

REVIEW

Lessons from next-generation sequencing analysis in hematological malignancies

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Next-generation sequencing has led to a revolution in the study of hematological malignancies with a substantial number of publications and discoveries in the last few years. Significant discoveries associated with disease diagnosis, risk stratification, clonal evolution and therapeutic intervention have been generated by this powerful technology. As part of the post-genomic era, sequencing analysis will likely become part of routine clinical testing and the challenge will ultimately be successfully transitioning from gene discovery to preventive and therapeutic intervention as part of individualized medicine strategies. In this report, we review recent advances in the understanding of hematological malignancies derived through genome-wide sequence analysis.

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INTRODUCTION

Over the past few years, a remarkable effort has been underway to identify the genetic basis of hematological malignancies catalyzed by increasing availability and more refined sequencing technologies (Figure 1). The growth in high-throughput sequencing, which has facilitated this effort, has been exponential with a dramatic increase in efficiency and correlating drop in price per base pair. Modern platforms can now perform whole-genome sequencing (WGS) of an individual for less than \$5000 and a few days of work; notable progress compared with the resources and time that were used just a few years ago by an international consortium when completing the first human genome.¹ Strikingly, this technological revolution occurred in <10 years.

A representative example of how increasingly more powerful technologies continuously improve our knowledge of the genetic basis of hematological malignancies comes from the study of the genome of an acute myeloid leukemia (AML) patient with normal cytogenetics, which was studied twice in a period of 2 years. The initial WGS analysis study only revealed small insertions and deletions affecting two genes, and nonsynonymous somatic mutations, in another eight genes.² Two years later, the same genome was resequenced utilizing more advanced sequencing technology and analytical methods resulting in the detection of a previously unidentified frameshift deletion in *DNMT3A*.³ After the initial discovery, *DNMT3A* mutations were screened in large cohorts and it is now known that 22–30% of AML patients have mutations in this gene, being currently one of the most relevant and potentially targetable mutations found in AML.

Next-generation sequencing (NGS) encompasses several different methodologies that allow the investigation of genomics, transcriptomics and epigenomics. A summary of the different sequencing approaches is briefly described below and summarized in Table 1. For more in-depth information, we direct the reader to a number of excellent reviews.^{4–10}

WHOLE-GENOME SEQUENCING

Two major approaches are utilized in the preparation of DNA libraries for WGS. The first is called paired-end sequencing, where ~100 bp are sequenced from each end of ~400-bp DNA fragments. By this method, single nucleotide variants (SNV), insertions and deletions and copy-number changes can be identified. Paired-end WGS needs low-input quantities of DNA (<1 µg) for generating the libraries, which is a critical advantage in the study of hematological malignancies, where the amount of tumor tissue is usually scarce.

The second approach used in the DNA library preparation is named mate-pair sequencing. Mate-pair is based on the generation of much larger DNA fragments than paired-end sequencing with fragments ranging in length from 1 to 10 kb. Longer distance between the read pairs enables improved detection of structural rearrangements because the read pairs can span repeat and duplicate regions, thereby capturing regions not adequately captured with smaller insert sizes utilized with paired-end sequencing. Very low coverage of the genome is enough for studies focused on the detection of structural abnormalities, thus reducing costs and complexity of the analysis. On the other hand, if the genome is covered in enough depth (>30X mean coverage), mate-pair sequencing can be used for the simultaneous detection of mutations, copy-number changes and structural abnormalities. A disadvantage of the mate-pair approach is that quite a large quantity of DNA is required for the library preparation, thus limiting its use in a significant number of tumors.

WHOLE-EXOME SEQUENCING

Whole-exome sequencing (WES) is useful for those interested in studying only what lies within the exome (coding genome) and untranslated regions. This method is based on an initial enrichment step of exonic regions followed by targeted

