

Correspondence: Dr. Manuel Martínez Mancilla, Depto. de Genética y Biología Molecular, CINVESTAV-IPN. Av. Instituto Politécnico Nacional 2508 Col. San Pedro Zacatenco 07300 México, D.F. Telephone/Fax: international +52.57477000/5313. E-mail: vidal@lambda.gene.cinvestav.mx

#### Manuscript processing

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#### Significance of real-time quantitative polymerase chain reaction detection of p16 gene deletions in childhood acute lymphoblastic leukemia

We show that real-time polymerase chain reaction can detect the majority (40/55; 80%) of patients with homozygous deletion of the p16 gene as well as those with germline p16 configuration. The remaining samples (11/55; 20%) should be interpreted with caution. Patients with p16 deletion show a worse prognosis and TEL/AML1-positive children do not accumulate in any p16-defined group.

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Deletion of p16ink4A gene is one of the most frequent genetic alterations in childhood acute lymphoblastic leukemia (ALL) with a particularly high incidence in T-ALL.

Recently, an effort has been made to examine this abnormality in ALL patients by new technology, real-time quantitative polymerase chain reaction (RQ-PCR).<sup>1-3</sup> The published data concerning the usefulness of this method are to some extent contradictory. Carter *et al.*<sup>1</sup> are strongly convinced that RQ-PCR is able to distinguish not only between samples with germline and homozygously deleted p16 genes but, moreover, can reveal those patients with hemizygous deletion. The alternative view of Einsiedel *et al.*<sup>2</sup> suggests that the RQ-PCR technique is suitable only for the detection of homozygous deletion. The data published in the study by M'Soka *et al.*<sup>3</sup> also show some technical ambiguity.

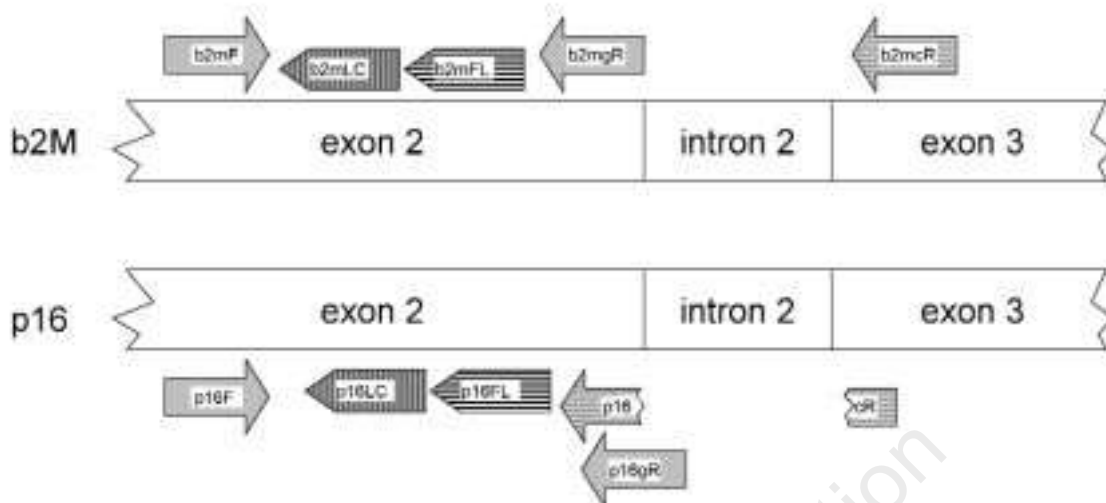
We used RQ-PCR with LightCycler™ technology to examine 56 children with ALL (54 newly diagnosed patients and 2 children at relapse) diagnosed in 1999 in the Czech Republic. The genomic DNA (gDNA) and complementary DNA (cDNA) levels of p16 gene were successfully analyzed in 55 and 54 patients, respectively. The amount and quality of gDNA and cDNA were determined using RQ-PCR amplification of  $\beta$ -2-microglobulin ( $\beta$ 2M) gene in two separate systems. Sequences of oligonucleotides used for RQ-PCR are listed in Table 1.

Examination of genomic DNA and normalized values of [gDNA p16]/[gDNA  $\beta$ 2m] ratio adjusted to the percentage of leukemic blasts at diagnosis divided patients into three groups: (1) patients with a low p16 content in leukemic blasts (<20%) due to homozygous deletion (22/55 patients; 40%); (2) patients with normal p16 content (>80%) and germline p16 (22/55 patients; 40%); (3) patients with a medium p16 content (30-65%) (11/55 patients; 20%). The distribution of p16 gDNA subgroups among patients with different immunophenotype was as follows: mature B-ALL (n=1): medium p16 content; pre-B and common ALL (n=43): low p16 content 14/43=33%, medium 9/43=21%, normal 20/43=47%; pro-B ALL (n=2): low p16 content 2/2=100%; T-ALL (n=9): low p16 content 6/9=67%, medium 1/9=11%, normal 2/9=22%.

