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CAMKIIγ, HSP70 and HSP90 transcripts are differentially expressed in resistant mutated patients with Chronic Myeloid Leukemia

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Short running title: *CAMKIIγ, HSP70* and *HSP90* gene profile

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Key words: Chronic Myeloid Leukemia, *BCR-ABL1*, *CAMKIIγ, HSP70, HSP90*, gene expression.

ABSTRACT

Chronic Myeloid Leukemia (CML) patients can develop resistance to tyrosine kinase inhibitors (TKI) therapy, which is mainly attributable to the presence of point mutations in the tyrosine kinase domain of *BCR-ABL1*. In order to examine suitable markers to monitor treatment efficacy, we investigated transcript expression profiles of genes known to be involved in myeloid cell proliferation, such as CAMKIIγ and KI67, and in protein stability and ultimately for cell survival under physiological and stress conditions, such as heat shock proteins HSP70 and HSP90. We studied 101 CML patients in different stages of disease and responses to TKI treatment. The results of qPCR analyses showed that the expression level *of CAMKIIγ, KI67, HSP70* and *HSP90* genes were upregulated in patients at diagnosis and in cases with signs of treatment resistance both in chronic phase and advanced phases (accelerated and blastic phase) with respect to chronic phase in remission and healthy donors.

When only 56 resistant cases divided in 31 with mutations (MT) and 25 without mutations (WT) in the *BCR-ABL1* tyrosine kinase domain were considered, the transcript expression profile showed an unexpected significant increase in *CAMKIIγ* and *HSP70,* and a significant decrease in *HSP90* in MT *versus* WT cases. This differential transcript expression prompted us to design an expression *score* LOG (CAMKIIγ x HSP70 / HSP90), which can be used to provide rapid screening to discriminate the presence or absence of mutations in resistant patients and to monitor TKI treatment efficacy in CML patients.

INTRODUCTION

Chronic Myeloid Leukemia (CML) is a clonal hematological malignancy characterized by the Philadelphia chromosome (Ph´) (1) resulting from a balanced translocation between the long arms of chromosomes 9 and 22, t (9;22)(q34;q11.2). This translocation results in the juxtaposition of *BCR* and *ABL1* genes, generating a chimeric oncogenic protein $P210^{BCR-ABL1}$ with upregulated tyrosine kinase activity altering basic cellular processes such as proliferation, differentiation and apoptosis leading to malignant transformation. Despite the fact that, there is huge evidence that deregulation of kinase activity of BCR-ABL1 oncoprotein directly contributes to the pathogenesis of CML, the mechanism by which CML malignant transformation occurs is not entirely understood. According to this paradigm, tyrosine kinase inhibitors (TKIs) used in Ph' positive leukemia patients blocks the ATP binding site of $P210^{BCR-ABL1}$ with high affinity and specificity resulting in an efficient specific therapy. However, CML patients can develop mechanism of resistance (dependant of BCR-ABL1) which is mainly attributable to the presence of point mutations in the TK domain of *BCR-ABL1* (2), or, alternatively, to other molecular mechanisms such as gene amplification of *BCR-ABL1* (3), or *BCR-ABL1* transcript overexpression (4).
In order to examine suitable markers to monitor TKI treatment efficacy and primary and/or acquired resistance, several selected gene expression profiles were studied. This hunting of treatment efficacy-markers in CML mainly focused on a group of heat shock proteins (HSP70 and HSP90) and typical markers of cell proliferation, such as KI67 and CAMKIIγ. KI67 is commonly studied to detect and quantify proliferating cells used as a prognostic marker in several tumor types (5). CAMKIIγ protein plays an important role in proliferation of myeloid cells. Previous studies demonstrated an increase in CAMKIIγ autophosphorylation in myeloid cells lines (K562) (6). In different *in vitro* cell models the treatment with imatinib triggers terminal differentiation and growth arrest. This loss of proliferative ability of leukemic cells is accompanied by a marked reduction in CAMKIIγ activation. CAMKIIγ regulates multiple signaling pathways involved in the proliferation of tumor cells, including MAPK, JAK/STAT and GSK3β/β-catenin. HSPs function as molecular chaperones assisting in protein folding and translocation, thus maintaining cellular homeostasis. HSPs prevent misfolding and protein aggregation, and facilitate refolding of denatured proteins under physiological and stress conditions (7, 8). HSP90 is an ATP-dependent molecular chaperone, which binds and releases client proteins (9). HSP70 is also an ATP-dependent molecular chaperone, which is induced by cellular

stress due to misfolded and denatured proteins. HSP70 protein is abundantly expressed in most cancer cells (10, 11); whereas in normal cells, its expression is mainly stress inducible (12-14). Ectopic overexpression or induced endogenous levels of HSP70 not only inhibits apoptosis (15,16) but also enhances the tumorigenic potential of cells *in vitro* and *in vivo* (17), eventually leading to an invasive phenotype.

In this context, the aim of this study was to analyze the expression of genes involved in signaling processes activated or mediated by P210 $^{BCR-ABL1}$ oncoprotein in different stages of CML (at diagnosis, remission, relapse and progression). We considered that the levels of *BCR-ABL1, CAMKIIγ, KI67, HSP70* and *HSP90* transcripts may define differential expression profile characteristics of different stages of the disease. Therefore, this profile could be useful prognostic factors, easily detectable, adding up valuable information for monitoring the response to TKI treatment and progression to more advanced stages of the disease.

