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REVIEW ARTICLE

Applications of Next Generation Sequencing in Haematological Disorders—Indian Status: Updates from ISHBT 2018

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Abstract Research in India based on next generation sequencing (NGS) has been plentiful over the past few years. Significant progress in research both in benign and malignant haematology can be attributed to this technique. It has now progressed to both diagnostic and a theranostic modality in many areas of not only haematology but also other medical specialities. Papers presented at the annual conference of the Indian Society of Haematology and Blood Transfusion, ISHBT (Haematocon 2018) highlighted a snapshot of the research activities using NGS that are ongoing in various academic and other centres. This review summarizes the salient findings of the original research abstracts presented. The papers are divided into two broad subsections of non-malignant and malignant haematology.

Keywords Next Generation Sequencing (NGS) · Targeted sequencing · Benign haematology · Malignant haematology

Introduction

Demand has never been greater for revolutionary technologies that deliver fast, inexpensive and accurate genome information. This has led to a revolution in sequencing technologies resulting in a better understanding of genetics and genome biology. Next generation sequencing (NGS) allows for a broad analysis of a genome by whole-genome sequencing (WGS), exome sequencing, transcriptome sequencing, and epigenomics. There is a great potential for NGS in clinical application, including its use for the efficient detection of either inherited or somatic mutations in cancer genes. Translation of this knowledge to bed-side clinical utility by improving diagnostics, prognosis, monitoring of minimal residual disease, and the identification of new targets for therapy is the goal. However, a significant amount of research needs to be undertaken before these discoveries can fully integrate into clinical medicine and help clinicians with their management of patients.

Research in India has also shifted to this technique since a few years now, with a few centres offering it as a diagnostic modality in both benign and malignant haematologic disorders. Papers and posters presented at the annual national conferences of the ISHBT (Haematocon 2018) focus on the ongoing contemporary research activities in various academic and other centres. This review summarizes the salient findings of the original research abstracts presented.

Methodology

The abstracts presented in the Haematocon 2018 (Kochi) on Next generation sequencing (NGS) technology and its application in haematology were reviewed. This year there were a total of 21 abstracts of which 11 were for non-malignant and 10 in malignant haematology. All these abstracts highlight the use of NGS technology in the molecular diagnosis of haematological diseases.

NGS in Non-malignant Conditions

A spectrum of benign haematological diseases diagnosed by NGS ranging from various red cell disorders, bleeding disorders and primary immune deficiency disorders were presented in this conference. Various approaches such as

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whole exome, targeted panel approach and a novel integrated approach such as long amplicon based NGS (LAP-NGS) were used.

Congenital Haemolytic Anaemias

Hereditary haemolytic anaemia encompasses genetically, and phenotypically heterogeneous disorders characterized by increased red cell destruction. Diagnosis by routine morphology and biochemical analysis may be difficult, particularly in transfusion-dependent children. Causal genes implicated in congenital anaemias are numerous, making a gene-by-gene approach time consuming, expensive and labour intensive. Use of targeted sequencing can expedite the molecular diagnosis where the cause for haemolysis remains unexplained after routine laboratory tests. There were 3 studies on the diagnosis of red cell disorders resulting in haemolytic anaemia by various approaches of NGS.

Jamwal et al. [1] used the targeted gene panel approach to identify variants causing haemolytic anaemia (HA)/congenital dyserythropoietic anaemia (CDA). Patients with clinical and laboratory evidence suggestive of HA/CDA were enrolled after excluding common causes by various tests. Streamlined testing with a customized panel of 55 genes was then performed on 49 patients. Enzymopathies were identified in 17 cases and membrane disorders were identified in 6 cases. Of the 17 cases clinically suspected to be CDA, only 7 cases had disease causing mutations (*SEC23B*-6 cases & *GATA1*—1 case). Six out of seventeen cases had mutations resulting in PK deficiency and membrane defects (*MTRR*, *SPTB* and *PIEZO1*) instead of CDA associated genes. This study demonstrates high diagnostic utility of NGS for molecular diagnosis of unexplained anaemias with an overall $\sim$ 70% of cases being diagnosed confidently.

More et al. [2] also used the targeted gene panel approach of NGS in identifying disease causing mutations in unexplained haemolytic anaemias/dyserythropoietic anaemia. Variants in *Piezo1* gene causing rare haemolytic anaemia such as hereditary xerocytosis (HX) could be identified in five patients of unexplained haemolytic anaemia. Of the 6 variants identified, four were novel.