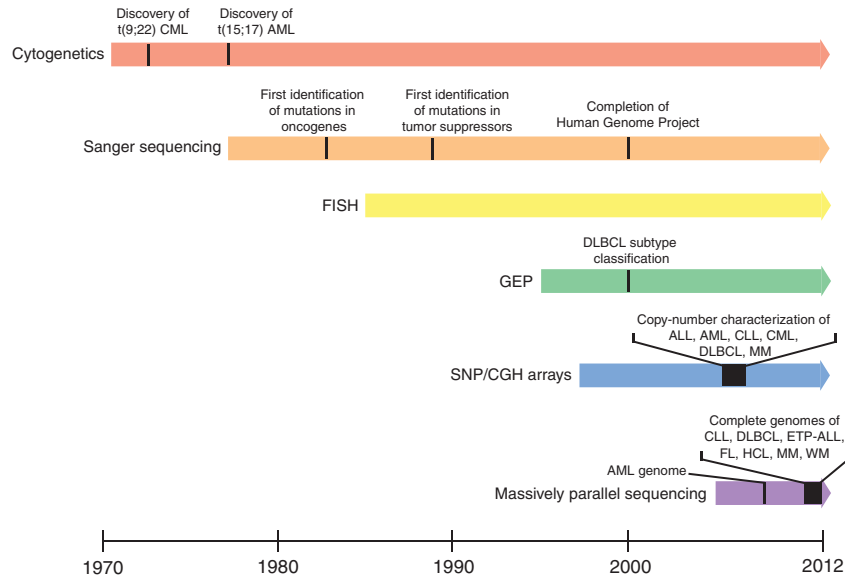


Figure 1. Evolution of genetic detection methods and discoveries. Landmark findings from each method are indicated.

Table 1. Summary of high-throughput sequencing methods

Method	Minimum input quantity ^a	Strengths	Weaknesses
Whole genome	10 ng–1 µg genomic DNA	Small input DNA requirement Variant detection in all regions of the genome	Lower DNA input may reduce library complexity and representation PCR duplicates can impact accuracy of variant detection software Computationally intensive analysis
Mate pair	5–10 µg genomic DNA	Identification of large structural rearrangements	Large input DNA requirement High false discovery rate
Whole exome	1 µg genomic DNA	Deep coverage of exome enabling precise interrogation of coding regions Multiple samples can be pooled and run together reducing time and cost per sample	Non-coding regions excluded Standard capture kits do not capture all exons
mRNASeq	100–400 ng total RNA ^b	Dynamic range of expression detection can be much broader than using microarrays Detection of rare and hybrid transcripts Precise quantitation of highly expressed transcripts and multiple isoforms Investigation of 3'UTR and promoters	RNA fragmentation methods can bias the resulting library Artifacts from amplified cDNA libraries ⁸ Appropriate normal controls may be difficult to obtain for tumor/normal comparison
ChIPSeq	10 ng ChIP enriched DNA	Detection of DNA–protein interactions Discovery of new interactions in regions not represented on microarray chips Avoids hybridization problems associated with array-based ChIP assays	Quality of sequencing results dependent on the quality of ChIP assay Library preparation can introduce GC-rich region bias
Single molecule	1 µg genomic DNA	No amplification step resulting in no PCR duplicates Long-read length (> 1 kb)	High error rate Throughput not comparable to current platforms

Abbreviations: ChIPSeq, chromatin immunoprecipitation sequencing; UTR, untranslated region. Cost per sample is highly variable depending on the platform and on the amount of multiplexing utilized. ^aMay vary by platform and approach. ^bMay require polyA RNA- or rRNA-depleted total RNA.

sequencing. As the exome represents only 1.4% of the genome, multiple samples can be pooled and sequenced together in a single instrument run. The major weakness of WES is the inability of the available enrichment kits to capture the totality of the exome.

Ideally a non-tumoral, reference DNA sample from the individual patient is simultaneously analyzed with each tumor. The amount of normal variation between individuals is in the order of thousands of variants, and performing a paired analysis enables subtraction of the nontumor-specific from the tumor-specific

variants. In the event that normal tissue is not available for comparison, an increased number of publicly available databases, such as dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) and 1000 genomes (<http://www.1000genomes.org/>), can be utilized to identify and clean previously reported variants in the general population that are normal genetic variation rather than somatically acquired mutations.

MESSENGER RNA SEQUENCING

Besides the detection of mutations, messenger RNA sequencing is also a powerful tool for gene expression analysis. The dynamic range of expression obtained by messenger RNA sequencing can be much broader than that obtained by gene expression microarrays, allowing the detection of rare transcripts and more precise quantitation of expressed transcripts.^{8,11} In addition, messenger RNA sequencing can be utilized for the identification of hybrid transcripts and quantitation of multiple isoforms resulting from alternative splicing. Major weaknesses include the biases in library preparation caused by the RNA fragmentation methods utilized, introducing artifacts into the resulting reads.

