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Differential gene expression changes and their implication on the disease progression in patients with Chronic Myeloid Leukemia

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ABSTRACT

The molecular mechanisms responsible for disease progression of CML are not conclusive. The main functional changes associated with disease evolution in CML was high proliferation rate, decreased apoptosis, blockade of differentiation, and strong resistance to chemotherapeutic agents. The current study analyzed the relative expressional profiles of genes related with proliferation, apoptosis, differentiation, and resistance to chemotherapeutic agents such as *c-MYC*, *BAD*, *BCL-2*, *C/EBPα/-β* and *ABCB1* respectively in different clinical stages of CML by SYBR Green I quantitative real-time (qRT) PCR. We selected a total of 183 CML patients and 30 healthy control samples. The study populations were classified into four groups, including de novo CML-CP (50/183), CML-AP (32/183), CML-BC (51/183) and Imatinib Mesylate or IM resistant CML-CP (50/183) groups. qRT PCR analysis revealed that significant overexpression of *c-MYC*, *ABCB1* and *BCL-2* was observed in advanced phases and IM resistant CP of CML compared to healthy controls. Likewise, the mean expression level of *BAD*, *C/EBPα/-β* genes were found to be significantly down regulated. Present study concluded that the complex interplay of several candidate genes like overexpression of *c-MYC*, *ABCB1*, *BCL-2* and down regulation of *BAD*, *C/EBPα/-β* played a significant role in the disease evolution and development of drug resistant in CML.

1. Introduction

Malignant progression of Chronic Myeloid Leukemia (CML) from benign chronic phase (CP) to fatal blast crisis (BC), through an intermediate accelerated phase (AP), is accompanied by several drastic phenotypic and genotypic transformations in blast cells and these changes were mirrored by differences in gene expression profile [1]. Thus far, multiple downstream signaling pathway rather than single were responsible for the erratic acceleration of the disease. Moreover, genomic instability in advanced phases of CML boosts the aggressiveness of disease at its end stages. These cascades of molecular events will alter the expression profile of various important genes that may perform a crucial role in the pathogenesis, evolution and resistance to therapy in CML. The main obvious functional changes associated with disease evolution in CML was high proliferation rate, decreased apoptosis, blockade of differentiation, and strong resistance to chemotherapeutic agents. Therefore better understanding of the variations in the expressional profiles of several candidate genes involved in these specific pathways will provide new insights into the disease anatomy of CML and it will help in the proper identification of potential prognostic markers of CML evolution and thereby enlighten the new therapeutic strategies.

Active efflux of chemotherapeutic drugs from the target cells as a mechanism of drug resistance was first described in the 1970s. Overexpression of ABCB1 (ATP binding cassette sub family B member-1), a cell-surface trans-membrane protein conferred insensitivity to various chemotherapeutic drugs, a mechanism known as multidrug resistance, by active drug efflux [2]. This efflux end results in the decreased intracellular drug accumulation, which is known to be one of the main drug resistance mechanism adopted by the CML blast cell clone [3,4].

Similarly, *c-MYC*, an oncogenic transcription factor of the helix-loop-helix/leucine zipper protein family, found to be altered in half of human cancers and frequently involved in cancer progression [5]. The

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harmonic function of *c-MYC* is essential for normal hematopoiesis; any signs of *c-MYC* deregulation would ultimately results in the malignant transformation of hematopoietic cells [6]. Recently, it was reported that Philadelphia chromosome (Ph) positive leukemic cell clones with high *c-MYC* mRNA levels were selected and amplified during disease progression of CML [7].

In CML, increased insensitivity towards apoptosis was considered to be the final consequence of constitutive tyrosine kinase activity of *BCR-ABL1*, which leads to the aberrant expression of the B-Cell Lymphoma 2 family of apoptosis regulator proteins, such as the anti-apoptotic members *BCL-2*, *BCL-xL* and *MCL-1* and the pro-apoptotic members *BAD* and *BIM* [8]. In vivo studies showed that the dysregulated expression of *BCL-2/BAD* played a major role in the disease transformation of CML [9,10]. Interestingly, expression profile analysis of CML patients suggested that disease progression did not seem to be solely depend on *BCL-2* levels [11], however the expression level or activity of *BCL xL*, and its negative regulator *BAD*, might also played an important role in both CML-BC development and Tyrosine Kinase Inhibitor (TKI) resistance [12,13].

CCAAT/enhancer binding proteins (C/EBPs) are a family of six proteins including *C/EBPα* and *C/EBPβ* isoforms. Both *C/EBPα* and *C/EBPβ*, members of the basic region leucine zipper (bZIP) family of transcription factors, considered as the critical component for myeloid differentiation and which are shown to be down regulated in CML-BC by *BCR-ABL1* [14].

Little is known about the expression profile of *c-MYC*, *BAD*, *BCL-2*, *C/EBPα*, *C/EBPβ* and *ABCB1* genes in patients with CML and their involvement in the disease progression of CML. Thence, the current study opted to examine the expressional variations in the genes related with proliferation, apoptosis, differentiation, and resistance to chemotherapeutic agents such as *c-MYC*, *BAD*, *BCL-2*, *C/EBPα*, *C/EBPβ* and *ABCB1* respectively in all the clinical stages of CML to elucidate the molecular mechanism responsible for disease progression in CML.

2. Methods

2.1. Samples

Fifty newly diagnosed and untreated patients with CML in chronic phase [de novo CML-CP], fifty CML-CP cases with drug resistance to Imatinib Mesylate (IM) therapy [IM resistant CML-CP, the current study examined both patients with primary and secondary resistance to IM], 32 patients with CML in accelerated phase [CML-AP], and 51 cases with CML in blast crisis (CML-BC), who attended the Medical Oncology outpatient clinics of Regional Cancer Center (RCC), Kerala, India were recruited for the study. Thus, a total of 183 patients from the four different clinical stages of CML were selected for the study during the period from January 2013 to January 2016. The details of the samples were listed in Table 1. The diagnoses of all patients were based on cytomorphology, immunohistochemistry, and molecular analysis. All the study subjects possessed *BCR-ABL1* fusion gene which was confirmed both by GTG-banding and Fluorescent In Situ Hybridization (FISH) analysis using LSI BCR/ABL1 dual-color dual-fusion translocation probe. Peripheral blood mononuclear cells (PBMCs) from 30 healthy individuals (HI) served as controls.