Jajodia et al. [3] introduced a novel, high-throughput and sensitive long amplicon-PCR Next-Generation Sequencing (LAP-NGS) method to characterize the spectrum of red cell membrane and enzyme disorders. Disease-causing mutations were identified in all the 10 patients studied, of which 2 mutations were novel. Among the five patients presenting with unexplained haemolytic anaemia, 3 cases showed mutations in the genes causing RBC membranopathies (*ANK1* and *SPTB*) and 2 patients showed mutations in the gene associated with pyruvate kinase deficiency. This technique was validated by sanger sequencing. This study showed that LAP-NGS has an advantage of ultra-deep sequencing of the amplicons allowing efficient variant identification, reduces the sequencing costs and turnaround time compared to exome sequencing. These studies show that the hitherto diagnostically unapproachable group of inherited haemolytic anaemias can now be detected with cost effective robust molecular methods.

Thalassaemias and Haemoglobinopathies

Thalassaemias and other haemoglobinopathies are mainly caused by mutations in α (*HBA1* and *HBA2*) and β (*HBB*) globin genes. Co-inheritance of various polymorphisms in the globin genes like *HBG1* and *HBG2* can modulate the phenotype of homozygous β thalassaemia or sickle cell disease.

Senthil Kumar et al. [4] used a LAP-NGS method targeting the β, δ, α and γ globin genes to identify the mutations/rare genetic variants or polymorphisms that can modulate the clinical phenotypes of homozygous β-thalassaemia or sickle cell disease. A total of 15 pathogenic variants were identified in heterozygous or homozygous states in the 24 samples analysed in this study. Apart from identifying all the *HBB* mutations detected by Sanger sequencing in the 10 patients, this method detected 3 mutations in *HBA1* and 2 mutations in *HBA2* and a few missense variants in *HBD, HBG1* and *HBG2* genes like *HBG1*:c.410C > G (rs56205611), *HBG1*:c.227C > T (rs1061234)and *HBD*:c.61G > A (rs369305779) along with intronic and untranslated region (UTR) variants like *HBD*:c.93-16G > A (rs539587172), *HBD*:c.-118C > T (rs549964658) and *HBG1*:c.-158C > T (*Xmn1* polymorphism).

The presence of coexisting variants in beta thalassaemia/sickle cell anaemia cases can modify the phenotype of the patient and studying all the associated alpha and beta globin cluster genes simultaneously by NGS helps to this end.

Inherited Bone Marrow Failure Syndromes (IBFMS)

Fanconi anaemia (FA) is the most common bone marrow failure with an increased risk of malignancies. Joshi et al. [5] presented a comprehensive analysis in 76 patients with Fanconi anaemia by exome sequencing. This study aimed to identify disease modifiers effectively by correlating genotype-phenotype. Seventy-five out of 76 cases sent for exome sequencing showed disease-causing mutations in various genes associated with FA pathway. The frequency of the patients with mutations in *FANCA* gene was

relatively lower (52.6%), compared to previous reports. Also, a common mutation in *FANCL* gene was identified in 18.4% of the patients and *FANCG, FANCC* mutations were identified in 11.8% and 3.8% respectively.

Although FANCD2 ubiquitination is a critical event in ICL repair which is defective in $\sim$ 95% of FA cases, and can be identified by western blotting, exome sequencing can identify disease-causing mutations and various other disease modifiers implied in the pathogenesis of fanconi anaemia.

Bleeding Disorders

Three papers described the role of NGS in the diagnosis of inherited bleeding disorders including rare platelet disorders. Maddali et al. [6] compared the conventional Sanger sequencing method with a novel integrated approach of LAP-NGS in the diagnosis of Haemophilia A & B in terms of cost effectiveness and turn-around time. DNA from 10 patients with haemophilia A, who were negative for *F8* intron 22 and intron 1 inversions, and 5 patients with haemophilia B (*F9*) were included in this study. There was 100% concordance in the results obtained from Sanger Sequencing and the LAP-NGS. The type of mutations included missense and frameshift mutations caused by small deletions and duplications. LAP-NGS was found to be highly reproducible and was superior to Sanger sequencing, in terms of faster turnaround time and considerably lower cost.

Shanbag et al. [7] highlighted the utility of NGS in the diagnosis of unexplained bleeding disorders, where they reported mutations resulting in Glanzmann's thrombasthenia (GT) by clinical exome sequencing in two cases. The first case was type III or variant GT due to a compound heterozygous mutation in exon 4 of *ITGA2B* in a 7-year-old boy with easy bruisability. In the second case, a 13-year-old with recurrent gum bleed and ecchymotic patches was diagnosed to have a coexisting mild FVII deficiency with GT. NGS study attributed the mild phenotype of FVII deficiency in this case to the presence of three polymorphisms in *F7* gene.