MATERIALS AND METHODS

Cell lines, patients and controls

K562 cell line (ATCC catalog No. CCL-243), derived from a patient with chronic myeloid leukemia *BCR-ABL1* positive (18), was used as control, to estimate amplification efficiencies and as a calibrator. These cells were maintained in 5% CO_2 atmosphere at 37º C in RPMI 1640 medium supplemented with 10% of fetal bovine serum and 1% glutamine.
A total of 101 peripheral blood samples from CML patients in different phases of the disease (patient group characteristics in Table 1) and from 20 healthy individuals were studied. The study was approved by our Local Ethics Committee (*Comité de Ética de los Institutos de la Academia Nacional de Medicina, CEIANM)* and a written informed consent was obtained in all cases. The definitios of complete hematologic response (CHR) and of cytogenetic response (CyR) used were published by Baccarani et al. (19, 20). Fifteen patients´ samples were taken at diagnosis (Dx) prior to treatment. The remaining samples were obtained from patients treated with TKIs including 30 patients in chronic phase with hematological and cytogenetic remission (CP Remission); 26 patients in chronic phase resistant to treatment with TKIs (CP Resistant) and 30 patients in advanced phases, AP, (including accelerated and blast phase).
Total RNA was extracted and purified from peripheral blood leukocytes by TRIZOL method according to manufacturer's instructions (Invitrogen). Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using random hexamer primers and MMLV reverse transcriptase at 37°C for 1 hour, followed by incubation at 72° C for 5 min and stored at minus 20° C until use. Aliquots of 5 µl were used for quantitative real-time PCR (qPCR) experiments.

Quantitative real-time PCR

Molecular response is assessed according to the international scale (IS) as the ratio of BCR-ABL1 to ABL1 transcripts and it is expressed and reported as BCR-ABL1 (%) on a log scale, where 10%, 1%, 0.1%, 0.01%, 0.0032%, and 0.001% correspond to a decrease of 1, 2, 3, 4, 4.5, and 5 logs, respectively (21).
The relative quantification of *BCR-ABL1* and *ABL1* was performed using the One-Step qRT-PCR *BCR-ABL* Kit (Molecular MD) based on Taqman chemistry, according to

manufacturer's instructions. The values obtained by this method are expressed in IS. The conversion factor was determined by the reference laboratory at the Institute of Medical and Veterinary Science, Adelaide, Australia (22, 23).

The Quantitative real-time PCR (qPCR) for *CAMKIIγ,* KI-*67, HSP70, HSP90* was performed with LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) using the SYBR-Green method. Real time PCR amplifications were carried out in 20 µl of total volume containing 5 µl of sample cDNA, 1x master mix (LC FastStart DNA Master SYBR Green I, Roche Diagnostics), 3.5 mM $MgCl_2$ and 0.25 µM of each primer.

Specific oligonucleotides for qPCR amplification of target genes *CAMKIIγ,* KI-*67, HSP70, HSP90* and reference gene *β2M* were designed using PrimerSelect software (DNA Star Lasergene version 7.0) (Table 2). The primers were designed across exon boundaries to ensure no DNA contamination.

Optimal reaction conditions for qPCR amplifications were 50 cycles with four steps, of 95°C 5 sec, 62°C 3 sec, 72°C 16 sec and 79°C 1 sec; after an initial denaturing step of 95°C 10 min.

Dilutions of K562 cells were used for establishing standard curves and as a calibrator to test reproducibility and accurateness of each experiment to measure gene expression levels. Leucocyte samples from healthy donors were included as controls**.** Calibration curves were performed to estimate qPCR amplification efficiencies and to quantify all the transcripts within the dynamic range (Supplementary Figure SF1)**.**

Point mutation detection in the BCR-ABL1 transcript

Total cDNA samples from patients showing resistance to TKI treatment were subjected to a first round of *BCR-ABL1* specific PCR amplification yielding a product of 1,327 bp (24). A nested second round of PCR amplification of 579 bp fragment encompassing exon 4 to exon 7 covering amino acids residues 220-411 of the ABL kinase domain was analyzed. This process guaranteed the detection of mutations associated with the *BCR-ABL1* chimeric gene and not with the non-involved *ABL1* counterpart (normal allele). DNA sequencing of the latter product allowed characterization of different domains of the protein: P-loop, C-Helix, ATP binding, Catalytic and Activation loop. DNA sequencing was performed by the fluorescent-labeled dideoxi chain termination method (Sanger sequencing) using the ABI 3130 XL (Genetic Analyzer, Applied Biosystems) equipment and the ABI BigDye chemistry (Applied Biosystems).

Statistical analysis

Hypothesis tests for quantitative variables (e.g., relative gene expression levels) were performed using Student *t* tests and Fisher's exact tests for categorical variables. Cutoff values of gene expression to discriminate between mutated and non-mutated populations were assessed by use of Receiver Operating Characteristics (ROC) analysis. Statistical analysis was performed using GraphPad software version 4.0.