Table 1
Details of samples used in the study.

Diagnosis	Numbers			Age range (years)
	Total	Male	Female	
De novo CML-CP	50	28	22	16–81
IM resistant CML-CP	50	26	24	22–72
CML-AP	32	20	12	23–65
CML-BC	51	27	24	18–72
HI	30	16	14	19–69

CML-chronic myelogenous leukemia, CP-chronic phase, AP-accelerated phase, BC-blast crisis, IM -Imatinib Mesylate, HI-healthy individual.

The study was approved by the Institution review board and Human Ethics committee of Regional Cancer Centre (RCC HEC No-06/2010). After getting written informed consent, 2–3 ml of Peripheral blood (PB) samples was collected aseptically in the heparinized vacutainer from the study subjects. PB samples were subjected to gradient centrifugation using Ficoll-Histopaque reagent to isolate PB mononuclear cells (PBMCs) from the study specimen.

2.2. RNA extraction and cDNA synthesis

RNA extraction was carried out using the Trizol kits (RNeasy Mini Kit, Quiagen, US and innuPREP RNA Mini Kit, Analytik Jena AG, Germany). The extracted RNA from the study subjects were then reverse-transcribed into first-strand cDNA using random hexamer primers and the High Capacity cDNA reverse transcription kit (Applied Biosystems, US) according to the manufacturer's instructions.

2.3. Real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

The relative expression profiles of *c-MYC*, *BAD*, *BCL-2*, *C/EBPα*, *C/EBPβ* and *ABCB1* genes were examined by SYBR Green I real-time PCR chemistry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control gene. In brief, PCR reactions were performed in a final volume of 20 µl containing 1 µL of cDNA, 9 µL of 1X Takyon Rox SYBR Master Mix (Eurogentec, Belgium), and 100 nM primer pairs. In order to prevent the genomic DNA amplification exon/exon junction spanning primers were designed (the details regarding the qRT PCR primers are given in the Table 2). After initial denaturation at 95 °C for 10 min, 40 cycles consisting of the following procedure were performed using Step One Real Time PCR System (Applied Biosystem, US): 15 s at 95 °C, 1 min s at 60 °C. Each PCR reaction was done in triplicates. The data are presented as the relative expression of the genes of interest relative to the internal control gene as determined by the $2^{(-\Delta\Delta CT)}$ method [15,16]. The fold changes values were further converted to the log 10 scale. The mean values of relative fold changes observed among the study groups and healthy control were calculated, analyzed and compared. In addition, the specific amplification of the PCR products was analyzed by melting curve analysis and agarose gel electrophoresis. The primers used for real-time PCR for all gene amplifications were synthesized by Eurogentec, Belgium (Table 2).

2.4. Treatment response analysis

The hematological and cytogenetic data of study subjects were documented initially at the time of diagnosis and these parameters were further evaluated periodically at 3-month intervals for the first year and at 6-month intervals for the next 2 years after the initiation of targeted therapy.

Patients with Complete Hematologic Remission (CHR) would exhibit a WBC count $< 10 \times 10^9$/L and platelet count $< 450 \times 10^9$/L in their PB with complete disappearance of blast cells in association with entire reversal of splenomegaly. Partial Hematologic Response (PHR) is similar to that of CHR, except that there could be a perseverance of blast cells, or platelet count < 50% of the pre-treatment count but $> 450 \times 10^9$ cells/L, or perseverant splenomegaly but > 50% of the pretreatment extent.

Cytogenetic response (CyR) is denoted by the percentage reduction of Ph positive metaphase cells in the bone marrow (BM). The complete disappearance of Ph positive cells from the BM is defined as complete cytogenetic response (CCyR), patients whose BM showed the presence of 1–35% of Ph positive metaphase cells is considered as major cytogenetic response (MCyR), presence of 36–65% of metaphase cells with

Table 2
Primer sequences and the PCR product size of various genes studied.

Genes	Primer sequence	Amplimer size (bp)	Accession no
c-MYC	F 5′GCG ACT CTG AGG AGG AAC 3′ R 5′CCA GCA GAA GGT GAT CCA 3′	106	NM_002467.4
BAD	F 5′GAC TCC TTT AAG AAG GGA CTT 3′ R 5′ACC AGG ACT GGA AGA CTC 3′	96	NM_004322.3
BCL-2	F 5′CTG GAG AGT GCT GAA GAT TG 3′ R 5′CTA CTT CCT CTG TGA TGT TGT ATT 3′	120	NM_000633.2
C/EBPα	F 5′GCA ACT CTA GTA TTT AGG ATA A 3′ R 5′ACC TCC AAA TAA AAT GAC AA 3′	108	NM_001285829.1
C/EBPβ	F 5′GTG GTG TTA TTT AAA GAA GAA AC 3′ R 5′ACC GAT TGC ATC AAC TTC 3′	119	NM_001285878.1
ABCB1	F 5′GTG AGT TGG TTT GAT GAC 3′ R 5′GAA CCT ATA GCC CCT TTA 3′	98	NM_000927.4
GAPDH	F 5′AAT CCC ATC ACC ATC TTC CAG3′ R 5′AAA TGA GCC CCA GCC TTC 3′	122	NM_002046

Gene accession number was assigned as per NCBI data base, bp-base pair.

Ph in the BM is defined as partial cytogenetic response (PCyR) and minor or minimal cytogenetic response (mCyR) is the BM having 66–95% of Ph positive metaphase cells. Patients whose BM revealed > 95% of Ph positive metaphase cells were considered as IM non responders.

Primary resistance or intrinsic resistance to IM is defined as the absence of hematologic response within three months, having incomplete hematologic response or the absence of cytogenetic response within six months and having less than partial cytogenetic response within 12 months. Secondary resistance or acquired resistance is the loss of hematologic response, loss of MCyR or CCyR after 12 months of treatment with IM.

2.5. Statistical analysis

Data was analyzed using the SPSS software 21. Analysis of variance (ANOVA) was used to find the association in the expression profile of *c-MYC*, *BAD*, *BCL-2*, *C/EBP-α*, *C/EBP-β* and *ABCB1* genes between the control and study groups and Bonferroni multiple comparison tests was used to identify the significantly differing gene profiles. A P value < 0.05 is considered to be significant.

