A Single Institution Experience Using Next Generation Sequencing (NGS) for Analysis of Immunoglobulin Heavy Chain Variable Region (IGVH) Somatic Hypermutation in Chronic Lymphocytic Leukemia

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Introduction

Somatic hypermutation (SHM) status of the immunoglobulin heavy chain variable region (IGVH) gene is an important prognostic marker in B-cell chronic lymphocytic leukemia (CLL). SHM, defined as a mutation load ≥2% (compared to germ line sequence), is associated with a better prognosis, whereas an unmutated status (mutation <2%) is associated with adverse prognosis. SHM status is commonly detected by PCR amplification of the monoclonal CLL IGVH-JH region followed by Sanger sequencing, but this process is relatively labor intensive. Recently, Next Generation Sequencing (NGS) technology has been used to detect clonal IGVH rearrangements and to determine SHM status in CLL patients. NGS represents a significant improvement over Sanger sequencing by simultaneously detecting and characterizing IGVH rearrangements using a single multiplex PCR library, thereby allowing direct identification of the SHM status in CLL patients. This study presents our experience using NGS for the detection of IGVH SHM status in CLL.

Materials and Methods

Patient Samples and RNA Preparation

Peripheral blood and bone marrow aspirate samples with at least 5% clonal B cells were obtained and RNA was isolated using the ZR Whole-Blood RNA MiniPrep kit (Zymo Research Corp., Irvine, CA) for peripheral blood and miRNeasy Mini Kit (Qiagen USA, Valencia, CA) for bone marrow samples. Reverse transcription was performed using the Multiscribe Reverse Transcriptase and Random primer cDNA synthesis kit (Life Technologies, Grand Island, NY).

Sanger Sequencing

Leader PCR was performed with 7 individual PCR primer sets for each patient (7 consensus V-family primers and JH primer). A rapid capillary gel electrophoresis step was used to assess the quality and presence of clonal bands using the QIAxcel DNA High Resolution Kit (Qiagen USA, Valencia, CA). If a distinct clonal leader band was not present, FR1 PCR was then pursued using 6 individual PCR primer sets (6 consensus V-family primers and 1 JH primer) and assessed for the presence of a clonal band, following which the positive PCR product was treated with exoSAP-IT (Affymetrix, Santa Clara, CA) to remove excess nucleotides and primers. The purified PCR product was sequenced using the BigDye Terminator kit version 1.1 (Life Technologies, Carlsbad, CA) per manufacturer's instructions. Sequencing reactions were purified using EdgeBio Performa DTR Ultra 96 well plates (EdgeBio, Gaithersburg, MD) and analyzed by capillary electrophoresis on the ABI 3130xL (Applied Biosystems, Foster City, CA).

Materials and Methods

Sequences were analyzed using Sequencher software version 5.0 (Gene Codes Corporation, Ann Arbor, MI) and the consensus contig sequence was analyzed using IMGT/V-QUEST software (IMGT, Montpellier, France).

Next Generation Sequencing

Multiplex leader PCR was performed including adapter ligation and sample indexing using the LymphoTrack IGHV Leader Somatic Hypermutation Assay Panel for Miseq (Invivoscribe, San Diego, CA). A check gel was run to assess the quality and presence of clonal PCR product using the QIAxcel DNA High Resolution Kit. If no band or an indistinct band was present, FR1 multiplex PCR was performed using the LymphoTrack IGH FR1 Panel for Miseq (Invivoscribe, San Diego, CA). PCR product libraries were purified using the Agencourt AMPure XP Purification kit (Beckman Coulter, Brea, CA) and subjected to next generation sequencing (NGS) on the MiSeq platform (Illumina, San Diego, CA), after normalization and sample pooling. NGS generated data was evaluated for sequence quality and converted to FASTQC files. After de-multiplexing, individual patient samples were analyzed and graphically displayed using the Invivoscribe LymphoTrack IGHV SHM Software for MiSeq (Invivoscribe, San Diego, CA). A minimum total read (coverage) depth of 100,000X and a clonal IGVH fraction of at least 10% was required to adequately evaluate clonal rearrangements for SHM status. Recurrent patterns of clonal gene rearrangements and potential artifacts were determined. In some cases, additional assessment using IMGT/V-QUEST was performed.

