EXPRESSION OF ACTIVE BCR RELATED (ABR) GENE IN CHRONIC MYELOID LEUKEMIA (CML)

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ABSTRACT

Chronic myeloid leukemia (CML) is a clonal proliferative disorder of the haemopoietic stem cell cytogenetically characterized by the Philadelphia (Ph) chromosome, a result of chromosomal translocation t(9;22)(q34;q11). At the molecular level, the Ph chromosome results in a fusion gene, the chimerical BCR-ABL which has constitutive tyrosine kinase activity and is detected in virtually all cases at diagnosis. Indeed, the BCR-ABL gene expression has a pivotal role in the known pathogenetic mechanisms in CML cell proliferation and disease progression. Conversely, BCR-ABL inhibition with imatinib mesilate efficiently produces disease remission, since it is capable of selectively block the protein through occupying its ATP binding site. However, resistance to imatinib mesilate do occur and, although acquired mutations in the tyrosine kinase domain of BCR-ABL have been described, it seems that the appearance of acquired mutations, which result in gain of function, does not suffice for the resistant phenotype. Active BCR Related ABR gene is similar to BCR and both have a GTPase-activating protein (GAP) domain. Increased ABR activity has been detected in different solid tumors and more recently we detected over-expression of ABR in CML cDNA (EST) library. The aim of this study was to investigate the expression levels of the ABR gene in peripheral blood and/or bone marrow samples of CML patients with real-time quantitative RT-PCR in different time points at diagnosis and after starting treatment. Fourteen CML patients were included and BCR beta-actin and GAPDH genes (geometric mean; GeNorm algorithm) were used as reference. Sensitivity dynamic range and efficiency were tested for all primers. Preliminary results showed that ABR gene expression was elevated at least five fold in CML samples at diagnosis. Currently, BCR-ABL expression is being detected and quantified for establishing a correlation between ABR expression levels and treatment with imatinib mesilate in CML patients.

METHODS

Samples. A total of 14 bone marrow samples of BCR-ABL-positive CML patients were investigated at varying stages of CML. A pool of three bone marrow donors was used as control.

RNA extraction and reverse transcription. Total RNA extraction was performed using Trizol reagent (invitrogen). Samples of 5x10^6 cells were pelleted and ressuspended in 1mL of Trizol and incubated for 5 minutes at room temperature. Chilled chloroform (0.5 mL) was added to the suspension, which was incubated for 5 minutes at room temperature and centrifuged at 13,500 rpm to achieve phase separation (organic phase from aqueous phase). The aqueous phase, containing the RNA, was transferred to a new Eppendorf tube, and RNA was recovered by precipitation with isopropyl alcohol. The RNA integrities were analyzed by electrophoresis in 1.2% denaturing agarose gel and the RNA concentration was quantified using a GeneQuant UV spectrophotometer (Pharmacia). Five-microgram RNA samples were icubated with 1U DNaseI (Invitrogen) for 30 minutes at room temperature, and EDTA was added to a final concentration of 2mM to stop the reaction. The enzyme was subsequently inactivated for 10 minutes at 65°C . The DNaseI-treated RNA samples were reverse transcribed with 200 U SuperScript III (Invitrogen) for 50 minutes at 50°C and for 5 minutes at 85°C. 2 U of Rnase H (Invitrogen) were subsequently added, and the samples were incubated at 37°C for 20 minutes. The cDNA samples were quantified using a GeneQuant UV spectrophotometer (Pharmacia).

Cloning of PCR fragments. The size-selected fragments ABR, ACTB, BCR and GAPD were cloned into pUC18 PCR was performed at the conditions 94°C for 3 min, 35 cycles of 94°C for 20s, 58°C for 15s, and an extension step of 72°C for 1 min. PCR reactions with M13 specific primers were performed for confirmation of sizes of DNA fragments and the PCR products were sequenced for target sequence validation.

Sequencing Reactions. Sequencing reactions were analyzed by using the capillary sequencer Mega-BACE 1000 (Amersham Pharmacia Biotech) by using standard protocols of the ThermoSequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech). Each sequencing reaction was performed with a in a 15 µL volume containing 1.5 µL M13 PCR, 4 -µL pre-mix, 8.5 -µL sterile distilled water and 1µL 5pmol of each insert specific primer M13-F and M13-R used in two separated reactions. PCR was performed at the conditions 94°C for 3 min, and 35 cycles of 94°C for 20s, 55°C for 15s, and 60°C for 1 min.