3. Results

3.1. Relative gene expression profile of c-MYC and ABCB1

Significant *c-MYC* overexpression pattern was found in CML-BC (mean, 0.601; P = 0.003) and in IM-resistant CML-CP (mean, 0.702; P < 0.0001) patients in comparison with the healthy control samples (mean, 0.152). Even though compared to HI, the mean expression level of *c-MYC* was found to be high in CML-AP patients (0.344 vs 0.152, P = 1.00), it was not statistically significant. However, the transcript level of *c-MYC* in de novo CP patients and in HI were almost equal (0.141 vs 0.152, P = 1.00). We next compared the expression level of *c-MYC* between different CML study groups and it was found that mean expression level of *c-MYC* was high in IM resistant CP, while it was low in de novo CP patients. These findings pointed the fact that, overexpression of *c-MYC* was predominant in therapy resistant chronic phase and in advanced phases of CML. Moreover, patients in IM resistant CP showed a significant overexpression of *c-MYC* compared to AP (0.702 vs 0.344, P = 0.034) and de novo CP patients (0.702 vs 0.141, P < 0.0001). Likewise, significantly high expression was obtained in BC patients compared to de novo CP patients (0.601 vs 0.141, P < 0.0001). Compared to BC patients, the mean expression level of *c-MYC* was relatively high in IM resistant CP (0.702 vs 0.601, P = 1.00), however the difference was not statistically significant. Similarly, in comparison with CML-AP, patients with CML-BC exhibited comparatively high *c-MYC* transcript level (0.601 vs 0.344, P = 0.338), though differences were not statistically significant [Fig. 1A & B].

The relative expression level of *ABCB1* gene was high in advanced phases and IM resistant CP of CML compared to HI. However, the pattern of expression was almost similar in de novo CP (mean, −0.015) and in HI (mean, −0.128). The highest expression of *ABCB1* was observed in therapy resistant CP while de novo CP patients showed the lowest. The *ABCB1* expression level was significantly high in IM resistant CP compared to HI (0.565 vs −0.128, P < 0.000) and de novo CP patients (0.565 vs −0.015, P < 0.000). Likewise, the mean expression level of *ABCB1* was significantly higher in CML-BC patients in comparison with HI (0.344 vs −0.128, P = 0.024). Compared to BC and AP, IM resistant CP patients possessed an elevated amount of *ABCB1* transcript, 0.565 vs 0.344, P = 0.981 and 0.565 vs 0.254, P = 0.410 respectively, but these differences were without any statistical significance. Similarly, in comparison with de novo CP, patients in both AP (0.254 vs −0.015, P = 0.765) and BC (0.344 vs −0.015, P = 0.075) had relatively a high level of *ABCB1* transcript; however the differences were not with any statistical significance. Furthermore, the mean expression level of *ABCB1* in AP and BC phases of CML were almost equal and comparable (0.344 vs 0.254, P = 1.00) [Fig. 1C & D].

3.2. Relative gene expression profile of BCL-2 and BAD

These relative fold change calculation revealed that compared to healthy controls overexpression of *BCL-2* gene was observed among the study groups. Mean *BCL-2* expression was significantly higher in CML-BC (0.752 vs −0.141, P = 0.003) and in IM resistant CML-CP (0.693 vs −0.141, P = 0.007) compared to HI. Similarly, de novo CP (mean, 0.280; P = 0.853) and AP (mean, 0.598; P = 0.064) patients also possessed a high mean *BCL-2* expression in comparison with HI (mean, −0.141), but it was not with any statistical significance. Even if the mean expression level of *BCL-2* was high in BC compared to de novo CP (0.752 vs 0.280, P = 0.255) and AP patients (0.752 vs 0.598, P = 1.00), the differences were not at all statistically significant. Although, the mean expression level of *BCL-2* was higher in IM resistant CP patients compared to de novo CP (0.693 vs 0.280; P = 0.515) and AP patients (0.693 vs 0.598; P = 1.00), differences were not with any statistical significance. Moreover, it should be noted that there was a gradual increase in the expression level of *BCL-2* from CP to BC stages of CML, with a notable increase in the therapy resistant CP compared to HI [Fig. 2A & B].

The expression level of *BAD* appeared to be low in different phases of CML compared to healthy controls. *BAD* expression level was significantly reduced in de novo CML-CP (mean, −0.286; P < 0.000), CML-AP (mean, −0.550; P < 0.000), CML-BC (mean, −0.338; P < 0.000) and IM resistant CML-CP (mean, −0.147; P = 0.005) in