RESULTS

TK resistance classification: with/without BCR-ABL1 point mutations

A total of 101 CML patients were treated with TKIs. Among these, 56 (26 patients in chronic phase and 30 in advanced phases) presented treatment failure or suboptimal response and were therefore classified as resistant patients according to international criteria (25). Among the resistant patients, 31/56 (55%) showed *BCR-ABL1* point mutations while the remaining 25/56 (45%) did not show mutations as detected by nested-PCR cDNA-amplification and Sanger sequencing. Nine patients (9/31, 29%) showed mutations in the phosphorylation site (P-loop). Three patients (3/31, 10%) showed mutations in the C-helix loop. Eight patients (8/31, 26%) showed mutations in the ATP binding site (ATP-binding). Ten patients (10/31, 32%) showed mutations in the tyrosine kinase catalytic domain (C-loop) and one patient (1/31, 3%) showed a mutation in the A-loop (Table 3).

Analysis of gene expression

Measurements of *BCR-ABL1*/*ABL1* relative expression.

Relative quantification of *BCR-ABL1/ABL1* was evaluated in each group of CML patients. The transcripts level at diagnosis [median (interquartile interval)] was 217.0 (108.0 - 268.5); in CP Resistant to imatinib 23.0 (1.9 - 41.5); and in AP 18.5 (3.0 - 75.9) which were all found to be significantly higher than those in patients in CP in Remission 0.018 (0.002 - 0.085) ($p<0.0001$) (Figure 1A).

Expression level of CAMKIIγ, *KI67, HSP70* and *HSP90* genes.

For practical purposes gene expression values were Log_{10} transformed.

The gene expression level of *CAMKIIγ* in CML patients at diagnosis [median (interquartile interval)] 1.4 (0.2 - 1.8); in CP Resist 0.5 [(-0.5) - 1.7] and in AP 0.6 [(-0.08) - 1.2] showing increased values compared to the CP Remission group -0.7 [(-1.1) - (-0.3)] ($p <0.0001$) (Figure 1B).

KI67 gene expression in patients at diagnosis -2.1 [(-4.6) – (-1)]; in CP Resist -3.1 [(-4.1) - (-2.1)] and in AP -2.5 [(-4.3) – (-2)] had an expression level respectively higher than CP Remission group -3.7 [(-5.0) – (-3.0)] ($p <0.01$) (Figure 1C).

HSP70 gene expression in patients at diagnosis, in CP Resist and in AP 0.5 (0.07 - 1.7); -0.2 [(-1.2) -0.4] and -0.15 [(-0.7) - 0.5], respectively, were significantly higher that the CP Remission group -0.85 [(-1.1) – (-0.5)] ($p < 0.0001$, $p < 0.03$ and $p < 0.001$, respectively) (Figure 1D).

HSP90 gene expression in CP Resist -4.5 [(-6.3) - (-3.4)] and in AP -6.5 [(-9) – (-3.7)] were found to be significantly higher than CP Remission -5.2 [(-7.2) – (-4.6)] ($p < 0.02$) (Figure 1E), indicating that the measurement of the levels of transcript expression of these genes could be a potential marker for resistance to TKI treatment.

In order to explore the level of expression of *CAMKIIγ, KI67, HSP70* and *HSP90* genes within the cohort of TKI resistant patients, we classified them taking into account the presence of mutations in the kinase domain of *BCR-ABL1*, and grouped the cases as mutated (MT) and non mutated or wild type (WT).

BCR-ABL1 and *KI67* showed no differences between both groups (i.e., MT and WT) (Figure 2A and 2B).

Notably, *CAMKIIγ* and *HSP70* were significantly increased in the MT group (mean ± SE) 0.831 ± 0.155 and 0.012 ± 0.1618, respectively, with respect to those in the WT group 0.273 ± 0.2 and -0.575 ± 0.1 ($p < 0.03$ and $p < 0.04$, respectively) (Figure 2C and D).

Conversely *HSP90* expression was found to be significantly lower in the MT group -7.064 ± 0.424 than the WT group -5.522 ± 0.5 ($p < 0.03$) (Figure 2E).

Use of the differential transcript expression of CAMKIIγ, HSP70 and HSP90

Since genes *CAMKIIγ*, *HSP70* and *HSP90* showed differential expression between MT and WT in TKI resistant patients, we designed a score using the formula Log (CAMKIIγ x HSP70 / HSP90) in order to discriminate the presence of mutations and/or to evaluate the CML progression.

To explore the behavior of this score, it was evaluated in the five groups: healthy donors (HD), CML patients in chronic phase at diagnosis (Dx); patients in chronic phase in remission (CP Remission), patients in chronic phase showing TKI resistance (CP Resist) and patients in advanced phases (AP). We observed a significant higher score in CML patients at Dx, CP Resist and AP than patients in CP Remission (Figure 3A). This analysis suggested a direct correlation between the score and the efficacy to TKI treatment.

The score value showed a significant increase in CML Resistant MT patients (0.97 ± 0.5) versus CML Resistant WT (-1.42 ± 0.6) ($p = 0.011$) (Figure 3B).