NEW APPROACHES AND FRONTIERS

It is still the subject of debate which approach is the most appropriate for studying the cancer genome. WGS is the most inclusive approach, but major limitations remain related to the high cost and the difficulty associated with managing the data storage and intensive computational analysis. The study of the exome reduces these limitations. WES is a well-established strategy for analyzing coding regions at low cost, making this approach the most popular in the analysis of the tumor genome nowadays. However, eliminating 98% of the genome from the analysis brings the obvious risks associated with omitting crucial information. This concern is supported by recent findings performed by the Encyclopedia of DNA Elements Project, where integrated analysis demonstrated that >80% of the genome is biochemically active.¹² This new paradigm will require reconsideration of the best strategic approach to optimize the cost/benefit ratio in the analysis of the cancer genome.

Single-molecule, long-read, sequencing approaches are now available and allow the simultaneous search for single-allele mutations and methylation profiles. Furthermore, several new platforms are currently under development that promise to sequence the whole genome in few hours for less than \$1000. These technological advances open a new world of opportunities and soon will put the use of WGS within reach of the clinical labs.

CHALLENGES IN DATA ANALYSIS

Data generation is just a small facet of the much bigger challenge associated with data analysis. The goal of data analysis is to utilize bioinformatics tools in a data analysis 'pipeline' (Figure 2) to transform the raw data into results that can ultimately be seen in a user-friendly visualization tool. Detailed information about the most commonly utilized alignment and functional prediction tools are beyond the scope of this manuscript, and thus we direct the readers' attention to several informative manuscripts.^{13–16}

The ability to quickly generate large quantities of data at relatively low cost is limited by these constantly evolving data analysis pipelines, failure to report analytical methods with the level of detail expected from traditional experimental data and the lack of consensus regarding which tools to use when transforming the data into a useable form. Nekrutenko and Taylor¹⁷ reviewed 50 papers that used the Burrows–Wheeler Aligner for mapping sequencing reads, and they found that most of the papers neither provide access to the raw data nor specify the parameters utilized

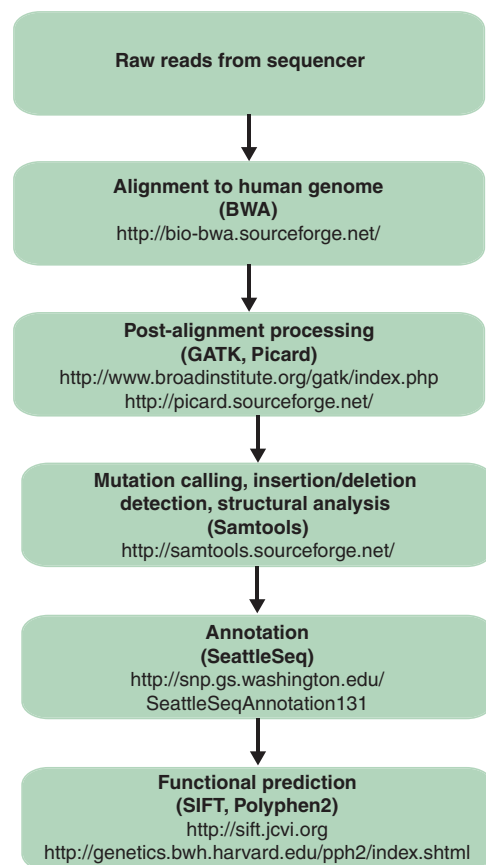


Figure 2. Schematic of a bioinformatics pipeline. Examples of the most commonly used publicly available software programs utilized at a particular step are in parentheses. The programs listed were the most commonly used in 2012 hematological malignancy sequencing analyses. These are only examples and are not intended to be an exhaustive list. The number of publicly available tools is rapidly expanding and review of these tools is beyond the scope of this report.

or identify the precise version of the genomic reference sequence. From the remaining analyses, only four provide settings, eight list the version used and seven list all necessary details. Furthermore, of 19 sequencing articles that cited and had a similar experimental design to that of the 1000 Genomes project, only 4 used the workflow recommended. Different analysis pipelines can yield similar, yet different results suggesting that more than one pipeline may be currently necessary to successfully analyze the data and ultimately introduce this technology into the clinic.

