The impact of PCR thermocycling conditions, DNA template concentration and inter-operator variation on determining allelic ratio in the detection of *FLT3*-ITD mutations in AML

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Abstract

The recent availability of fms like tyrosine kinase 3 (FLT3) inhibitor treatment for acute myeloid leukaemia (AML) and the updated European Leukaemia Net (ELN) recommendations have meant that accurate determination of allelic ratio (AR) for FLT3 mutations is now an important variable in determining patient risk and disease management strategy. Currently, PCR amplification of patient DNA followed by capillary electrophoresis fragment analysis is commonly used to determine AR and length of FLT3 internal tandem duplication (ITD) mutations, but this assay is not standardised. This study assessed the impact of the following variables; patient DNA template concentration, PCR cycle number and operator handling variations in determining AR values. DNA from bone marrow and peripheral blood samples known to contain FLT3-ITD mutations were amplified by PCR over a range of different cycle numbers, at different concentrations by different operators. Initial DNA concentration, inter-operator and inter-run variation showed little impact in determining AR. In five of the six samples PCR cycle number variation showed no impact in determining AR, although AR variation was observed in a sixth sample, where the patient had a much higher disease burden. No sample tested crossed the AR 0.5 boundary set by the ELN 2017 guidelines for AML risk stratification as a result of the variables tested. Consequently, data from this study suggests that current methodology is a robust method in determining AR values and AML risk stratification.

Introduction

In acute myeloid leukaemia (AML), fms like tyrosine kinase 3 (*FLT3*) mutations are present in about 25-45% of cases.[1] Historically, although allelic ratio (AR) and insertion site of internal tandem duplications (ITDs) has been shown to have prognostic effect on disease only the presence or absence of a mutation was important for disease management.

Accurate determination of AR for FLT3-ITD, as outlined in the most recent European Leukaemia Net (ELN) AML recommendations, is now an essential component in determining AML risk stratification; and for disease management with recent availability of FLT3 inhibitors.[2,3] Typically, *FLT3*-ITD mutation length and disease burden are determined by capillary electrophoresis fragment analysis following PCR amplification of patient DNA. However, this assay is not standardised between laboratories, raising concerns that differing protocols may cause variations in AR determination. This study aimed to determine whether PCR cycle number, patient DNA template concentration and inter-operator handling variations, impacted on calculated AR values.

Methods and Materials

To investigate the potential impact of PCR conditions and DNA concentration in determining AR, six bone marrow or peripheral blood samples previously obtained at diagnosis, from patients entering the National Cancer Research Institute AML trials, and containing *FLT3*-ITD mutations of varying length (Table 1) and disease burden, were identified.

Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque (BD Biosciences) and DNA extracted using the QIAcube Robot (Qiagen) as per manufacturers instructions. These were amplified by PCR as previously described,[4] over a range of PCR cycle numbers and at different DNA concentrations. The PCR product (1µl) was mixed with Genescan ROX500 size standard per the manufacturer's protocol, heated to 95°C for 2 minutes, and placed on ice for at least 1 minute before electro-kinetic injection into a 3130xl genetic analyser capillary electrophoresis instrument (Applied Biosystems) and the AR determined. AR was calculated as the ratio of the area under the curve of mutant to wild-type alleles.

To determine the inter-operator effect on AR variation, PCR was performed as previously described,[4] on the patient samples identified above. Patient DNA (1µl) was amplified multiple times under identical PCR conditions and the variation in AR determined.

Further to establish whether any inter-run variation was introduced by the 3130xl genetic analyser instrument, one PCR product per patient sample was analysed multiple times by capillary electrophoresis and the variation in AR values also determined.

Patient Sample	Length of internal tandem duplication (base pairs)
1	33
2	66
3	30
4	9
5	102
6	123

Table 1. Patient samples taken at diagnosis as part of the National Cancer Research Institute AML trials and their associated FLT3-ITD mutation lengths.