In order to use the score Log [CAMKIIγ x HSP70 / HSP90] as a tool to classify patients with mutations (MT) and those without mutations (WT) (i.e., patients who developed other mechanisms of resistance), we applied ROC curves to assess a critical value that allowed maximum discrimination between MT and WT groups (Supplementary Figure SF2A and SF2B). This analysis estimated an optimal cut-off score value of 1.1 (Supplementary Figure SF2A and SF2B). Patients with score values greater than 1.1 are six times more likely to present mutations than patients with score values lower ($p < 0.002$) (Table 4). This score value limit of 1.1 estimates the probability to present mutations with 60% sensitivity and 80% specificity.

DISCUSSION

Most patients with CML achieve cytogenetic and molecular remission with TKI treatment. However, development of drug resistance by different molecular mechanisms (e.g., point mutations, *BCR-ABL1* amplification, *BCR-ABL1* over expression) has become a major obstacle for an effective treatment of patients with CML. TKIs transiently inhibit kinase activity of BCR-ABL1 but do not reduce the level of the P210 $^{BCR-ABL1}$ protein. The elimination of BCR-ABL1 protein, including those with mutations in the tyrosine kinase domain of *BCR-ABL1*, by use of HSP inhibitors provides a promising therapeutic strategy for the treatment of Ph^+ leukemia.

Peng et al (26) showed that inhibition of HSP90 by a novel inhibitor, IPI-504, associated with BCR-ABL1 protein degradation decreased the number of leukemia stem cells and prolonged patient overall survival.

The literature showed a differential protein expression level of *CAMKIIγ*, *HSP70*, *HSP90*, and *KI67* in patients with CML and *in vitro* in CML derived-cell lines (27, 28). Our aim in this scenario was to demonstrate the differential expression of *CAMKIIγ*, *HSP70*, *HSP90*, *KI67* genes but at the transcriptional level by qPCRs. This study was conducted to detect markers that may allow the discrimination of different CML stages, monitoring TKI treatment efficacies and to achieve an empirical classification of the molecular mechanism responsible for treatment failure.

This study demonstrated differential expression levels of *CAMKIIγ*, *HSP70*, *HSP90*, *KI67* and *BCR-ABL1* in CML patients undergoing different phases of the disease.

KI67 and CAMKIIγ expression were found to be significantly higher in patients at diagnosis, in chronic phase with resistance to TKI treatment and in advanced stages of the disease, with respect to the group of patients in CP with cytogenetic and molecular remission. This evidence indicated a correlation of KI67 and CAMKIIγ expression with the clinical course of CML. Taking into account that CAMKIIγ was implicated as a key regulator of the proliferation, via phosphorilation of proteins, experiments conducted for its specific silencing may be valuable prospects to control the dynamics of CML progression.

HSPs are known to play important roles in human tumorigenesis (29-31) through stabilization of various proteins involved in signaling pathways leading to malignant transformation.

Zackova et al (28) analyzed the HSP70 and HSP90 protein levels in patients with CML with different responses to TKIs therapy to find out whether this expression protein level

may characterize the state and prognosis of CML. However, as they did not find differences in HSP70 protein expression between the different stages, they ruled it out as a suitable marker reflecting response to treatment with TKIs. Interestingly, Yeh et al (27) found differences in HSP70 plasma level between CML patients and healthy individuals and also clinical correlation between HSP70 protein level and progression to accelerated/blast phase. Therefore, plasma level of HSP70 can be used as a biomarker of disease progression in patients with CML and supports the concept that HSP70 may be crucial in developing the resistance to treatment with imatinib (27). Moreover, Zackova et al (28) found that total leukocyte's HSP90 level showed good correlation with both the clinical phase of CML patients and with *BCR-ABL1* transcripts level. They determined that HSP90 protein level has a predictive value for response to TKI therapy.

From another perspective but in agreement with these previous studies, our data indicate that there is a significant increase in *HSP70* and *HSP90* expression level in CML patients at diagnosis, in chronic phase with resistance to TKI treatment and in advanced stages of the disease.

These interesting results suggest the use of these chaperones´ transcript levels as markers of resistance to TKI therapy. In addition, our findings on *HSP90* and *HSP70* expression confirmed that inhibitors against the HSPs may be helpful to overcome TKI resistance in patients with CML; and therefore they can be used as a biomarker of disease progression.

TKI treatment resistant CML patients, either in CP or in AP, grouped according to the presence or absence of point mutations in the tyrosine kinase domain of *BCR-ABL1* gene, showed significantly increased expression of *CAMKIIγ* and *HSP70* in the group of patients with mutations with respect to those without mutations, whereas the level of expression of *HSP90* was significantly lower in the mutated group as compared with the non mutated group. *BCR-ABL1* and *KI-67* did not show significant differences between both groups.

These results regarding the differential transcript levels of *CAMKIIγ, HSP70* and *HSP90* in resistant patients with and without point mutations and the cost-effectiveness of its specific assessment prompted us to elaborate the score, LOG (CAMKIIg x HSP70 / HSP90), which can be used to discriminate with minor experimental efforts, the presence or absence of mutations in resistant patients and to help in monitoring the efficacy of TKI treatment. We defined an optimal cut-off score value of 1.1, which allowed us to estimate the probability to present mutations in the *BCR-ABL1* tyrosine kinase domain with 60% sensitivity and 80% specificity.