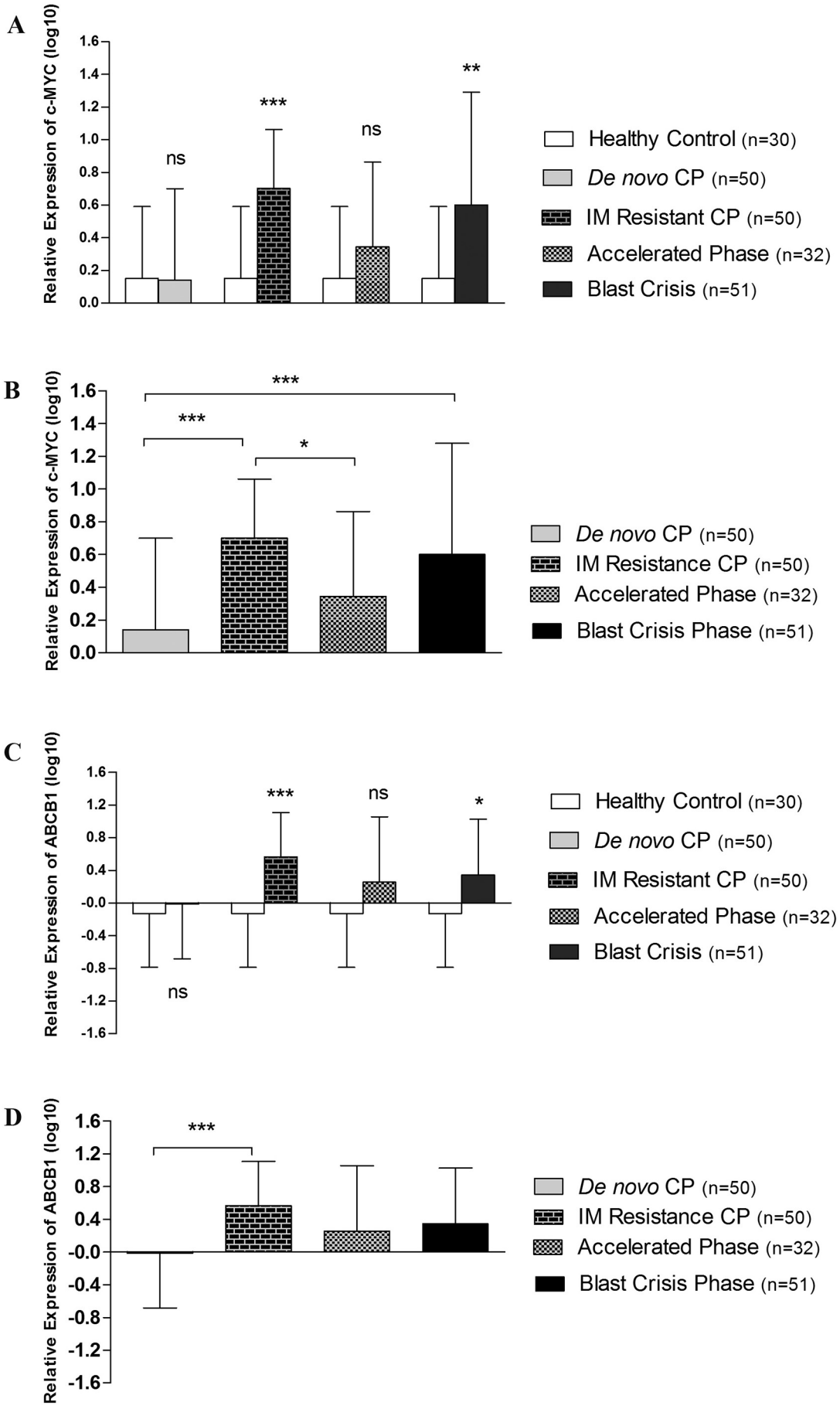

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Fig. 1. A) Comparison of the mean expression level of *c-MYC* between different study groups of CML & healthy individuals. B) The data showing the mean transcript level of *c-MYC* in different study groups of CML. C) Comparison of the mean expression level of *ABCB1* in different study groups of CML & healthy individuals. D) The data showing the mean transcript level of *ABCB1* in different study groups of CML (***; P-value < 0.001, **; P-value < 0.01, *; P value < 0.05).

comparison with HI (mean, 0.473). There was not any significant difference of *BAD* expression level was observed between de novo CP and BC and their expression level was comparable (−0.286 vs −0.338, P = 1.00). Likewise, the transcript level of *BAD* seemed to be similar between BC and IM resistant CP (−0.338 vs −0.147, P = 1.00). In comparison with BC, AP patients displayed relatively lower level of *BAD* expression (−0.550 vs −0.338, P = 1.00), however the difference was not statistically significant. In the same manner, patients with de novo CP exhibited reduced level of *BAD* expression compared to therapy resistant CP (−0.286 vs −0.147, P = 1.00). Among the study groups, patients with CML-AP showed the lowest level of *BAD* expression [Fig. 2C & D].

3.3. Relative expression profile of C/EBPα and C/EBPβ

The expression level of *C/EBPα* gene was significantly lower in de novo CP (mean, −0.279; P < 0.000), AP (mean, −0.652; P < 0.000), BC (mean, −0.976; P < 0.000) and IM resistant CP (mean, −0.626; P < 0.000) phases of CML compared to HI (mean, 0.532). Low expression levels of *C/EBPα* were more prominent in advanced phases and therapy resistant chronic phase of CML. Compared to de novo CP, the mean expression level of *C/EBPα* was significantly decreased in BC-CML (−0.976 vs −0.279; P < 0.000). Likewise, the transcript level of *C/EBPα* was markedly reduced in AP (−0.652 vs −0.279; P = 0.109) and IM resistant CP (−0.626 vs −0.279, P = 0.074) compared to de novo CP, but the differences were not at all statistically significant. The transcript level of *C/EBPα* in AP and IM resistant CP was appeared to be comparable (−0.652 vs −0.626, P = 1.00). The mean expression level of *C/EBPα* was remarkably low in BC in comparison with AP (−0.976 vs −0.652, P = 0.271) and IM resistant CP (−0.976 vs −0.626, P = 0.073), however the differences were not statistically significant [Fig. 3A & B].

Like *C/EBPα*, the expression level of C/*EBPβ* was found to be low in different phases of CML compared to HI. C/*EBPβ* expression level was significantly reduced in de novo CP (mean, −0.650; P < 0.000), AP (mean, −0.483; P < 0.000), BC (mean, −0.855; P < 0.000) and IM resistant CP (mean, −0.319; P < 0.000) compared to HI (mean, 0.327). Among the study group analyzed, lowest expression of C/*EBPβ* was observed in CML-BC. Unlike *C/EBPα*, the expression level of C/*EBPβ* was significantly lower in BC patients compared to IM resistant CP (−0.855 vs −0.319, P = 0.001). The transcript level of C/*EBPβ* was notably declined in de novo CP in comparison with AP (−0.650 vs −0.483, P = 1.00) and IM resistant CP (−0.650 vs −0.319, P = 0.136), yet the differences were not significant. Although CML-BC patients displayed a marked reduction in the expression level of C/*EBPβ* over de novo CML-CP (−0.855 vs −0.650, P = 1.00) and CML-AP patients (−0.855 vs −0.483, P = 0.138), differences were not statistically significant [Fig. 3C & D].