Results

Six patient samples containing FLT3-ITD mutations were amplified over 27, 31 and 35 PCR cycles and the AR for each sample determined. In five of the six samples tested the impact of PCR cycle number in determining sample AR was minor (standard deviation (SD) ≤ 0.1), however sample 3, with the highest disease burden (AR > 1.5) showed considerable fluctuation (SD = 1.19) when PCR cycle number was varied (Fig 1A). In this sample the AR was calculated at 3.83 over 27 cycles, 1.68 over 31 cycles and 1.91 over 35 cycles. The effect of DNA concentration on AR was also determined. Two concentrations of patient DNA, 10ng and 100ng as well as our standard operating protocol of 1µl patient DNA were tested. Only minor variations in AR were observed (SD ≤ 0.15) (Fig 1B) across all of the samples tested. Finally the impact on AR of operator variation and variance caused by the 3130xl genetic analyser was also assessed. When the same patient sample was analysed under the same PCR conditions by different operators, minor AR variation was seen in all six samples (SD ≤ 0.2), whilst variation in AR as a result of repeat runs on the 3130xl genetic analyser was negligible (SD ≤ 0.05). Whilst one sample tested showed AR variation when PCR cycle number was varied, no sample tested crossed the 0.5 (high vs low) AR boundary set by ELN 2017 guidelines for AML risk stratification as a result of varying any of the test conditions. Overall this data suggests that the current methodology for determining FLT3-ITD AR values and hence AML risk stratification is robust.

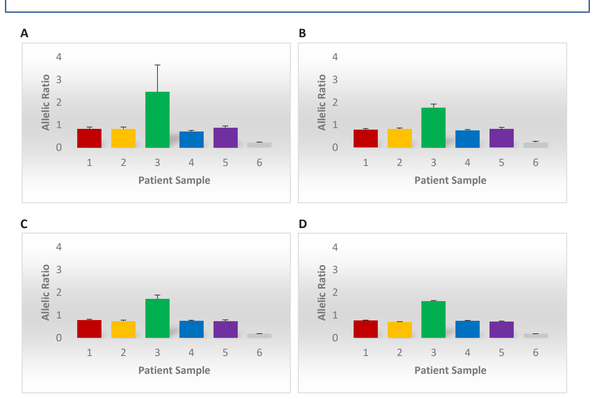

Figure 1. Effect of **(A)** PCR cycle number variation, **(B)** patient DNA template concentration, **(C)** inter-operator variation and **(D)** inter-run variation, when determining allelic ratio of FLT3-ITD mutations in AML patient samples. **(A)** 1µl DNA patient template run over 27, 31 and 35 PCR cycles. **(B)** 1µl, 10ng and 100ng DNA patient template run over 35 PCR cycles. **(C) & (D)** 1µl DNA patient template run over 35 PCR cycles. N=3. Data represents mean ±1SD.

Summary and Discussion

As per the most recent AML ELN recommendations, the accurate assessment of the AR of FLT3-ITD mutations in AML patients is now crucial in informing disease management. Whilst PCR amplification of patient DNA, followed by capillary electrophoresis fragment analysis has proven successful in determining the presence/absence of FLT3-ITD mutations, it's robustness for determining disease burden is not known. This is particularly important given that this assay is not standardised across different laboratories and variation in PCR conditions or between operators may lead to different AR determinations.

In this study the impact of a number of assay variables on AR determination were tested. We show that different initial patient DNA template concentrations, different operators and variation between repeat runs, all had a negligible impact on AR determination. Additionally, in all but one of the patient samples tested variation of PCR cycle number also had negligible impact on AR value. However, one sample (sample 3) did show considerable variation in its AR, when the PCR cycle number was varied. The reason for this variation is not known, however it seems unlikely to be attributable to its ITD base pair length as sample 1 showed no variation despite having an ITD of similar length (Table 1). It may be that the observed variation is a consequence of high disease burden, as this sample had a considerably higher AR than other samples tested. In support of this hypothesis the large discrepancy observed between the AR when 27 PCR cycles were employed (3.83 compared with 1.68 (31 cycles) and 1.91 (35 cycles)) was also observed in this sample when PCR cycle number was varied at different initial DNA starting concentrations (data not shown). However, further work will be required to fully test this hypothesis.

ELN guidelines for allelic ratio AML risk stratification set the boundary between low and high risk at an AR value of 0.5. Importantly in all samples tested, no sample crossed this boundary as a result of variation in any of the variables tested. Currently, the presence and length of *FLT3*-ITD mutations in patient samples are determined by PCR amplification followed by fragment analysis by capillary electrophoresis. Data from this study suggests that this methodology is also a robust method for determining AR values and hence AML risk stratification.

References

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Acknowledgements

The authors would like to thank the patients, clinicians and research nurses who provided samples for the NCRI AML17 trial. We would also like to thank Novartis Pharmaceuticals UK Ltd for their generous financial support for the conduct of this research project.