We believe that the score could be integrated in a rational monitoring algorithm, in CML cases in chronic phase with persistent high levels of *BCR-ABL1* transcripts over 0.1% or loss of molecular response achieved (confirmed in two consecutive studies).

The transcriptional levels of CAMKIIγ, HSP70 and HSP90 genes reflect key elements that are associated with the clinical, cellular and molecular status of CML biology, and they provide valuable information for the hematologist who has to start treatment. In order to determine the ultimate clinical usefulness of the score, and due to the availability of RNA samples collected to test BCR-ABL1 transcript level, we dare to ask the scientific community in this field to include the score testing not only in intermediate “warning” patients (32) but also in longitudinal studies for detecting in advance patients who will develop ITK resistance.

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All authors report no conflict of interest.

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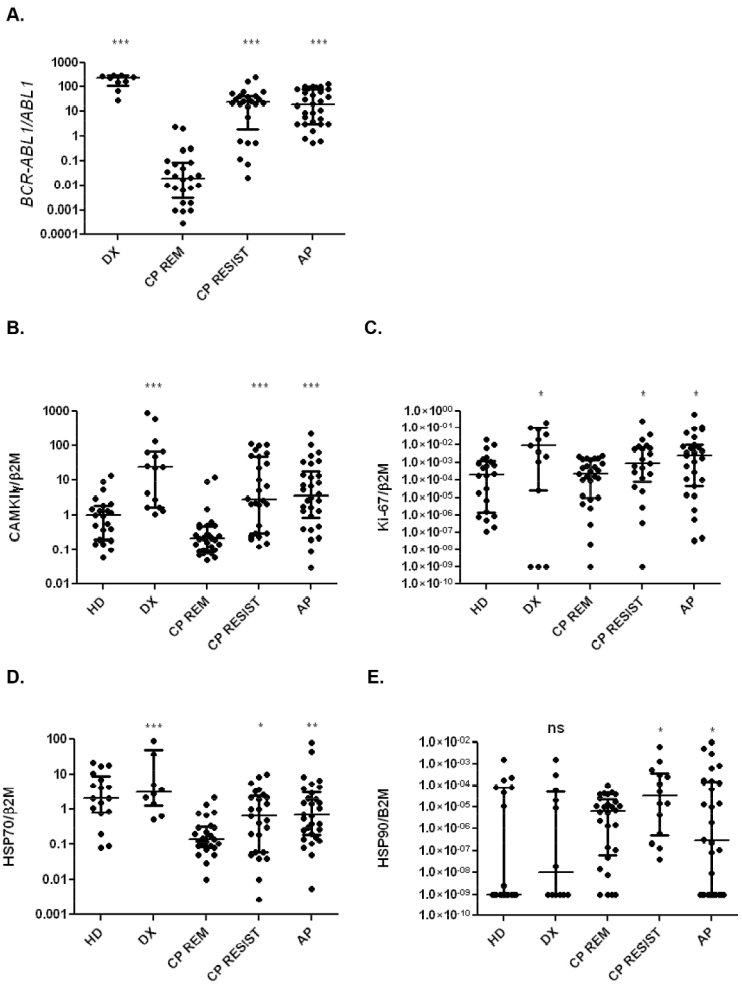
LEGENDS

Figure 1. Transcript differential expression in CML patients in different phases. (A) *BCR-ABL1*, (B)*CAMKIIγ,* (C) *KI67, (D) HSP70 and (E) HSP90.* Gene expression in patients at different stages of CML: HD: Healthy donors; Dx: diagnosis; CP Rem: chronic phase with molecular remission; CP Resist: chronic phase with resistance to TKIs and AP: advanced phases. ***P<0.0001, **P<0.001, *p<0.01 (respect to the CP Rem).

Figure 2. Transcript expression levels in TKI resistant CML patients in MT (with point mutations) *versus* WT (without mutations). (A) *BCR-ABL1,* (B) *KI67.* (C) *CAMKIIγ,* (D) *HSP70* and (E) *HSP90*. ns: no significant, *P<0.05.

Figure 3. Treatment efficacy *score* using gene expression values LOG (CAMKIIγ x HSP70 / HSP90). (A) *Score* values in patients at different stages of CML and different causes of TKI treatment failure. HD: Healthy donors, Dx: CML patients at diagnosis; CP Rem: patients in chronic phase with molecular remission; CP Resist: chronic phase with resistance to TKIs and AP: advanced phases. **P<0.001 (respect to the CP Rem). (B) Statistical analysis between groups of TKI resistant CML patients with mutations (MT) and without mutations (WT). *P <0.01.