4. Discussion

The stereotypical march of disease progression in CML involves alterations in genomic stability and DNA repair, proliferation, differentiation, apoptosis and sensitivity to chemotherapeutic agents and therefore it may serve as a unique model of cancer evolution and progression. Thus the genetic and molecular approach to address the biology of blast crisis with a special emphasis on what genes or pathways may be the future destiny of predictive assays or therapy of progression is of profoundest important particularly in the TKI era. The molecular and genetic mechanisms responsible for the progression of CML are still unknown or not fully understood. The remarkable functional changes associated with disease progression of CML are variations in proliferation, differentiation, apoptosis and cell adhesion. All these functional changes lead to decreased treatment response in advanced phases of CML [17]. Thus it can speculate that proper identification of molecular mechanisms responsible for blastic transformation of CML will help in the elucidation of novel therapeutic agents and which will aid to alleviate therapeutic challenges associated with advanced phases of CML. With this notion, the present study is proposed to focus on the different molecular genetic alterations in various genes like *ABCB1*, *c-MYC*, *C/EBPα*, *C/EBPβ*, *BAD* and *BCL-2* involved in major candidate pathways that have been elucidated to provoke a critical role of leukemogenesis in CML including (i) drug resistance, (ii) activation of oncogenes, (iii) differentiation arrest, (iv) inactivation of tumor suppressor genes and (v) decreased apoptosis/increased proliferation.

For this, we had selected a total of 183 patients from different clinical stages of CML. Patients were selected depending on the availability and integrity of the samples for the expression analysis. Among the 183 cases, 50 patients each from de novo and IM resistant CP, 32 were in AP and remaining 51 cases were in BC phases of CML. Moreover, thirty healthy individuals (16 Males and 14 Females, M:F ratio = 1.14:1) with age ranging from 19 to 69 years were assigned as control samples. The healthy controls were selected from the same population as the study subjects.

c-MYC, an oncogenic transcription factor of the helix-loop-helix/leucine zipper protein family, was found to get deregulated in different phases of CML with marked increase in expression in patients at diagnosis and in IM non responders compared to healthy controls [7]. In agreement with this report, our study also showed that *c-MYC* was over expressed in advanced phases of CML and in IM resistant CP patients. However, in our analysis the *c-MYC* transcript level of de novo CP patients was seemed to be similar to that of healthy controls (0.141 vs 0.152, P = 1.00). Diaz-Blanco et al. [18] reported that compared to healthy bone marrow *c-MYC* mRNA was elevated in CP of CML, whereas Handa et al. [19] reported, in accordance with our results, that expression level of *c-MYC* was normal in CP CML. In the current study we observed a significant overexpression of *c-MYC* in therapy resistant CP-CML in comparison with HI (0.702 vs 0.152, P < 0.0001). Moreover, patients in IM resistant CP showed significantly a high transcript level of *c-MYC* compared to CML-AP (0.702 vs 0.344, P = 0.034) and de novo CML-CP patients (0.702 vs 0.152, P < 0.0001). Likewise, compared to HI the mean expression level of *c-MYC* was significantly higher in BC (P = 0.003). Even though AP patients had high amount of *c-MYC* mRNA compared to healthy controls, it was not statistically significant (P = 1.00). Previous studies showed that *c-MYC* was significantly elevated in CML AP/BC through trisomy 8 or gene amplification or overexpression than that in earlier phases of disease which was in agreement with our results [19,20].

Compared to CML-BC patients, the mean expression level of *c-MYC* was slightly higher in IM resistant CP (0.702 vs 0.601, P = 1.00), however the difference was not significant and it was not in agreement with previous study [7,21], where it observed that BC patients were with markedly higher *c-MYC* transcript level compared to IM non responders. Albajar et al. [7] had shown that ectopic expression of *c-MYC* in K562 cell lines resulted in aberrant DNA synthesis under IM stress and inhibit IM mediated differentiation of myeloid cells. The role of *c-MYC* in disease transformation was also supported by the reason that *c-MYC* was found to be one of the prime target gene of beta-catenin, which had shown to be elevated in BC-CML [22,23]. Similar to our findings, Albajar et al. [7] reported that compared to HI the mean transcript level of *c-MYC* was higher in patients with IM resistance and therefore their study conceived that clones with high amount of *c-MYC*

