 2013) were the first selective BRAF inhibitors clinically approved for the treatment of melanoma with \textit{BRAF} mutations.\textsuperscript{29} MEK inhibitors have also shown efficacy in \textit{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 \textit{BRAF}-mutant advanced melanoma.\textsuperscript{32} With these compounds, clinical response rates of around 50% and increased survival have been reported in \textit{BRAF}-mutant melanoma\textsuperscript{46} as well as in cases of HCL refractory to conventional therapy.\textsuperscript{44} 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.\textsuperscript{44} 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 \textit{BRAF} mutants has been described for vemurafenib and dabrafenib.\textsuperscript{34} 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.\textsuperscript{47}

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.\textsuperscript{41} We, therefore, consider ERK itself as one of the “best” nodes for effective disruption of ERK signaling. Our results demonstrated that ulixertinib (BVD-532), a potent and highly selective inhibitor of ERK1/2, was able to inhibit ERK phosphorylation \textit{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.\textsuperscript{49} 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.\textsuperscript{35}

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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