SEQUENCING IN HEMATOLOGICAL MALIGNANCIES

At this point, where medium-sized cohorts of most of the hematological malignancies have been sequenced, it is time to wonder what have these data revealed thus far in this group of malignancies and what are the opportunities ahead? As we expected, genes such as *TP53*, *ATM* and *RAS* among others were confirmed as mutated in a wide variety of malignancies. However, promising and exciting findings came from the discovery of a completely novel group of genes and pathways impaired in hematological malignancies. A few malignancies seem to be driven by mutations in only one or a few genes, suggesting a unique pathway, pathognomonic to the disease. However, most of the malignancies show considerable genetic heterogeneity, with multiple genes and pathways affected. In this review, we aim to summarize the current knowledge of the genetic background

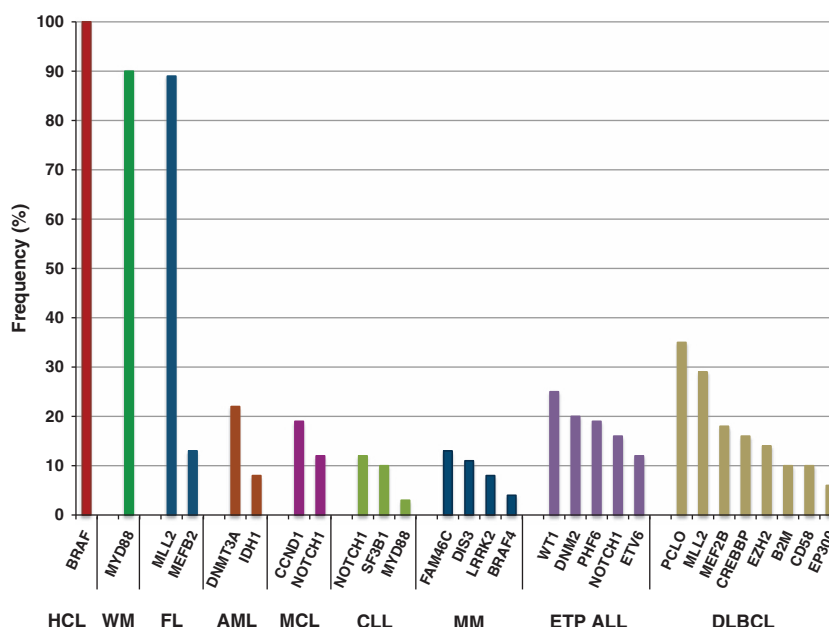


Figure 3. Most frequent somatic genetic mutations per hematological malignancy. Only original data from massively parallel sequencing were included, excluding confirmation data in previously mutated genes (for example, *ATM* and *TP53* in CLL, *FLT3* in AML, *RAS* and *TP53* in MM, *MYD88* in DLBCL, mutations in the nuclear factor- κ B pathways in DLBCL and MM). In cases where data were obtained from multiple studies, the data originating from the largest cohort were included.

on different hematological malignancies and how this knowledge could facilitate targeting of dysregulated signaling pathways by therapeutic targets. The most recurrent novel somatic genetic mutations per malignancy are summarized in Figure 3 and Table 2.

SINGLE CAUSATIVE MUTATIONS: HAIRY CELL LEUKEMIA AND WALDENSTRÖM'S MACROGLOBULINEMIA AS PARADIGMS

Probably, the most representative example of a single hit identified by sequencing are the hairy cell leukemias (HCL). Initially, WES was performed on a single HCL tumor/normal pair with somatic mutations identified in five genes: *BRAF*, *CSMD3*, *SLC5A1*, *CNTN6* and *OR8J1*.¹⁸ Another 47 HCL cases were screened for *BRAF* mutations and strikingly, the *BRAF* V600E substitution was found in all 47 patients evaluated. Conversely, *BRAF* mutations were absent in related peripheral B-cell lymphomas and chronic lymphocytic leukemia (CLL), and were only found in a small subset of multiple myeloma (MM) patients (4%).¹⁹ The same activating mutation and its damaging effect has been previously reported in solid tumors such as in melanoma²⁰ and papillary thyroid cancer.²¹ The presence of a common mutation across HCL provides a central novel therapeutic avenue in HCL based on V600E *BRAF* inhibitors^{22,23} alone or in combination with MEK or ERK inhibitors. The success of vemurafenib, a *BRAF* inhibitor, in the treatment of V600E *BRAF*-mutated melanoma patients led two groups to investigate the effectiveness of this small molecule inhibitor in one HCL case study each. In both cases, including one with a biallelic V600E *BRAF* mutation, treatment with vemurafenib resulted in successful disease treatment,^{24,25} thus providing evidence for clinical trials to evaluate the use of *BRAF* inhibitors in HCL.