Results

- 12 normal donor samples and 46 CLL samples with known IGVH status (by Sanger sequencing) were evaluated using the NGS methodology
  - All normal samples were negative for clonal gene rearrangements
  - 3/46 CLL samples were excluded from the study due to Sanger sequencing or NGS failure.
  - Concordance with Sanger method was observed in 41/43 of the samples (95.3%) using NGS.
  - Additional findings: a) Different VH gene designation between Sanger and NGS informatics; b) Different SHM status between NGS and Sanger methods; c) Occasional biclonal rearrangements variably detected between platforms
- Patterns of artifact and intraclonal heterogeneity were identified
- Successful PCR and NGS required a clonal B-cell population of at least 5%

Results

Figure 1. Representative data from a productive mutated NGS sample

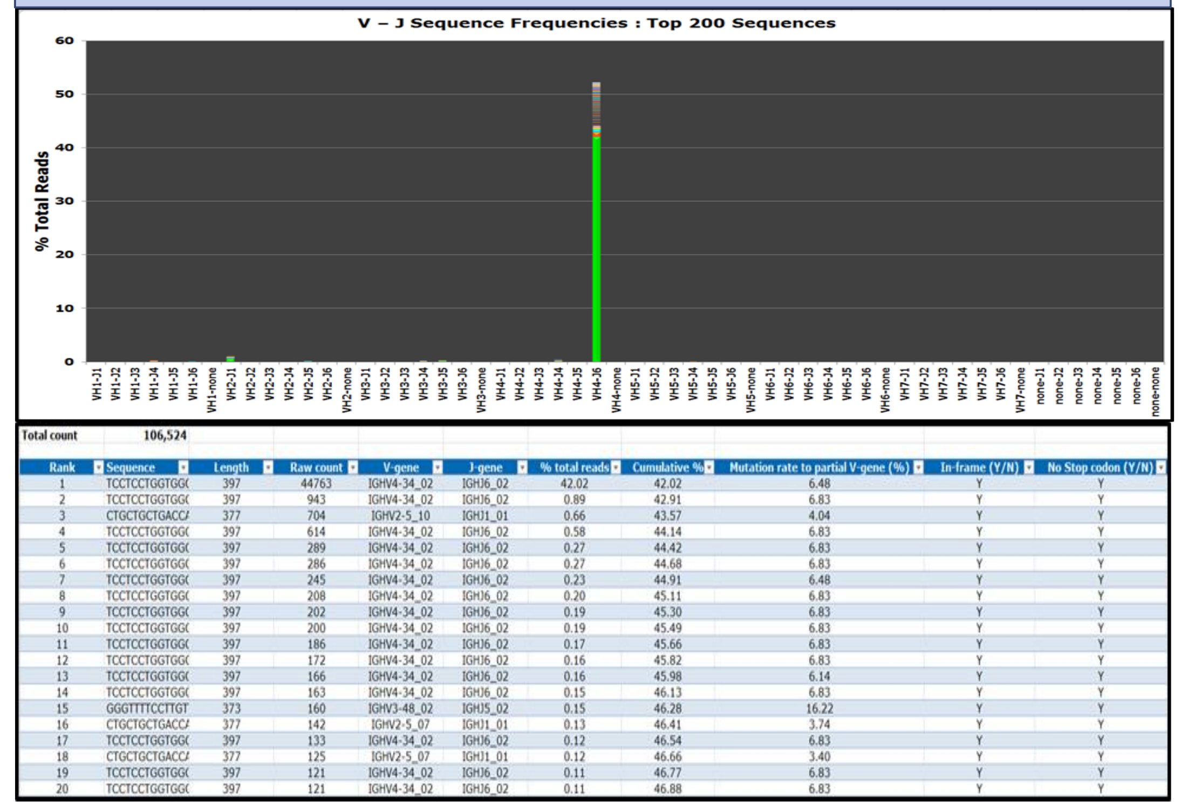


Figure 2. Representative data from a polyclonal NGS sample

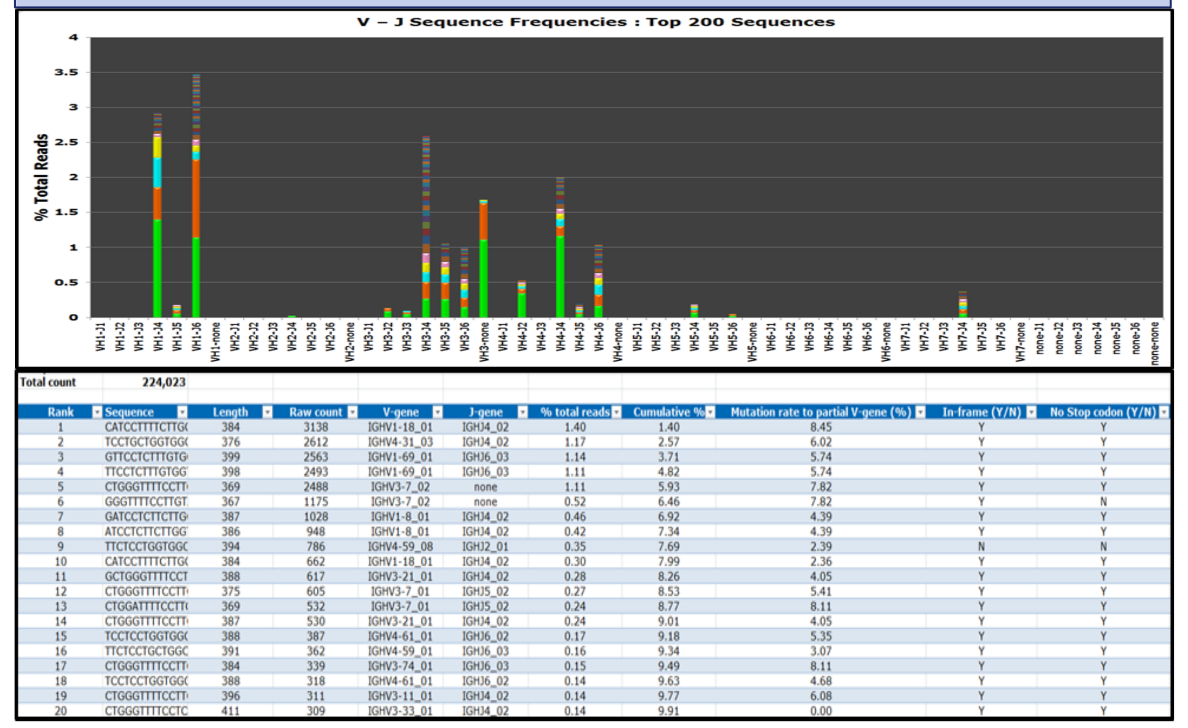
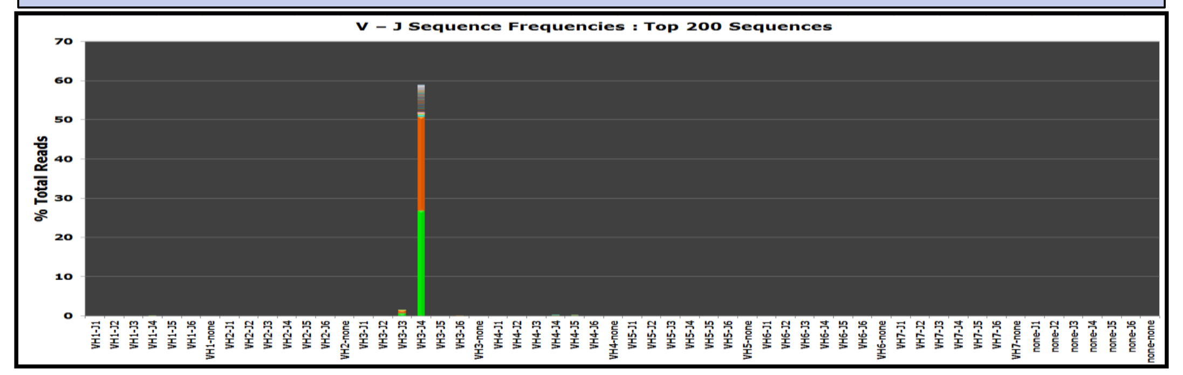


