**Role of Minimal Residual Disease Monitoring in Acute Promyelocytic Leukemia Treated with Arsenic Trioxide in Frontline Therapy**

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**Appendices:**

*Appendix A1:*

**RNA extraction, cDNA synthesis and RT-PCR:** Total RNA was obtained from leukocytes using Blood RNA mini Kit (Qiagen). The mRNA integrity was assessed by loading 5ul of the total RNA on a 1% agarose gel and checking for the 28S and 18S rRNA quality and quantity which reflects the intactness of the underlying mRNA population (Ambion, Tech Notes) [Figure A1]. Reverse transcription was performed with superscript RT (II) kit (Invitrogen). Approximately 500ng of total RNA were added to 20uL volume containing random hexamers as primers and 50U superscript RT enzyme. The mixture was incubated at 42ºC for 50 minutes, followed by 70ºC for 10 minutes and then stored at -20ºC prior to further analysis. The quality control of the cDNA preparation was assessed by amplification of the normal Gus gene [Figure A2, Lane 1-100bp molecular weight marker, Lane 2-7 patient cDNA, Lane 8- No template control].
Qualitative Reverse Transcriptase PCR (RTPCR) assays: The PML-RARA fusion gene transcripts bcr 1, 2 and 3 types were amplified by a two-step (nested) qualitative RTPCR as per the recommendations of BIOMED-1 Concerted Action\(^1\). A volume of 2ul of cDNA was diluted into 25ul of a PCR mixture containing 400uM primers, 200uM dNTPs, PCR buffer and 0.2 U of AmpliTaq\(^\text{TM}\) polymerase. PCR cycles included an initial denaturation at 95\(^0\)C for 10 minutes. Melting, annealing and extension were carried out at 95\(^0\)C for 30 sec, 65\(^0\)C for 1 min, and 72\(^0\)C for 1 min, respectively, for a total of 35 cycles. Nested PCR was performed under same conditions, but using 0.75ul of PCR product from the first round and internal primers. Finally, 10ul of the PCR products were analyzed in a 2.5% agarose gel stained with ethidium bromide and visualized under UV light [Supplementary figure A3]. We are part of an external quality assessment program conducted by the Royal College of Pathologist Australia (RCPA Quality Assurance Program Pty Limited (Northmead, NSW, Australia)).
Figure A3. Nested RTPCR for \textit{PML-RARA} bcr 1 transcripts

[Lane 1-8 patient samples, Lane 9- positive control for bcr 1 isoform, N- No template control, M- 100 bp molecular weight ladder].

Appendix A2:

\textit{Preparation of standards for RQ-PCR}: Standards for the control gene \textit{ABL} and target genes \textit{PML-RARA} bcr 1 and 3 assays were plasmids prepared from the PCR amplicons generated by the described primer sets from patients’ samples which were known to be positive with that particular type of transcript\textsuperscript{2}. Fresh PCR products were cloned into a pCR4 vector using the TOPO-TA cloning kit according to the manufacturer’s instructions (Invitrogen), and the plasmids were purified using the QIAprep mini-prep kit (QIAGen, Valencia, CA). The identities of the purified plasmids were confirmed by sequencing (ABI BigDye kit and ABI310, Applied Biosystems). The plasmids were linearized by PstI digestion (New England Biolabs, Beverly, MA) before quantitation and used. Each plate included a series of 6 standards: 10-fold serial dilutions starting at 10\textsuperscript{5} targets per well, down to 1 target per well, for each of the target and the control assay. An example
of the amplification plots with the associated standard curve is shown in the supplementary figure A4.

**Figure A4:** bcr 1 plasmid standards A4a. Amplification plot showing 1 log serial dilutions A4b. Standard curve with -3.05 slope and 0.99 regression.

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**Appendix A3:**

**Sensitivity assay for RT-PCR and RQ-PCR:** The sensitivity assay was performed on serial 10-fold dilutions of cells from a *PML-RARA* positive cell line (NB4, Kind gift from Lanotte) in a negative cells background of normal peripheral blood mononuclear cells (obtained from volunteers with informed consent). RNA was extracted from the dilutions. The undiluted cell line RNA was called 100% because we assumed all of the cells in NB4 cell line contained the *PML-RARA* mRNA. Following this model, the 10-fold dilution was called 10%, the 100-fold was called 1%, and so on. According to our procedure, 500ng of each RNA dilution was used for the reverse-transcription reaction. The serial 10-fold dilutions spanned 8 logs, from 100% to 0.00001%. The qualitative RT-PCR assay
sensitivity was $10^{-3}$ for the first round and $10^{-4}$ for the second round. The sensitivity of RQ-PCR was $10^{-4}$ which was equivalent to the nested round of RT-PCR.

Appendix A4:

**Cytogenetic analysis:**

The karyotypes were designated according to the International System for Human Cytogenetic Nomenclature. Karyotyping was done on unstimulated bone marrow cultures using GTG banding. Images were analyzed using Ikaros software (Metasystems, Germany). At least 20 metaphases were analyzed from each case, whenever possible. An abnormal clone was defined as either $\geq$ two metaphases with the same additional chromosome(s) / the same structural abnormality or $\geq$ three metaphases with a loss of the same chromosome. Based on the karyotype, patients were allocated into two groups, namely those with an isolated $t(15;17)$ and those with $t(15;17)$ and an additional CTG abnormality.