A.
BCR-ABL1/ABL1
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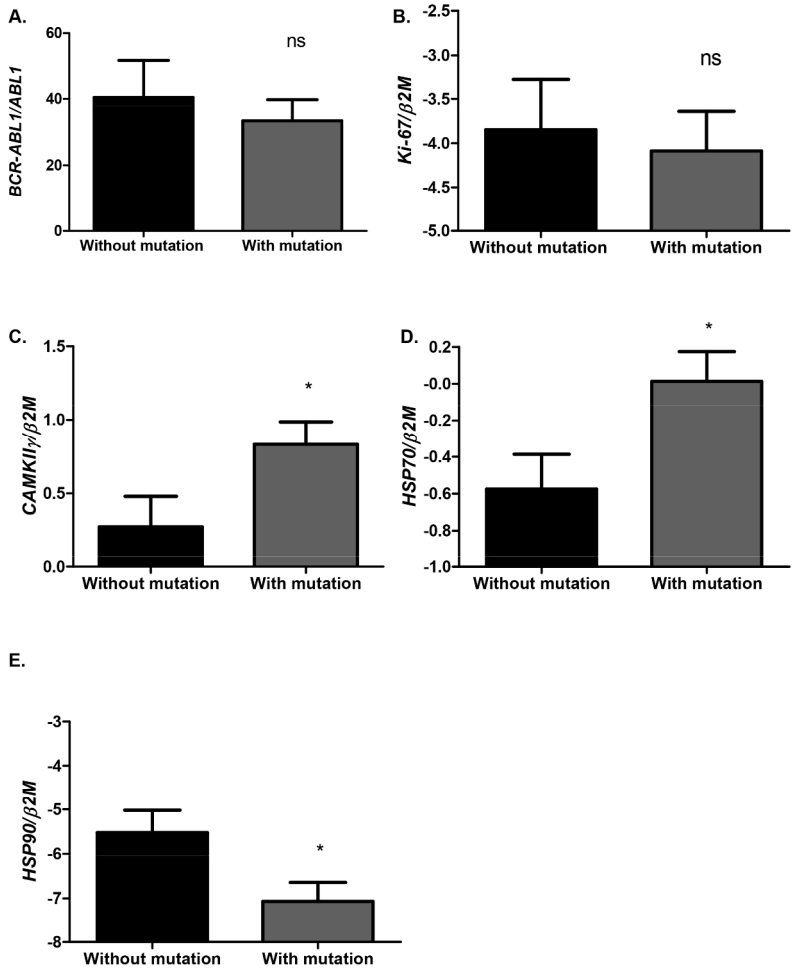
DX
CP REM
CP RESIST
AP
C.
Ki-67/β2M
HD
DX
CP REM
CP RESIST
AP
D.
HSP70/β2M
HD
DX
CP REM
CP RESIST
AP
E.
ns
HSP90/B2M
HD
DX
CP REM
CP RESIST
AP


A.
BCR-ABL1/ABL1
60
40
20
0
ns
Without mutation
With mutation
B.
Ki-67/β2M
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
ns
Without mutation
With mutation
C.
CAMKIIγ/β2M
1.5
1.0
0.5
0.0
*
Without mutation
With mutation
D.
HSP70/β2M
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
*
Without mutation
With mutation
E.
HSP90/β2M
-3
-4
-5
-6
-7
-8
*
Without mutation
With mutation

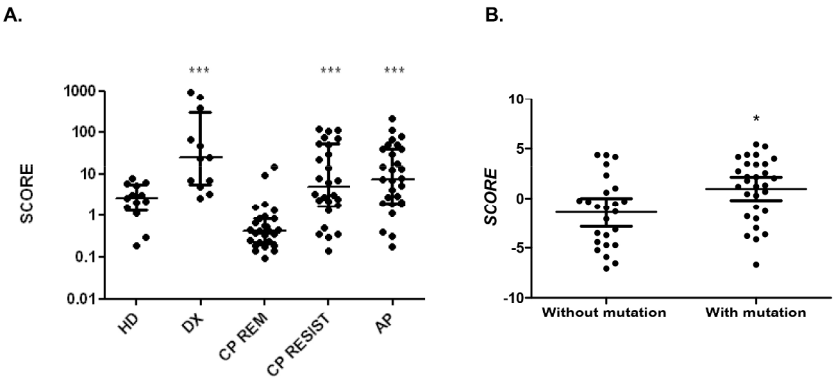

A.
SCORE
1000
100
10
1
0.1
0.01
***
***
***
HD
DX
CP REM
CP RESIST
AP
B.
SCORE
10
5
0
-5
-10
*
Without mutation
With mutation

Table 1. Patient's clinical characteristics.

Total Patients	101
Males/Females	54/47
Median age (years)	49
Range age (years)	23 -77
At diagnosis [1]	15
In CP Rem[2]	30
In CP Resist [2]	26
In AP (AcP and BC) [2]	30

[1] Untreated. [2] Treated with TKIs.

Table 2. Oligonucleotide primer sequences for qPCR analysis.

Official Symbol	**Legacy notation**	***primer sequences* (5' to 3')**	**Product size bp**
CAMK2G	*CAMKIIγ*	Fw 5´GCAGCAGGCTTGGTTTGGTTTTG 3´ Rv 5´ GTGATGCGCTTTGCTGGGTTTATG-3´	285
MKI67	*KI-67*	Fw 5´ GGCAAGAGGCAAATCATCCGAACC 3´ Rv 5' CCTCCGCTCTCCTCTGCCACCTTA 3´	417
HSPA41	*HSP70*	Fw 5´ GCTGATCGGCCGCAAGTTCG 3' Rv 5´ TGCCCCCGCCCAGGTCAAAGAT 3´	395
HSP90AA1	*HSP90*	Fw 5' TTATGAAACTGCGCTCCTGTCTT 3´ Rv 5' AGTGCACGTTACCCCAATCTGT 3´	448
B2M	*β2M*	Fw 5´ AAGATGAGTATGCCTGCCGTGTGA 3' Rv 5´ ACCTCTAAGTTGCCAGCCCTCCTA 3'	319

Fw: *Foward*, Rv: *Reverse*.