Real-time RT-PCR (qPCR). Quantification of ABR, BCR, ACTB and GAPD gene expressions was performed on a ABI 5700. QPCR reactions were performed with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Quantification of ACTB, GAPDH and BCR expressions were used as internal controls for the amount and quality of cDNA and for an accurate normalization of qPCR data. Gene expression was determined according to the 2($^{-ÄÄCt}$) algorithm after certifying that reaction efficiencies were similar and close to 100%.Reference samples (calibrators) constituted of RNA extracted from bone marrow donors. Samples were run in duplicates and melting curve analysis were also performed at the end of each run to check for nonspecific amplification. Each sample was also run twice, in indeoendent experiments. Primers sequences are ABR-F ACACAGACAGCCCGCTTTG, ABR-R GCTGGACTCAGGCGGAAA, ACTB-F AGGCCAACCGCGAGAAG, ACTB-R ACAGCCTGGATAGCAACGTACA, BCR-F CCTTCGACGTCAATAACAAGGAT, BCR-R CCTGCGATGGCGTTCAC, GAPD-F GCACCGTCAAGGCTGAGAAC, GAPD-R CCACTTGATTTTGGAGGGATCT.

qPCR efficiency rates: ABR 98,2%; BCR 99,8%; ACTB 100% e GAPD 100%

cDNA input with high linearity (Pearson correlation coefficient r=0.99). Sensibility.

Gene-stability measure (M) and ranking of housekeeping genes. To validate the presumed stable expression of the control genes, gene-stability measures were determined using the geNorm visual basic application (VBA) for Microsoft Excel. The mean Ct values of each sample for each control gene were used to calculate the input data, termed quantities (Q), using the delta-Ct formula for transforming Ct values to relative quantities with the highest expression level set to 1: Q = E (minCt - sampleCt). Q = sample quantity relative to sample with highest expression, E = amplification efficiency (2 = 100%), minCt = lowest Ct value = Ct value of sample with highest expression. The program enables elimination of the worst-scoring housekeeping gene, the one with the highest M value, and recalculation of new M values for the remaining genes. Pairwise variation (V) was determined with the control genes as the standard deviation of the logarithmically transformed expression ratios, and the gene-stability measures (M) were defined as the average pairwise variation of a particular gene with other control genes.

Normalization factor (NF) calculation. In order to measure expression levels accurately, three internal control genes ACTB, BCR and GAPD were used for calculation of an qPCR normalization factor (NFn, n=3). The Ct values were transformed to quantities, (Q), Q = E (minCt - sampleCt). Q=sample quantity relative to sample with highest expression; E = amplification efficiency (2 = 100%); minCt = lowest Ct value = Ct value of sample with highest expression. The highest relative quantities for each gene were set to 1. These raw -not yet normalized-housekeeping gene quantities are the required data input for geNorm. NF=geometric mean of control genes quantities.

Pairwise variation (V). To determine the possible need of including more control genes for normalization, the pairwise variation Vn/n+1 was calculated between the two sequential normalization factors (NF_n e NF_{n+1}), n=2, for all samples, reflecting the effect of adding an 3**rd** gene.

RESULTS

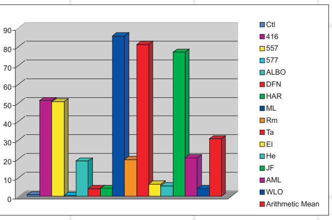


Figure 1 – ABR gene expression levels in CML bone marrow samples related to normal pool (Ctl) and normalized to the geometric mean of 3 control genes (ACTB, BCR and GAPD).

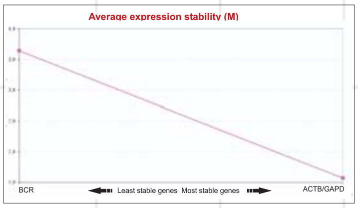
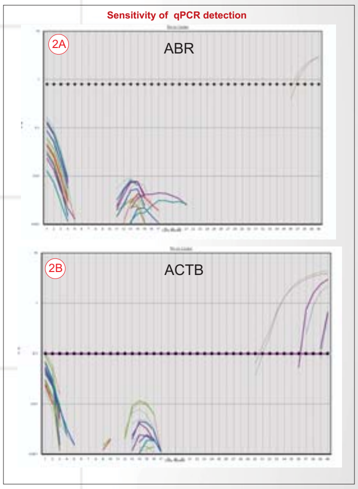


Figure 3 – Average expression stability values (M) of remaining control genes during stepwise exclusion of the least stable control gene (BCR) in bone marrow of CML patients.



Figures 2A e 2B – Amplification plot for reactions with serial dilutions of PCR product for todetermine the sensitivity of detection with qPCR.

CONCLUSIONS

- Although ABR gene expression individual levels were considerably variable (standard deviation = 31,82), the remarkable 30-fold average expression increase of ABR in CML patients correlates with our previous results (Alberto & Costa, 2003), suggesting a possible important function of ABR gene on disease evolution.
- Expression data apparently indicate that ABR gene expression has a tendency to be higher (greater than 5 fold) amongst chronic phase CML patients (10 of 11 FC patients).Whether or not ABR could be used for disease status monitoring remains to be further elucidated.
- The normalization strategy adopted in this work was helpful for accurate qPCR profiling, and is especially suitable for studying the relevance of biological signals of small intensities such as those observed in samples from patients in accelerated phase CML.

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