Kawankar et al. [8] using clinical exome sequencing detected two novel mutations in *RASGRP2* gene associated with platelet function disorder. Homozygous mutations in exon 13 (c.1433delA (p.Glu478Glyfs*41) and in exon 8 (c.782C > T (p.Thr261Ile) were identified in two patients with unexplained bleeding.

These reports conclude that atypical platelet disorders are difficult to diagnose, especially with milder bleeding phenotype and the best strategy to adapt in such cases is to perform clinical exome sequencing to understand the underlying molecular pathology leading to platelet function disorders.

Primary Immune Deficiencies (PID)

Primary immune deficiency diseases (PID) comprise a genetically heterogeneous group of disorders that currently includes 354 distinct disorders with 344 different gene defects (2017 IUIS classification). Clinical diagnosis is often difficult due to overlapping symptoms and laboratory findings in different PIDs.

Neelagandan et al. [9] analyzed the spectrum of mutations causing PID and created a stepwise approach to the molecular diagnosis of PID. A total of 161 patients and parents were evaluated for PID. Sanger sequencing for specific gene targets (familial Hemophagocytic Lymphohistiocytosis: *PRF1, STX11, UNC13D*, Hyper-IgE syndrome: *STAT3*, Severe Combined immunodeficiency: T-B+NK+ - *IL12RG* or T-B-NK+ - *RAG1/RAG2* or X linked agammaglobulinemia: BTK and Chronic Granulomatous Disease: CYBB) was done in 81 patients and mutations were identified in 27 of them. In 37 of the remaining patients who were mutation negative by Sanger sequencing, a customized NGS panel was used to identify mutations. Twenty-nine patients had mutations in genes which could be attributed to the pathogenesis of PID like *CD40LG, TBX1, DOCK2, TNFRSF11A, FTR*, to name a few.

Arun et al. [10] screened patients with a clinical diagnosis of congenital neutropenia with no *ELANE* gene mutations by sanger sequencing, using a custom NGS panel. In four among the ten patients studied, disease causing variants were identified in genes that have been associated with CN including *SBDS, WAS, GATA2* and *JAGN1*. Three other patients had mutations in genes like *CHD7, PAX1, FCGR3A* and *TNX1*, which are associated with immunodeficiency, although their role in causing in congenital neutropenia is unclear. Variants identified in the remaining 3 cases, however, could not be attributed to congenital neutropenia. In conclusion, diagnosis of PIDs which has a variable phenotypic spectrum is best possible by targeted gene sequencing.

Role of NGS in Malignant Haematology

Acute Leukaemia

Serial monitoring of *NPM1* mutations from the blood or bone marrow using mutation specific assays during chemotherapy of AML with mutated *NPM1* (NPM1mut AML) has been shown to be highly predictive of relapse. Bhanshe et al. [11] compared ultradeep NGS with flow cytometry (FCM-MRD) in MRD monitoring of *NPM1* mutated AML cases. A total of 137 samples obtained from 83 patients at the end of induction (PI) and consolidation (PC) were included. Exon 12 of *NPM1* was amplified and

sequenced by NGS and simultaneous FCM MRD was performed. The authors report that detection and monitoring 12 different types of *NPM1* mutations at a sensitivity of 0.001% was possible. This study suggested that NPM1 NGS MRD is a highly useful test for prediction of relapse and survival in *NPM1*mut AML.

The presence of *CEBPA* gene mutation is an important prognostication factor in AML. Naeem et al. [12] developed an in-house error corrected sequencing protocol to sequence the *CEBPA* gene. In 24 AML samples analyzed by both capillary electrophoresis and error corrected NGS methods, NGS could detect mutations in all 24 cases while capillary sequencing did not detect mutations in 2 cases. This assay could also detect single nucleotide variants as low as 1.95% and indels as low as 2.3%.

Conventional techniques such as FISH are not suitable as diagnostic strategy as they can neither consistently identify both partners involved in a gene fusion nor can they identify the exact gene sequences for disease monitoring. The role of RNA sequencing in the identification of unknown fusion genes was presented in three different studies.