**List of the additional cytogenetics observed**

<table>
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<th>S. No.</th>
<th>UPN</th>
<th>Additional CTG</th>
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<tbody>
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<td>1</td>
<td>21</td>
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<tr>
<td>2</td>
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<tr>
<td>5</td>
<td>33</td>
<td>46,XX,$t(15;17)(q22;q21)[9]$</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>47,XX,$+8,t(15;17)(q22;q21)[14]$</td>
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<td>7</td>
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<td>8</td>
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<td>86</td>
<td>46,XY,$t(15;17)(q22;q21),i(17)(q10)[20]$</td>
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</table>
**Results:** Of the 151 patients, karyotyping data was available in 135 cases. Of these 135 cases an isolated t(15;17) karyotype was seen in 93 (68.8%) while additional cytogenetic abnormalities were detected in 42 (31.1%) (Table A1). There was no significant difference in time to achieve CRm in both these groups. Presence of an additional cytogenetic finding was associated with a significant increase risk of relapse on a univariate analysis compared to those who did not (RR=3.58; 95% CI=1.61-7.98; P-value=0.002; figure 2F). This effect was not retained on a multivariate analysis.
Appendix A5:

**Determination of FLT3 mutation status:**

FLT3-ITD and FLT3-TKD point mutation involving codons D835/I836 were analyzed on archival genomic DNA or cDNA samples obtained at the time of diagnosis. For the detection of FLT3-ITD, the juxtamembrane domain of FLT3 was amplified using primers spanning the exonic region in order to obtain bands from both genomic DNA and cDNA, depending on availability of sample. With the exception of minor variations in the primers for amplifying the cDNA and forward primer was labeled with fluorescent dye for gene-scan analysis (forward: 5’FAM - AGC AAT TTA GGT ATG AAA GCC AGC TA -3’, reverse: 5’- CTT TCA GCA TTT TGA CGG CAA CC -3’) the rest of the procedure was based on standard established methods. The PCR product was mixed with de-ionized formamide and LIZ size standard as per the protocol, heated to 95°C for 5 minutes, and placed on ice for at least 1 minute before injecting into the ABI 3130 capillary electrophoresis (Applied Biosystems). ITD mutants yield FAM labeled PCR products that are greater in the size than the wild type.

The point mutations in the codon D835/I836 (abbreviated as FLT3-TKD mutation hereafter) was detected using a standard polymerase chain reaction (PCR) and a restriction-digestion using the EcoRV based on established protocols. The restriction digests were resolved by 3% agarose gel electrophoresis. The wild type yielded two fragments of 68 and 46bp whereas the mutated sequence remained uncut.

**Results:** Of the 151 patients, DNA/cDNA samples at diagnosis were available for 136 patients. Thirty six of these 136 patients (26.4%) had a detectable FLT3 mutation. Of these, FLT3-ITD was seen in 21 patients (15.44%) and a FLT3-TKD mutation was seen
in 16 (11.76%). One patient had both the FLT3-ITD and the FLT3-TKD mutation. For further analysis of the impact of these mutations on outcomes, patients with either a FLT3-ITD mutation or a FLT3-TKD mutation were combined and compared with the cohort that did not have a FLT3 mutation. At the end of induction there was no significant difference in the proportion that was RT-PCR positive among the FLT3 mutation positive and negative cohorts. A month later at the beginning of consolidation, post a 4 week break in therapy without any additional ATO, 11/30 FLT3 mutation positive patients (36.7%) had detectable PML-RARA transcripts by RT-PCR while 11/76 FLT3 mutation negative patients (14.5%) were RT-PCR positive at this time point (P-value=0.017). However, presence of a FLT3 mutation did not translate to an increased risk of relapse (Table 3).

Appendix A6:

Impact of age, PML-RARA isoforms, WBC and Platelet counts at diagnosis and risk group stratification on the PML-RARA clearance post initiation of induction therapy and risk of relapse:

It was noted that patient ≤ 10 years of age were significantly more likely to remain RT-PCR positive at the end of induction compared to the rest of the patients (P-value=0.018). The type of the PML-RARA isoforms (bcr 1, 2 or 3), WBC ≤ 5x10^9/Lt versus ≥ 5x10^9/Lt and risk group stratification (good risk = WBC ≤ 5x10^9/Lt and ≥ 20x10^9/Lt; versus high risk group = remaining patients) did not impact time to becoming RT-PCR negative. None of these parameters were associated with an increased risk of relapse.
**Appendix A7:**

**Kinetics of relapse:**

The graph shows the rise in median PML-RARA NCN as measured by the RQ-PCR in successive samples prior to frank hematological relapse Figure A5. The median log increase per month was 0.34(range: 0.12-1.17).

Figure A5.

**References**