

# REAL-TIME PCR GENE EXPRESSION ANALYSIS TO EVALUATE ADDITIONAL MOLECULAR MARKERS IN ACUTE PROMYELOCYTIC LEUKEMIA (APL) WITH MULTIPLE RELAPSES

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## Introduction (I)

### PML-RAR Acute Promyelocytic Leukemia (APL)

Qualitative PCR play a fundamental role, both at diagnosis and during follow-up, in the identification of PML-RAR rearrangement, the molecular marker of Acute Promyelocytic Leukemia (APL or APL-M3). However, PCR result value, though of great importance, maybe limited in case of persistent positivity, because of the impossibility to associate this result to a numerical index, yielding information about the effective amount of minimal residual disease (MRD). For this reason, real-time PCR methods aiming at PML-RAR quantification have been proposed. Even though ATRA therapy revealed an effective approach in APL treatment, inducing clinical and molecular remission through leukemic cells differentiation, an ATRA resistance, leading to relapse, may develop in some patients. Different mechanisms causing ATRA-resistant phenotype have been proposed. Research conducted in murine models suggest that PML-RARa fusion protein expression alone is not sufficient to induce APL and that additional mutations are requested for leukemia development.

## Introduction (II)

### FLT3

The observation, in Acute Myeloid Leukemia patients, of mutations affecting the sequence of FLT3 gene, a class III kinase receptor involved in the hemopoiesis, has suggested that this protein may play a role in AML pathogenesis. Such alterations are often presents as internal tandem duplications (ITD) in the kinase domain and are frequently (30-39%) observed in APL patients, particularly in the variant form of disease (65-80%). Research in murine models demonstrated that the contemporary presence of PML-RAR fusion gene and of ITD mutations induces the rapid onset of APL, with complete penetrance; the two alterations may accordingly exert a cooperative action in the disease development process. ATRA and chemotherapy resistant patients more frequently exhibit ITD mutations.

## Introduction (III)

### hTERT and telomerase

Telomerase is a ribonucleoprotein complex, providing to balance the replicative loss of telomeric sequence with telomeres elongation and consisting of two main component: hTERT, the catalytic subunit with reverse-transcriptase activity, and hTERC, the RNA template one. hTERT gene expression correlates with telomerase activity, specifically associated with cancer and immortal cells and increased in some hematological neoplastic disease, as AML. It has been understood that cancer cells utilize telomerase action mechanism to gain immortality, but telomerase role in disease pathogenesis and progression is still undefined. Recent findings indicate that this ribonucleoprotein confers additional tumorigenic functions independent of its ability to maintain telomeres and is also responsible for cellular resistance to antineoplastic drug treatments. A telomerase involvement has been observed in retinoids action in APL cells, in which ATRA mediated apoptosis induction achievement occurs through a telomerase repression mechanism independent of the maturation process.

## Aim of the study

In the present study we performed real-time PCR to analyse mRNA expression variations of three different genes (PML-RARa, FLT3 and hTERT) in consecutive samples of 5 APL (Acute Promyelocytic Leukemia) patients, 4 of them with multiple relapses and 1 in clinical and molecular remission. The study focuses on the application of real-time PCR to evaluate alternative genes, other than PML-RARa, as additional prognostic molecular markers in APL patients.

## Materials and methods

Gene expression analysis was conducted on 80 total RNA samples, previously isolated from mononuclear cells routinely separated by density gradient centrifugation from bone marrow or peripheral blood. All the samples were also submitted to reverse transcription and qualitative PCR for the detection of PML-RARa rearrangements and FLT3 ITD mutation. Relative quantifications of PML-RARa (BCR1 and BCR3 isoforms), FLT3 and hTERT transcripts were performed by real-time RT-PCR using SybrGreen Master Mix (Applied Biosystems). ABL mRNA expression was used to normalize target genes expression. For each assay, amplification efficiency (98-99%) and sensitivity (10<sup>-3</sup>-10<sup>-4</sup>) were evaluated by standard curve analysis of 5 serial dilution (1-10<sup>-4</sup>) of total RNA isolated from samples with known high expression of the gene of interest. Both DDct method and REST software were applied to calculate target genes mRNA levels, expressed as number fold in relation to a calibrator sample. Specificity of each amplification assay was demonstrated by dissociation curve analysis and by agarose gel-electrophoresis of PCR products. FLT3 and hTERT expression analysis was also conducted on a control group of normal sample.

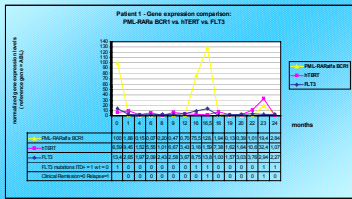
## Results (I)

### Real-time PCR assays results

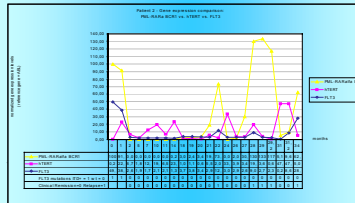
Gene	Amplification Tm (°C)	Calibrator	Efficiency (%)	Slope	Correlation	Sensitivity
PML-RARa BCR1	87	Patient basal sample	98	-3.3861	-0.9922	10 <sup>-3</sup>
PML-RARa BCR3	85	BCR3 patient basal sample*	98	-3.3823	-0.9968	10 <sup>-4</sup>
FLT3	77	Sample with FLT3 low expression	99	-3.3342	-0.9968	10 <sup>-4</sup>
hTERT	80	K562 cellular line	98	-3.3676	-0.9966	10 <sup>-4</sup>
ABL	79	target calibrator in the different assays	98	-3.3699	-0.9972	10 <sup>-4</sup>

\* PML-RARa gene expression analysis in the only patient with BCR3 isoform has been performed using a BCR3 patient basal sample as calibrator, because patient 3 was diagnosed in another Centre and came at our observation in clinical and molecular remission. For PML-RARa BCR1 and BCR3 values we expressed calibrator=100, supposing that diagnosis samples presented a basal cell population 100% PML-RARa positive.

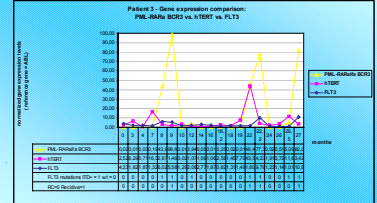
## Results (II)



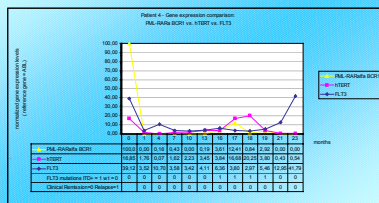
## Results (III)



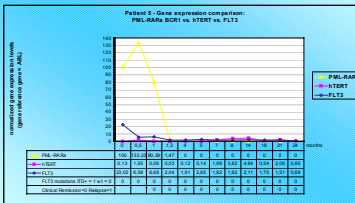
## Results (IV)



## Results (V)



## Results (VI)



## Conclusions

In all the relapsed patients (1-4): PML-RARa levels, as expected, increased at relapse; PML-RARa and FLT3 show a similar variation pattern during patients follow-up, but an increased expression of FLT3 does not always correspond to ITD reappearance; hTERT increase seems to precede or to accompany the increments of both PML-RARa and FLT3 expression.

In the patient in clinical and molecular remission (patient 5): high FLT3 levels not associated with ITD were observed at diagnosis; FLT3 levels decrease with PML-RARa reduction and remain constantly low during follow-up; hTERT levels are always low. Even if additional patients are undoubtedly required to confirm these preliminary observations, real-time PCR is a useful approach to investigate new potential molecular markers.