A similar situation was found in Waldenström's macroglobulinemia (WM). Remarkably, a *MYD88* L265P-activating mutation was recently found in 90% of WM cases.²⁶ *MYD88* encodes for an adapter protein that affects the interleukin-1 and toll-like receptor pathway, with the L265P mutation leading to the dysregulation of the nuclear factor- κ B and the JAK-signaling pathways.²⁷ The same mutation has been found, but to a lesser extent, in additional B-cell lymphomas, such as diffuse large B-cell lymphomas (DLBCL) of the

ABC type (~40%), MALT lymphomas and CLL (<10%), supporting the key role of *MYD88* in the pathogenesis of these neoplasias.^{27–29} Interestingly, a recent study evaluating the association between *MYD88* L265P and clinical characteristics of WM patients reported more involvement of the bone marrow disease, higher serum IgM, and lower IgA and IgG levels.³⁰ Another group conducting a case-control study evaluating the association between *MYD88* L265P and IgM MGUS patients progressing to WM or other lymphoproliferative disorders reported a trend toward progression in patients with the presence of the mutation when compared with patients with wild-type *MYD88*.³¹ These findings highlight the potential value of *MYD88* as a potential biomarker of disease progression in WM.

In CLL, Velsusamy *et al.*³² recently identified the presence of a YPEL5-PPP1CB RNA fusion in 95% of CLL patients screened. Interestingly, WGS in the two index cases possessing the chimera did not reveal the presence of a gene fusion at the DNA level.³² These findings emphasize the importance of concurrently utilizing multiple methodologies such as WGS and RNASeq when studying tumors to better screen for genetic abnormalities.

One of the recurrent findings of sequencing research efforts has been the epistatic nature of discoveries. This notion reinforces the thought of classifying disease more along the line of functional aberrant pathways, rather than on specific genetic changes. In fact, excluding HCL and WM, the majority of the malignancies show a considerable genetic heterogeneity, affecting multiple genes and pathways. Presented here are some of the most remarkable recent discoveries.

MUTATIONS AFFECTING THE SPLICING MACHINERY

Recent sequencing studies identified recurrent mutations affecting genes of the splicing machinery in myelodysplastic syndrome (MDS).^{33–35} Interestingly, six of these genes (*SF3A1*, *SF3B1*, *SRSF2*, *U2AF35*, *ZRSR2* and *PRPF40B*) affect the initial steps of RNA splicing; thus, mutations leading to the impaired recognition of the 3' splice site result in the production of abnormal mRNA splicing. Mutations of the spliceosome are highly prevalent in MDS and other myeloproliferative disorders, ranging from 44% of cases

Table 2. Summary of high-throughput sequencing studies performed so far in hematological malignancies