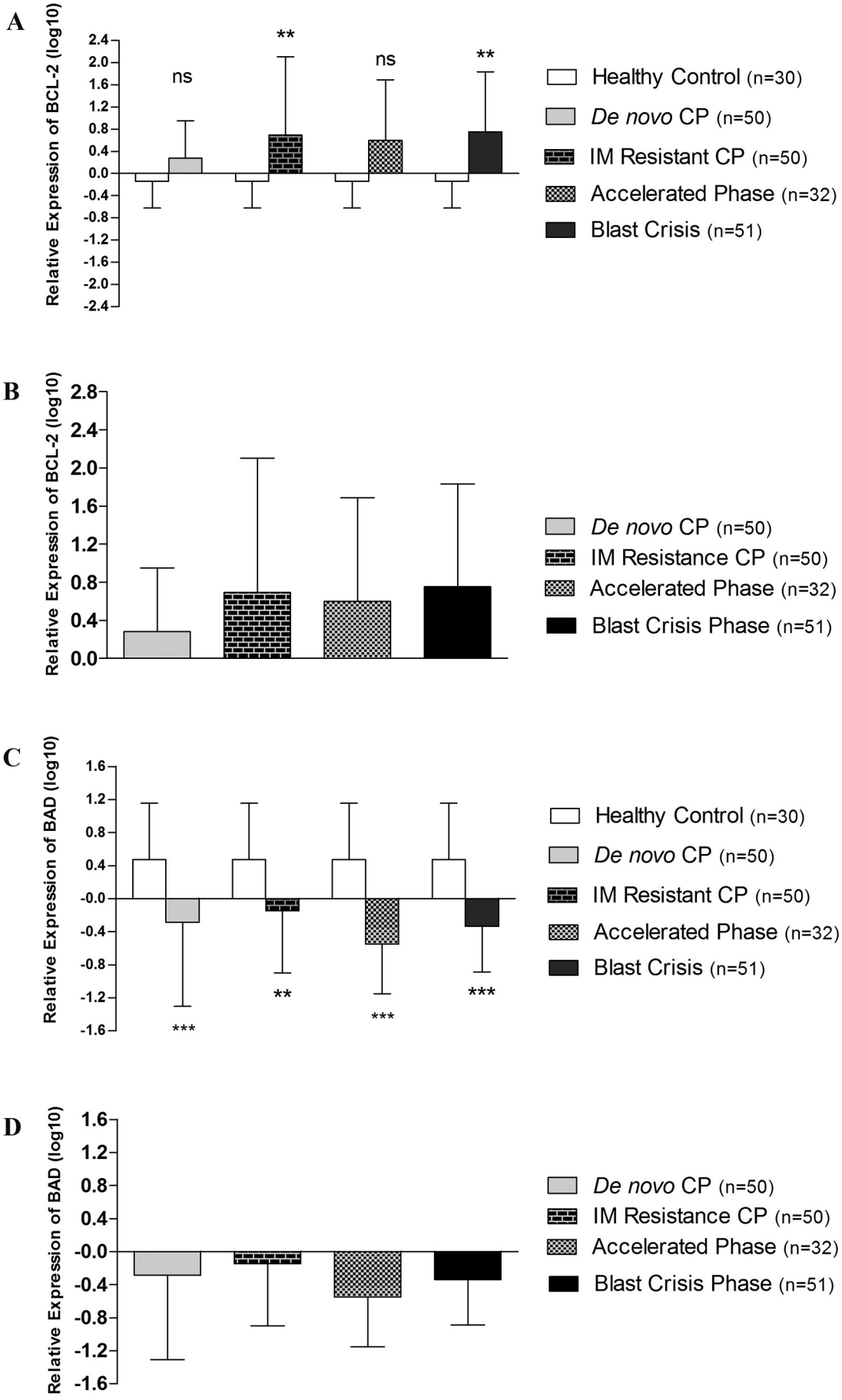

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Fig. 2. A) Comparison of the mean expression level of *BCL-2* between different study groups of CML & healthy individuals. B) The data showing the mean transcript level of *BCL-2* in different study groups of CML. C) Comparison of the mean expression level of *BAD* in different study groups of CML & healthy individuals. D) The data showing the mean transcript level of *BAD* in different study groups of CML (***; P-value < 0.001, **; P-value < 0.01, *; P value < 0.05).

were more likely to had poor response to IM treatment and they were selected for disease progression.

Correa et al. [24] and Jiang et al. [25] had shown that advanced phases of CML were more likely to show chemotherapy failure and it was mainly dependent on the overexpression of *ABCB1*, which ultimately led to multidrug resistance (MDR). In corroboration with these reports, current study revealed that *ABCB1* was overexpressed in IM resistant CP (P < 0.000) and BC (P = 0.024) of CML compared to HI. Although the transcript level of *ABCB1* was high in CML-AP in comparison with HI (0.254 vs −0.128, P = 0.251), it was not with any statistical significance. Previous studies proved that in CML-AP patients, IM therapy increased the amount of *ABCB1* positive cells with drug efflux activity [26,27,28]. However, the mean expression of *ABCB1* in de novo CP patients in our study was almost similar in comparison with HI and in both groups *ABCB1* was down regulated (mean, −0.015 vs −0.128; P = 1.00). As per previous reports, the expression of *ABCB1* was induced after a prolonged exposure with IM in IM resistant cells [29] and it was also reported that the expression levels of *ABCB1* was doubled in the BM mononuclear cells of TKI resistant patients with IM treatment [30]. These studies pinpointed the fact that expression of *ABCB1* was induced only after cells were treated with a particular chemotherapeutic drug and these might be the reason for down regulation of *ABCB1* in de novo CP and in healthy control samples.

In the current study, among the study groups analyzed, IM resistant CP patients showed the highest while de novo CP patients displayed the lowest level of *ABCB1* expression. Compared to BC and AP, IM resistant CP patients possessed an elevated amount of *ABCB1* transcript, 0.565 vs 0.344, P = 0.981 and 0.565 vs 0.254, P = 0.410 respectively, but these differences were without any statistical significance. In consistent with our results, previous studies also suggested that overexpression of *ABCB1* was seen in CML patients with intrinsic and acquired resistance to IM therapy [31,32]. These reports just opposed the findings from the previous study that patients in BC stage could only exhibit overexpression of *ABCB1* [28]. Eadie et al. [3] postulated that elevated expression of *ABCB1* resulted in declined intracellular levels of TKI by pumping out the therapeutic drugs, which lead to the creation of favorable environment for additional resistance mechanism to develop. They also proved that high fold increase in *ABCB1* transcript level was allied with blastic transformation. Altogether, these findings highlighted the fact that overexpression of *ABCB1* was preceded with development of mutations, disease evolution and IM therapy failure.

In our study, we found that anti-apoptotic gene *BCL-2* was significantly overexpressed in different clinical stages of CML compared to HI. Mean *BCL-2* expression was significantly higher in CML-BC (0.752 vs −0.141, P = 0.003) and in IM resistant CML-CP (0.693 vs −0.141, P = 0.007) compared to HI. Similarly, de novo CML-CP (mean, 0.280; P = 0.853) and CML-AP (mean, 0.598; P = 0.064) patients also possessed a high mean *BCL-2* expression in comparison with HI (mean, −0.141), but it were not statistically significant. Though the expression level of *BCL-2* was high in CML-BC compared to de novo CP (0.752 vs 0.280, P = 0.255) and CML-AP patients (0.752 vs 0.598, P = 1.00), yet the differences were not significant. Likely, the mean expression of *BCL-2* was higher in IM resistant CP patients compared to de novo CP (0.693 vs 0.280; P = 0.515) and AP patients (0.693 vs 0.598; P = 1.00), differences were not significant. In consistent with our findings, Goff et al. [33] and Quintas-Cardama et al. [34] reported that compared to healthy hematopoietic stem cells, patients with CML possessed high level of BCL-2 protein and its amount was further increased in BC-CML. Handa et al. [19], in consistent with our findings, have shown that *BCL-2* expression level was significantly higher in AP/BC phases than CP of CML. Thus it could say that *BCL-2* catalyzed the aggressiveness of myeloid leukemia by enhancing the survival of blast cells with *BCR-ABL1* and aided them to augment more and more genetic lesions [10].