Ghai et al. [13] presented an inexpensive laboratory developed targeted RNA sequencing for the detection of unknown fusions where only one of the fusion partners is known. Two AML cases confirmed by morphology and flowcytometry showed trisomy 8 and 3 copies of BCR allele/trisomy 22 in one patient and no abnormalities in other by FISH. A 34-gene myeloid panel was designed to detect any presumed partner of a fusion gene where one of the partners is known. Targeted RNA sequencing in both cases revealed a *NUP98-NSD1* fusion, product of the t(5;11) (q35;p15.5). Targeted DNA based assay detected *FLT3-ITD* in both cases and additional missense mutation (*NRAS* in one and *RUNX1* in the other).

Second study by Shaikh et al. [14] identified *BCR-JAK2* fusion with breakpoint at exon1 of *BCR* and exon19 of *JAK2* in a case of *BCR-ABL1* like BCP-ALL and a third presentation by Soni et al. [15] reported the identified *ETV6-ABL1* fusion with breakpoints at exon 5 in *ETV6* and exon 2 in *ABL1* in a case of Myeloid/Lymphoid neoplasm associated with eosinophilia.

Long noncoding RNA (lncRNA) have been recognized to play a major role in cancers. However, the role of lncRNAs in T-ALL is poorly understood. Chopra et al. [16], presented a profile of lncRNAs differentially expressed in immature T-ALL in a study that included a total of 36 T-ALL cases. A total of 1574 lncRNAs were found in T-ALL, most of which were previously unreported. Although the study concluded that this can serve as basis for the discovery of biomarkers and novel targets for immature T-ALL patients, functional studies should be carried out to understand the biological significance of these lncRNAs in T-ALL leukemogenesis.

Tyrosine Kinase Domain (TKD) Mutations in Chronic Myeloid Leukaemia (CML)

The current gold standard method for *BCR-ABL1* kinase domain mutation detection is Sanger sequencing (SS), which has analytical sensitivity limit of 10–20%. NGS is a promising tool that could identify mutations below the lower detection limits of SS. Benjamin et al. [17] investigated whether NGS could detect mutations in *BCR-ABL1* KD in mutation negative CML patients with suboptimal response to Imatinib. They reported 22/54 patients with KD mutations by NGS. Apart from identifying variants which were previously detected by SS, NGS additionally found 5 other reported mutations and 3 novel mutations in 4/54 patients and low-level mutations in 14/54 mutation negative Imatinib suboptimal responders in their cohort.

Ultra-deep error corrected NGS assay in the detection of *BCR-ABL1* KD mutations by Tripthi et al. [18] detected 50% additional mutations as compared to SS. Ten among the 22 cases with KD mutations detected by NGS had a VAF (Median VAF 1.23%, range 0.41–8.41) below detection limit of SS. Further, a higher coverage (X5000) identified 4 novel variants. This was validated in a cohort of 32 cases of CML with suspected imatinib resistance, of which 8 cases showed presence of mutations including 4 cases with novel mutations at VAF ranging from 2.187 to 5.38 (Median VAF 3.77%). A disadvantage of NGS assay is the inability to distinguish sequencing errors from true mutations especially at lower variant allele frequencies (VAF; $< 5\%$ VAF). This was overcome by using unique molecular identifiers (UMID) to libraries. However, its clinical relevance needs to be determined.

Mutation Profile in Triple Negative Myeloproliferative Neoplasms (TN-MPN)

Maddali et al. [19] used targeted sequencing approach in identifying possible driver mutations and other epigenetic mutations in triple negative myeloproliferative neoplasms (TN-MPN). A total of 21 variants, both reported and variants of undermined significance (VUS) were identified in 12 genes (*SETBP1, PDGFRA, BCORL1, NOTCH1, JAK3, ABL1, TET2, ASXL1, FLT3, PTEN, RUNX1* and *CUX1*) in 13 of the 14 patients evaluated. Most of these variants were found to be deleterious according to the prediction tools. However, the individual role of some of these variants in disease pathogenesis is to be determined by further studies. This study highlights the importance of NGS in identifying additional potential targets for therapy

by studying mutations in the genes involved in both driver and epigenetic pathways in triple negative MPN.

Conclusion

The papers presented in this annual conference of the Indian Society of Haematology and Blood Transfusion 2018 (Kochi) on the role of NGS in various areas of haematology reflect the rapid scientific advances in both research and diagnosis of haematological disorders today. Various approaches of NGS such as targeted panels, amplicon-based panels show that this technique is far more affordable than whole exome/genome NGS, making way for efficient diagnosis in certain inherited haematological disorders where classical methods are not only expensive but also laborious.

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to disclose.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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