Figure 3. Representative data from an unproductive unmutated Sanger sample



Results

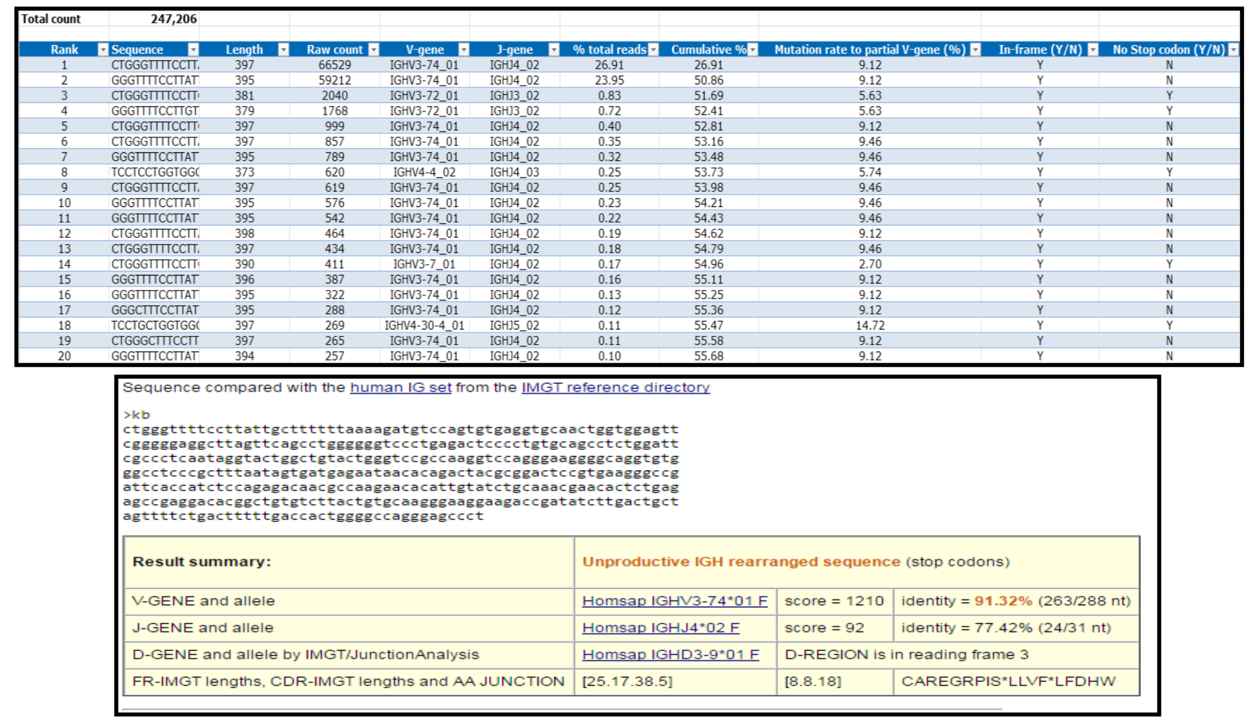


Figure 4. Representative data from a biclonal NGS sample

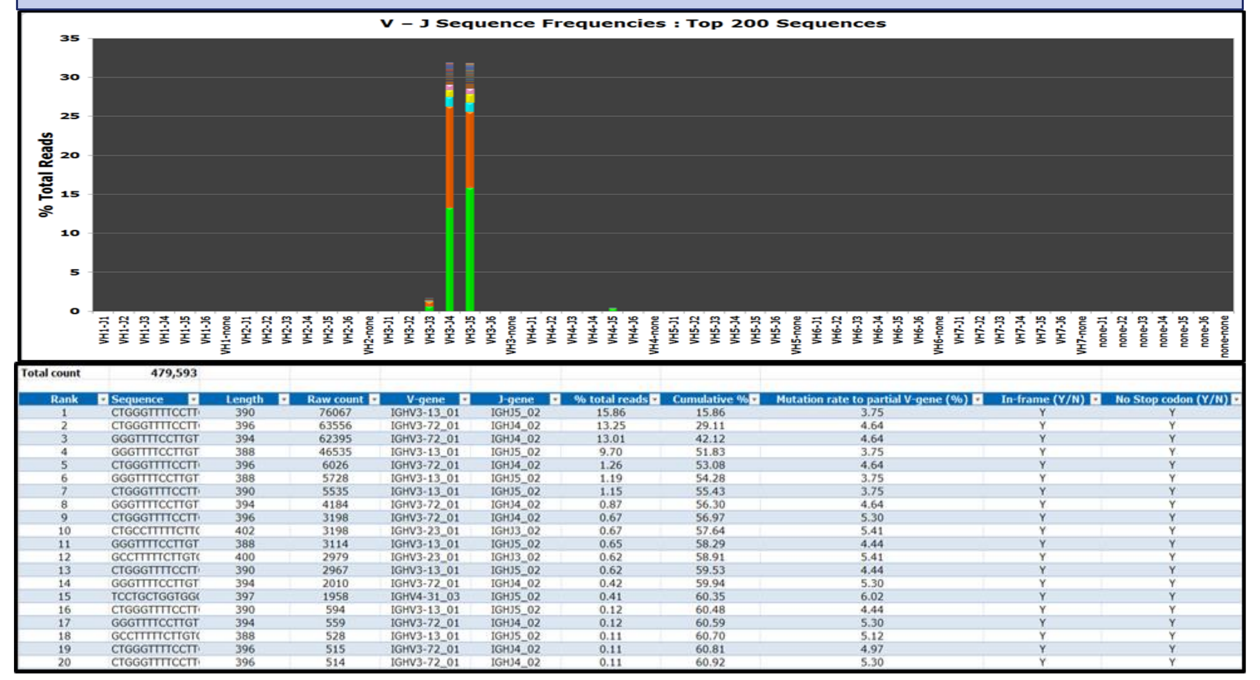
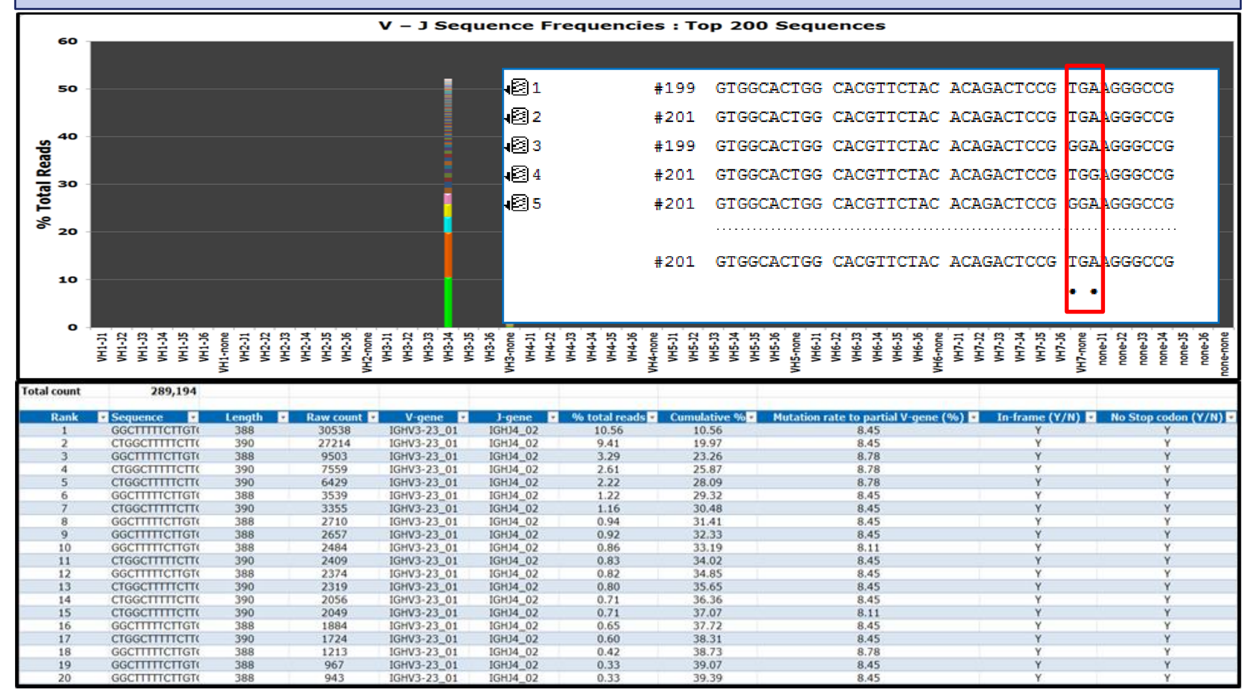


Figure 5. Representative data showing intraclonal heterogeneity in an NGS sample. Inset shows base differences between sequences 1-5.



Discussion

- Discordant findings were present in 2/43 (5%) of comparison samples; additional minor discrepancies identified in 8 cases
- Causes of discordant results:
  - NGS versus Sanger differences:
    - VH nomenclature (IMGT vs. IgBLAST)
    - Differing SHM status (M vs. UM), possibly from suboptimal Sanger data and sequence contig with increased ambiguous bases
    - NGS result negative and Sanger result positive for clonal IGHV: differences in multiplex versus single primer reaction PCR set up, or low clonal B-cell percentage
  - NGS leader versus FR1
    - Leader result negative and FR1 positive: leader primer site mutation or PCR efficiency
  - Other: "N" base artifact (sequencing platform/chemistry/low complexity region) vs. true intraclonal heterogeneity

Conclusions

- NGS methodology is accurate and acceptable for clinical use in the determination of somatic hypermutation status in CLL. Results obtained in our experience are highly concordant (41/43, 95.3%) in comparison to the current standard of PCR and Sanger Sequencing.
- Advantages of NGS include direct determination of the IGVH rearrangement and percent of somatic hypermutation; however, a clear understanding of the various rearrangement patterns, technical or informatics limitations, potential analytic artifacts, and adequate quality parameters is central to ensuring high quality results. Additional merits of NGS in this situation include the ability to batch multiple samples simultaneously and to potentially enable easier minimal residual disease monitoring.

References

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