We next analyzed the presence of TEL/AML1 and BCR/ABL fusion genes in our patients. Whereas both BCR/ABL-positive patients had normal p16 status, TEL/AML1-positive patients were distributed homogeneously within all three groups (4, 2 and 4 patients with low, medium and normal p16, respectively). Five patients from our cohort had an *event* (early death n=3; death in remission n=1; relapse n=1). All but one belonged to the p16 low gDNA group; the remaining *event* was early death of a BCR/ABL-positive patient with a normal content of genomic p16. There were only two patients examined at relapse in our original cohort and both had low p16 gDNA. This is in agreement with previously published data.<sup>4</sup>

**Table 1. The primers and probes for RQ-PCR amplification of p16 and  $\beta$ 2M genes.**

Oligonucleotide	5'_3' sequence	Application
p16-F	tggacctgctgaggagct	primer
p16-gDNA-R	tcctcaccctgaggaccttc	primer
p16-cDNA-R	tcaatcggggatgctgag	primer
p16-LC	ctgcctctgggcccccgc	probe
p16-FL	gcggcatctatgcggcatggt	probe
$\beta$ 2m-F	ccagcagagaatgaaagtc	primer
$\beta$ 2m-gDNA-R	agtgggggtgaattcagtgtag	primer
$\beta$ 2m-cDNA-R	gatgctgtacatgtctcg	primer
$\beta$ 2m-LC	atgaaccagacacatagcaattcag	probe
$\beta$ 2m-FL	ttcttcagtaagtcacttcaatgctgga	probe



**Figure 1.** The position of primers and probes for RQ-PCR amplification of  $\beta$ 2M and p16 genes. Hybridization probes were labeled with LC-Red 640 (LC) and fluorescein (FL). In both systems one forward primer ( $\beta$ 2mF, p16F) for both genomic DNA (gDNA) and complementary DNA (cDNA) amplification was used. Different reverse primers were designed to amplify gDNA and cDNA. In the  $\beta$ 2M system the cDNA primer ( $\beta$ 2mcR) was situated in exon 3, in the p16 system reverse cDNA primer (p16cR) overlapped the fusion of exons 2 and 3 to prevent possible amplification of potentially contaminating gDNA.

We also examined our patients for p16 transcript expression. The normalized cDNA levels varied considerably. Comparing the results with the gDNA analysis, there was significantly lower p16-mRNA expression within the *p16 low group* (median 109) within the *p16 normal group* (median 382). In conclusion, we found the p16 gene deletion in 67% of T-ALL patients and 36% of patients with B-cell precursor ALL. We could not statistically confirm the prognostic significance of the p16 deletion in our study due to the low number of patients but there seems to be a tendency to worse outcome in patients with p16 gene deletion. RQ-PCR is able to divide the vast majority of patients according to p16 status in the leukemic blasts (44/55; 80% in our study). Nevertheless, there remains ~20% of patients in whom the status of p16 is not absolutely clear. Imbalanced p16 to internal standard gene (e.g.  $\beta$ 2m) ratio can obscure the p16 status – and indeed 3/6 patients with available cytogenetic data in this subgroup were hyperdiploid. Technical limitations of the RQ-PCR method can also contribute to the seeming inaccuracy of the analysis.<sup>5</sup> Therefore, we believe that all data that classify patients solely on the basis of RQ-PCR p16 analysis, without any other correlation (Southern blot, fluorescent *in situ* hybridization), must be viewed with caution. Our data show that the group of patients with hemizygous deletion is unsuitable for analysis by RQ-PCR and we would strongly support the approach of Einsiedel *et al.*:<sup>2</sup> before p16 status is determined these patients should be screened by other molecular-genetic/cytogenetic methods.

Jan Zuna,\*<sup>o</sup> Katerina Muzikova,\*<sup>o</sup>  
Ondrej Hrusak,\*<sup>#</sup> Jan Sary,\*<sup>o</sup> Jan Trka\*<sup>o</sup>

\*CLIP - Childhood Leukemia Investigation Prague;  
<sup>o</sup>2<sup>nd</sup> Department of Pediatrics; <sup>#</sup>Institute of Immunology, 2<sup>nd</sup>  
Medical School, Charles University Praha, Czech Republic

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Correspondence: Jan Zuna, MD, PhD, 2<sup>nd</sup> Department of Paediatrics, 2<sup>nd</sup> Medical School, Charles University Praha, V uvalu

84, 150 06, Praha 5, Czech Republic.

Phone: international +420.2.24432280. Fax: international +420.2.24432268. E-mail: jan.zuna@lfmotol.cuni.cz

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