It was reported that *BCL-2* had played a pivotal role in primary and acquired drug resistance and it has been also proved that the expression of *BCL-2* was significantly elevated in IM resistant CML cell lines and busulfan resistant AML cell lines [35,36]. However, it has also been proved that silencing of *BCL-2* expression reverted the activity of IM in *BCR-ABL1* positive cells [37]. Similarly, in our study the transcript level of *BCL-2* was elevated in IM resistant CP-CML. However, Ferreira et al. [38] and Ravandi et al. [11] showed that patients with IM resistance reported to have reduced levels of *BCL-2* transcript and its expression not seemed to be associated with disease progression or any bad prognostic effect in CML.

Unlikely to *BCL-2*, the current study unveiled that the mean expression level of *BAD* appeared to be low in different phases of CML. That is, a significant down regulation of *BAD* was observed in all the study groups of CML compared to HI. The transcript level of *BAD* was significantly reduced in de novo CML-CP (mean, −0.286; P < 0.000), CML-AP (mean, −0.550; P < 0.000), CML-BC (mean, −0.338; P < 0.000) and IM resistant CML-CP (mean, −0.147; P = 0.005) in comparison with HI (mean, 0.473). The expression level of *BAD* in de novo CP and BC were seemed to be comparable (−0.286 vs −0.338, P = 1.00). However, compared to therapy resistant CP, patients with CML-BC reported to have remarkably low level of *BAD* transcript, but it was not statistically significant (−0.338 vs −0.147, P = 1.00). In the same manner, patients with de novo CP exhibited reduced expression of *BAD* compared to IM resistant CP (−0.286 vs −0.147, P = 1.00). Among the study groups, patients with CML-AP showed the lowest level of *BAD* expression.

Recently, it had shown that expression of the pro apoptotic gene *BAD* was not altered and it was almost similar in IM resistant and IM sensitive cases compared to HI [38], which was not in agreement with our findings. Similar to our findings, it was reported that the pro-death factor *BAD* was transcriptionally activated in CML patients during IM therapy [39,40]. They also pointed that TKI resistance in CML was mediated by overexpression of *BCL-2* or by down regulation of *BAD*. In contrast, Kohler et al. [41] found that patients with high levels of *BAD* and *BAX* mRNA were allied with failure to achieve complete remission and adverse prognostic effect in AML patients.

Earlier reports proved that the expression level of *C/EBPα* was significantly lower in myeloid leukemia and its deregulated activity eventually resulted in the differentiation arrest of myeloid cell, which was known to be one of the prime causes of disease progression in CML [42,43]. Likewise, the mean expression level of *C/EBPα* was significantly reduced in all the study groups of CML including de novo CP (mean, −0.279; P < 0.000), AP (mean, −0.652; P < 0.000), BC (mean, −0.976; P < 0.000) and IM resistant CP (mean, −0.626; P < 0.000) of CML compared to HI (mean, 0.532). The low expression levels of *C/EBPα* were more prominent in advanced phases and therapy resistant CP of CML. Compared to de novo CP, the mean expression level of *C/EBPα* was significantly decreased in BC-CML (−0.976 vs −0.279; P < 0.000). Similarly, the transcript level of *C/EBPα* was markedly reduced in AP (−0.652 vs −0.279; P = 0.109) and IM resistant CP (−0.626 vs −0.279, P = 0.074) in comparison with de novo CP. In accordance with our results [44], recently showed that the *C/EBPα* mRNA level was significantly lower in IM resistant and in advanced phase patients compared to CP patients prior to IM treatment. The level of *C/EBPα* expression in CML-AP and IM resistant CML-CP was almost similar and comparable (−0.652 vs −0.626, P = 1.00). However, CML-BC patients showed the lowest level of *C/EBPα*

