The Hong Kong Society of Haematology
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Title	**Acute Myeloid Leukaemia Genotyping by Nanopore Targeted Sequencing**
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Abstract

Background and Objective

Accurate and rapid genotyping of acute myeloid leukaemia (AML) is important for diagnostic, prognostic and therapeutic purposes. According to the 2022 edition of the European LeukaemiaNet (ELN) recommendations for diagnosis and management of AML, FMS-like tyrosine kinase-3 internal tandem duplication (FLT3-ITD), FLT3 tyrosine kinase domain (FLT3-TKD), IDH1, IDH2, NPM1 gene mutations should be identified within 3-5 days. Results of TP53 and other myelodysplasia-related gene mutations should be available within the first treatment cycle. Current methodologies for detecting these recurrent gene mutations in AML include polymerase chain reaction (PCR) followed by fragment size analysis with capillary electrophoresis, PCR-restriction fragment length polymorphism (RFLP) assay, Sanger sequencing and next-generation sequencing (NGS), which are tedious, time-consuming and not cost-effective. Oxford Nanopore Technologies (ONT) Nanopore sequencing is a rapidly growing technology that offers numerous advantages over traditional approaches. This study aimed to develop a Nanopore targeted sequencing assay for detecting FLT3-ITD, FLT3-TKD, IDH1, IDH2, NPM1 and TP53 gene mutations in AML cases.

Methods

A total of 67 AML blood samples from 2021 - 2023 were retrieved in the Haematology Laboratory at Tuen Mun Hospital. PCR covering FLT3 exon 14-15, FLT3 exon 20, IDH1 exon 4, IDH2 exon 4, NPM1 exon 11 and TP53 exons 2-11 was performed after DNA extraction. The library was prepared using ONT library preparation kit according to manufacturer's instructions. Sequencing was performed in the R10.4.1 flow cell on the ONT MinION device. An in-house developed bioinformatics pipeline was used for variant identification. Findings of FLT3-ITD and other gene mutations were compared with reference methodologies, fragment size analysis followed by Sanger sequencing and NGS respectively, previously performed in referral laboratories. Reported allelic ratio of FLT-ITD from referral laboratories was converted to variant allele frequency (VAF) for statistical comparison.

Results

The result concordance rate between Nanopore sequencing and reference methodologies was 100%, with a coefficient of correlation ≥ 0.7 and a bias of -8.4%. It was noted that the negative bias was mainly due to the underestimation of VAF in FLT3-ITD. After excluding the FLT3-ITD data, high correlation ($R \geq 0.9$) and minimal bias (-0.11%) were observed. Nanopore sequencing achieved a 100% concordance rate of repeatability and reproducibility analysed by running known positive and negative samples in triplicate within a single run and in three different days respectively in terms of positivity / negativity calls for each sample. A commercial DNA reference standard with known VAFs of different variants was used to validate the limit of detection of the assay. All defined variants were detected by Nanopore sequencing between 4% to 6% of VAFs, which were consistent with the stated VAFs of the reference standard (approximately 5% by NGS).

Conclusion

This study serves as a proof that the use of Nanopore sequencing is an alternative approach to the traditional methods for AML genotyping. However, fragment size analysis remains an indispensable tool for FLT3-ITD detection with accurate determination of VAF. On the whole, Nanopore sequencing is capable of generating accurate and reproducible results with acceptable sensitivity in a rapid and all-in-one fashion and hence helps to provide molecular information with diagnostic, prognostic and therapeutic values in AML patients.