Table 3. Mutations in the kinase domain of *BCR-ABL1*.

Site	Mutations	Nº cases
P-loop	L248V	1
	G250E	2
	Y253H	1
	E255K/V	5
C-helix loop	E279K	1
	V289F	1
	L298V	1
ATP- binding	T315I	5
	F317L	2
	L324M	1
C-loop	L348M	1
	M351I	3
	E355G	1
	N358S	1
	F359V/I/C	3
A-loop	L387M	1

P-loop: Phosphate binding loop*; C-loop:* tyrosin kinase catalytic domain*, A-loop:* activation loop.

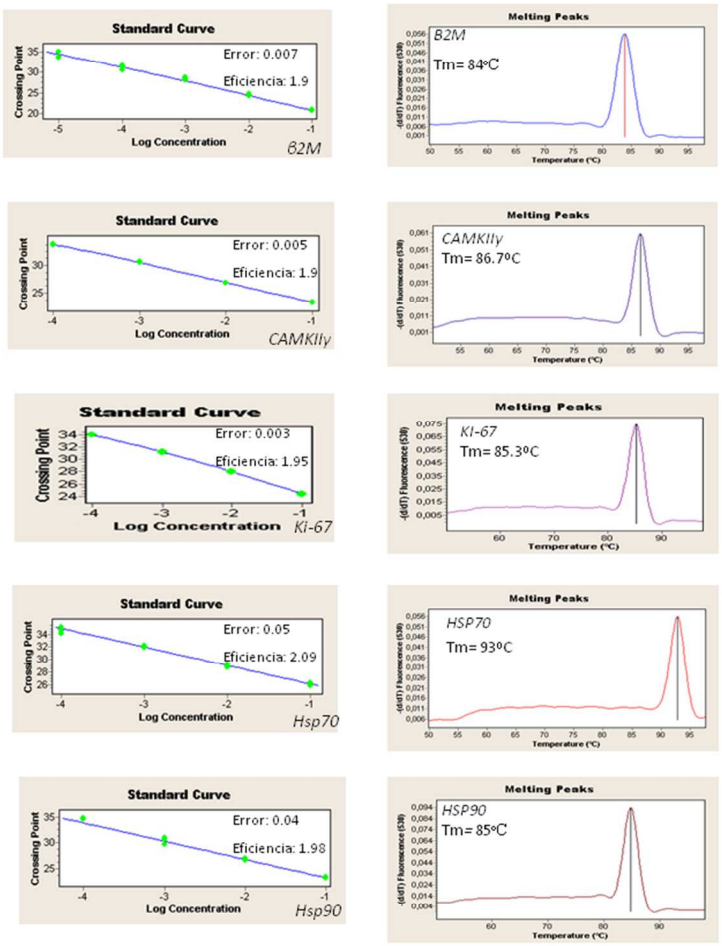
Table 4: Score based discrimination analysis of mutated and non-mutated TKI resistant patients.

Score cutoff class	With mutations MT	Without mutation WT
> 1.1	19	5
≤ 1.1	12	20

OR (CI95) = 6.3 (1.8-21.4) (p = 0.0027**)

Sensitivity = 80% (19/24)

Specificity = 63% (20/32)



Supplementary Figure SF1. (A) Left panel. Crossing point (Cp) *vs* cDNA concentration of K562 calibration curves. The qPCR amplification efficiency and average errors are shown. **(B) Right panel.** Specificity of qPCR products. Melting temperature peaks (Tm between 80-90° C) are shown (vertical lines).

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A.