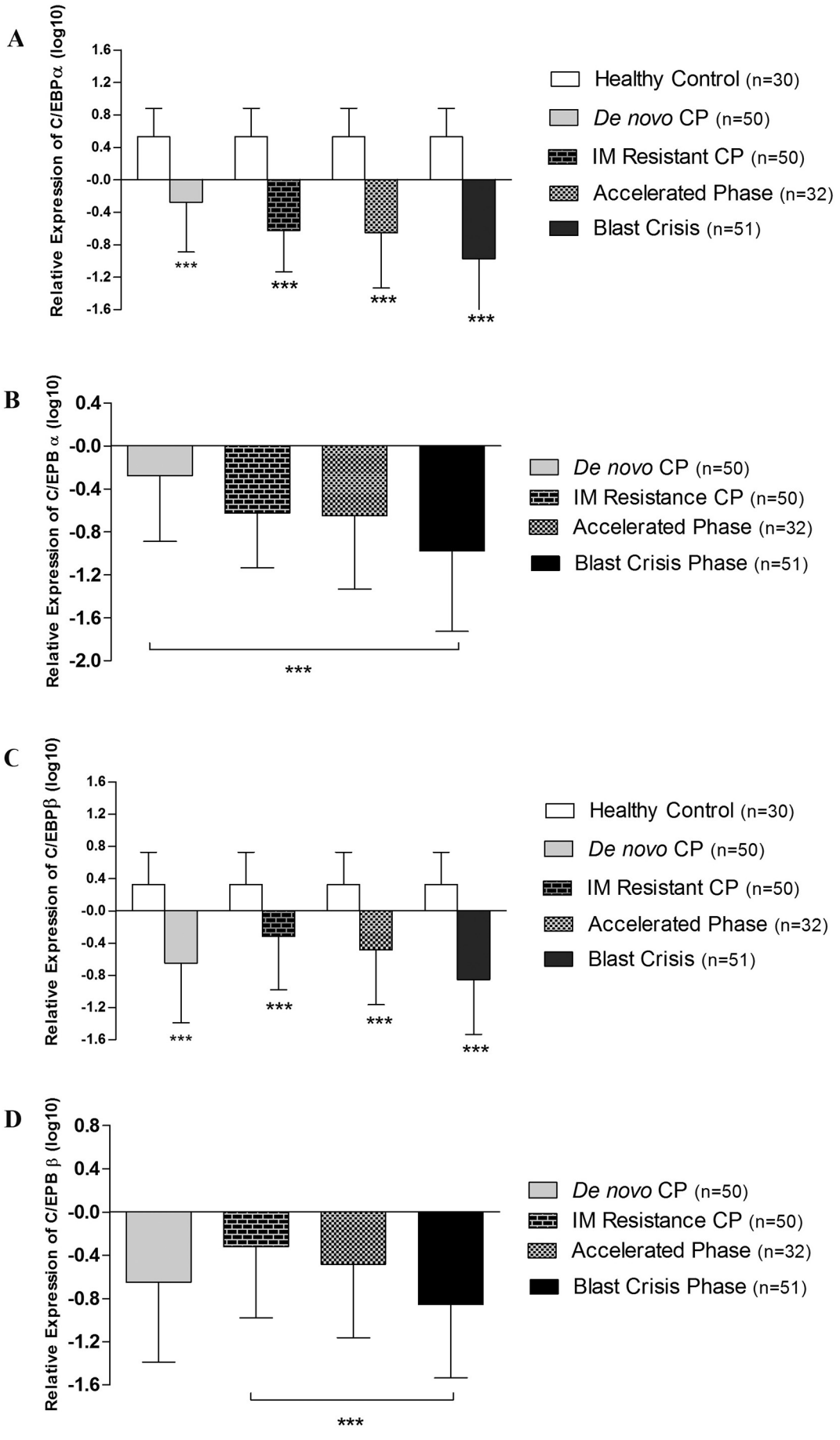

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Fig. 3. A) Comparison of the mean expression level of *C/EBPα* between different study groups of CML & healthy individuals. B) The data showing the mean transcript level of *C/EBPα* in different study groups of CML. C) Comparison of the mean expression level of *C/EBPβ* in different study groups of CML & healthy individuals. D) The data showing the mean transcript level of *C/EBPβ* in different study groups of CML (***; P-value < 0.001, **; P-value < 0.01, *; P value < 0.05).

expression with respect to CML-AP (−0.976 vs −0.652, P = 0.271) and IM resistant CP patients (−0.976 vs −0.626, P = 0.073).

Contradictory to our findings, recent study indicated that *C/EBPα* mRNA level in CP and advanced phases of CML were seemed to be similar [45]. However, in consistent with our findings, it was reported that in CML myeloid differentiation arrest due to the reduced expression of *C/EBPα* was associated with disease evolution [46,42]. Moreover, it was found out that the decreased expression of *C/EBPα* in IM resistant CP and late phases of CML were significantly correlated with high *BCR-ABL1* activity and *BCR-ABL1* extended this activity through MAPK-hnRNP-E2 pathway [46,47,48].

Like *C/EBPα*, the expression level of C/*EBPβ* was found to be low in different phases of CML compared to HI. C/*EBPβ* expression level was significantly reduced in de novo CP (mean, −0.650; P < 0.000), AP (mean, −0.483; P < 0.000), BC (mean, −0.855; P < 0.000) and IM resistant CP (mean, −0.319; P < 0.000) compared to HI (mean, 0.327). Hayashi et al. [49], in contrast to our results, reported that expression of C/*EBPβ* was up regulated in hematopoietic stem cells and myeloid progenitors of CP-CML. The lowest expression of C/*EBPβ* in our study group was observed in CML-BC. Although CML-BC patients displayed a marked reduction in the expression level of C/*EBPβ* over de novo CP (−0.855 vs −0.650, P = 1.00) and AP (−0.855 vs −0.483, P = 0.138) patients, differences were not statistically significant. Indeed, the expression level of C/*EBPβ* was significantly lower in BC patients compared to IM resistant CP (−0.855 vs −0.319, P = 0.001). In agreement with these data, Guerzoni et al. [50] explained that progression of CML towards BC was associated with down regulation of C/*EBPβ* and it might be a consequence of genetic or epigenetic changes. Moreover, in the present study, the level of C/*EBPβ* expression was notably declined in de novo CP in comparison with AP (−0.650 vs −0.483, P = 1.00) and IM resistant CP patients (−0.650 vs −0.319, P = 0.136), yet the differences were not significant.

According to Hayashi et al. [49], *BCR-ABL1* induced myeloid differentiation and proliferation was significantly diminished in *C/EBPβ* deficient bone marrow cells both in vitro and in vivo and they inferred that *C/EBPβ* was required for the *BCR-ABL1* mediated myeloid expansion. They also suggested that STAT5, one of the downstream targets of *BCR-ABL1* kinase pathway, might up regulate *C/EBPβ* expression. In contrast, Guerzoni et al. [50] and Schuster et al. [51] proved that the expression of *C/EBPβ* was low in CML-BC and its expression was inversely correlated with BCR-ABL1 levels. Furthermore, they portrayed that the ectopic expression of *C/EBPβ* in differentiation arrested murine 32D-BCR/ABL1 and K562 cell lines promoted morphologic differentiation and granulocytic lineage pathway. Taken together, like *C/EBPα*, the mean expression of *C/EBPβ* was often suppressed in blast cells of CML-BC patients, pointing the fact that loss of *C/EBPα* and *C/EBPβ* activity might contributes to differentiation arrest and aggressive behavior of CML-BC cells. Similarly, in the current study *C/EBPβ* expression was significantly low in CML-BC, where BCR-ABL1 tyrosine kinase level was high, compared to all other disease phases of CML.

From the present study, it was evident that the complex interplay of several candidate genes like overexpression of *c-MYC*, *ABCB1*, *BCL-2* and down regulation of *BAD*, *C/EBPα* and *C/EBPβ* played a significant and magnificent role in the disease evolution and development of drug resistant clone in CML. The current study pinpointed towards the possibility that during the blastic transition of CML from the benign chronic phase, the oncogenic, anti-apoptotic/proliferative and drug resistant signaling pathways might get activated with subsequent inactivation of both differentiation and pro-apoptotic pathways. In addition, the current study also highlighted the fact that TKI resistant CML-CP patients had disease biology similar to that of advanced phases of disease and hence warrants that the emergence of drug resistance in the early phase of CML could serve as an early warning system for CML progression. Together, these molecular signatures will help in the better prognostication of the patient and which will end results in the proper delineation of high and low risks groups among the CML patients. Altogether, our study recommends the necessity of a comprehensive genetic and molecular characterization of CML patients at the time of diagnosis to predict the probability of blastic transformation and drug resistance.

Conflict of interest disclosures

The authors have no conflict of interest to declare.

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