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> -6.881	100,0	88.43% to 100.0%	4,000	0.1012% to 20.35%	1,04
> -6.629	96,67	82.78% to 99.92%	4,000	0.1012% to 20.35%	1,01
> -6.248	96,67	82.78% to 99.92%	8,000	0.9840% to 26.03%	1,05
> -5.589	96,67	82.78% to 99.92%	12,00	2.547% to 31.22%	1,10
> -4.992	96,67	82.78% to 99.92%	16,00	4.538% to 36.08%	1,15
> -4.738	96,67	82.78% to 99.92%	20,00	6.831% to 40.70%	1,21
> -4.582	96,67	82.78% to 99.92%	24,00	9.356% to 45.13%	1,27
> -4.293	96,67	82.78% to 99.92%	28,00	12.07% to 49.39%	1,34
> -3.995	93,33	77.93% to 99.18%	28,00	12.07% to 49.39%	1,30
> -3.790	90,00	73.47% to 97.89%	28,00	12.07% to 49.39%	1,25
> -3.700	90,00	73.47% to 97.89%	32,00	14.95% to 53.50%	1,32
> -3.591	86,67	69.28% to 96.24%	32,00	14.95% to 53.50%	1,27
> -3.344	86,67	69.28% to 96.24%	36,00	17.97% to 57.48%	1,35
> -3.084	86,67	69.28% to 96.24%	40,00	21.13% to 61.33%	1,44
> -2.562	83,33	65.28% to 94.36%	40,00	21.13% to 61.33%	1,39
> -2.097	83,33	65.28% to 94.36%	44,00	24.40% to 65.07%	1,49
> -1.965	80,00	61.43% to 92.29%	44,00	24.40% to 65.07%	1,43
> -1.606	76,67	57.72% to 90.07%	44,00	24.40% to 65.07%	1,37
> -1.338	76,67	57.72% to 90.07%	48,00	27.80% to 68.69%	1,47
> -1.148	73,33	54.11% to 87.72%	48,00	27.80% to 68.69%	1,41
> -0.9401	73,33	54.11% to 87.72%	52,00	31.31% to 72.20%	1,53
> -0.8674	70,00	50.60% to 85.27%	52,00	31.31% to 72.20%	1,46
> -0.6954	70,00	50.60% to 85.27%	56,00	34.93% to 75.60%	1,59
> -0.4388	70,00	50.60% to 85.27%	60,00	38.67% to 78.87%	1,75
> -0.3054	70,00	50.60% to 85.27%	64,00	42.52% to 82.03%	1,94
> -0.2550	70,00	50.60% to 85.27%	68,00	46.50% to 85.05%	2,19
> 0.04470	70,00	50.60% to 85.27%	72,00	50.61% to 87.93%	2,50
> 0.3851	66,67	47.19% to 82.71%	72,00	50.61% to 87.93%	2,38
> 0.5315	63,33	43.86% to 80.07%	72,00	50.61% to 87.93%	2,26
> 0.6468	63,33	43.86% to 80.07%	76,00	54.87% to 90.64%	2,64
> 0.8538	60,00	40.60% to 77.34%	76,00	54.87% to 90.64%	2,50
> 1.103	60,00	40.60% to 77.34%	80,00	59.30% to 93.17%	3,00
> 1.203	56,67	37.43% to 74.54%	80,00	59.30% to 93.17%	2,83
> 1.275	53,33	34.33% to 71.66%	80,00	59.30% to 93.17%	2,67
> 1.565	50,00	31.30% to 68.70%	80,00	59.30% to 93.17%	2,50
> 1.921	46,67	28.34% to 65.67%	80,00	59.30% to 93.17%	2,33
> 2.102	43,33	25.46% to 62.57%	80,00	59.30% to 93.17%	2,17
> 2.171	40,00	22.66% to 59.40%	80,00	59.30% to 93.17%	2,00
> 2.254	36,67	19.93% to 56.14%	80,00	59.30% to 93.17%	1,83
> 2.443	36,67	19.93% to 56.14%	84,00	63.92% to 95.46%	2,29
> 2.651	33,33	17.29% to 52.81%	84,00	63.92% to 95.46%	2,08
> 3.075	30,00	14.73% to 49.40%	84,00	63.92% to 95.46%	1,87
> 3.427	26,67	12.28% to 45.89%	84,00	63.92% to 95.46%	1,67
> 3.457	26,67	12.28% to 45.89%	88,00	68.78% to 97.45%	2,22
> 3.474	23,33	9.934% to 42.28%	88,00	68.78% to 97.45%	1,94
> 3.747	20,00	7.713% to 38.57%	88,00	68.78% to 97.45%	1,67
> 4.095	16,67	5.642% to 34.72%	88,00	68.78% to 97.45%	1,39
> 4.181	13,33	3.755% to 30.72%	88,00	68.78% to 97.45%	1,11
> 4.274	13,33	3.755% to 30.72%	92,00	73.97% to 99.02%	1,67
> 4.370	13,33	3.755% to 30.72%	96,00	79.65% to 99.90%	3,33
> 4.380	13,33	3.755% to 30.72%	100,0	86.28% to 100.0%	
> 4.390	10,00	2.112% to 26.53%	100,0	86.28% to 100.0%	
> 4.807	6,667	0.8178% to 22.07%	100,0	86.28% to 100.0%	
> 5.320	3,333	0.08436% to 17.22%	100,0	86.28% to 100.0%	

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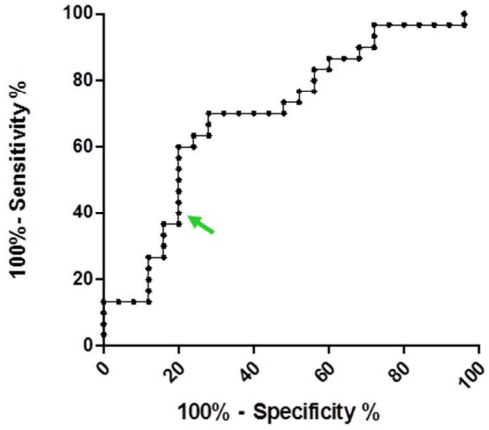
B.



Supplementary Figure SF2. Receiver Operator Curve (ROC) for discrimination of populations of TKI resistant CML patients with mutations (MT) and without mutations (WT) based on *score* values. (A) ROC analysis table showing cutoff values *versus* Likelihood ratios. Notably, 1.1 value shows the highest discrimination for MT to WT patient groups (highlighted in green). **(B) ROC graphics.** Specificity versus Sensitivity of each score value for discriminating MT and WT groups in TKI resistant patients with CML. Green arrow represents 1.1 score value.

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