Disease	Discovery cohort (N)	Validation cohort (N)	Method	Platform	Mean coverage depth (X)	Highlights	Year	Reference
ALL	1	24	mRNASeq	GAll	NR	DPEP1 (4%), longitudinal detection of PLXNB2 and CXorf21	2012	80
ALL(Phlike)	15	231	WGS/mRNASeq	GAllx/HiSeq	NR	NUP214-ABL1 fusion (2%), IKZF1 (67%)	2012	81
ALL (ETP)	12	94	WGS	GAll	33	RAS pathway (67%), hematopoiesis and lymphoid development (58%), histone modification (42%)	2012	40
ALL (T)	11	—	WES/mRNASeq	HiSeq	55/15	DNMT3A (17%), JARID2 (8%), IDH2 (8%), EZH2 (17%)	2012	82
AML	1	—	WES	HiSeq2000	NR	Leukemic transformation from SCN to AML	2012	83
AML	8	—	WGS/deep sequencing	GAllx	25/590	Clonal evolution	2012	73
AML	5	160	WES	GAllx	NR	GATA2 (39%) with biallelic CEBPA mutation	2012	84
AML	2	3	ChIPSeq	GAll/HiSeq	NR	Differential H3K4me3/H3K27me3 gene enrichment of stem and progenitor cells	2013	85
AML-CN	1	95	mRNASeq	GAllx	NR	TLE4 (2%), SHKBP1 (2%)	2011	86
AML-CN	1	262	WES	GAllx	69	BCOR (4%), DNMT3A (13%)	2011	87
AML-CN	7	230	mRNASeq	HiScanSQ	36	CBFA2T3-GLIS2 fusion (8%)	2013	88
AML-M1	1	—	WGS	GA	33	First genome sequenced	2008	2
AML-M1	1	187	WGS	GAll	23	IDH1 (8%)	2009	50
AML-M1	1	281	WGS	GAll	39	DNMT3A (22%)	2010	3
AML-M5	14	98	WES	GAllx	97	DNMT3A (21%)	2011	89
sAML	7	200	WGS/WES	GAllx/HiSeq	34	UMODL1 (29%), SMC3 (14%), CDH23, ZSWIM4 (14%)	2012	90
BL	28	78	mRNASeq	HiSeq2000	NR	ID3 (59%), TCF3 (29%), CCND3 (15%)	2012	91
BL	4	97	WGS/WES/ mRNASeq/ MethylSeq	GAll/HiSeq	32/121	ID3 (42%)	2012	92
BL	14	45	WES	GAllx/HiSeq	47	ID3 (34%)	2012	62
CLL	4	363	WGS/WES	GAllx	40/119	NOTCH1 (12%), MYD88 (3%)	2011	59
CLL	5	226	WES	Genome Sequencer FLX	10	NOTCH1 (17%)	2011	63
CLL	3/88	101	WGS/WES	GAll	38/132	SF3B1 (15%), MYD88 (10%)	2011	36
CLL	105	279	WES	GAllx	62	SF3B1 (10%), NOTCH1 (10%)	2012	37
CLL	7	103	RNASeq/WGS	GAll	NR/12	YPEL5-PPP1CB fusion (95%)	2013	32
CLL	160	—	WES	GAllx/HiSeq	112	Patterns of clonal evolution	2013	76
DLBCL	6	105	WES	Genome Sequencer FLX	10	MLL2 (24%), regulation of immune response (63% ABC, 31% GCB)	2011	29
DLBCL	13/83	37	WGS/mRNASeq	GAllx/HiSeq	32/41	MLL2 (32%) MEF2B (11%), histone modification (13%), lymphocyte activation ^a , differentiation ^a and apoptosis ^a	2011	28
DLBCL	49	—	WES	HiSeq	150	Histone H1 proteins (69%), ACTB (10%), P2RY8 (12%), PCLO (35%)	2012	38
DLBCL	34	39	WGS/WES	GAll/HiSeq	29/47	Signal transduction ^a , chromatin modification ^a	2013	39
FL	1/12	35	WGS/mRNASeq	GAllx/HiSeq	9/28	MLL2 (89%), MEF2B (13%), histone modification (15%), lymphocyte activation ^a , differentiation ^a and apoptosis ^a	2011	28
HCL	1	47	WES	GAllx	71	BRAF V600E (100%)	2011	18
MCL	18	108	mRNASeq	GAll	NR	NOTCH1 (12%), CCND1 (19%)	2012	60
MM	23/16	161	WGS/WES	GAll	33/104	Protein translation (42%), HOX9 pathway (29%)	2011	19
MM	22	127	WES	GAllx	61	Distinct mutation patterns between t(4;14) and t(11;14)	2012	75
MM	1	—	WGS	SOLiD/HiSeq	30	Genomic evolution and clonal tides over course of disease	2012	74
MDS	9	354	WES	GAllx	NR	SF3B1 (67%)	2011	34
MDS	29	582	WES	GAllx/HiSeq	134	RNA splicing (55%)	2011	33
MDS	1	150	WGS	GAllx/HiSeq	39	U2AF1 (9%)	2012	43
MDS/MPN	15	310	WES/mRNASeq	HiSeq	NR	RNA splicing, SRSF2 (24%), clinical outcomes associated with mutations	2012	35

Table 2. (Continued)

Disease	Discovery cohort (N)	Validation cohort (N)	Method	Platform	Mean coverage depth (X)	Highlights	Year	Reference
MPN	40	—	WES	HiSeq	NR	SUZ12 (3%)	2011	93
NHL(B-cell)	2	263	mRNASeq	GALL	NR	CIITA translocations (16%)	2011	94
PCNSL	4	25	WES	GALLx	NR	MYD88 L265P (38%), TBL1XR1 (14%)	2012	95
SMZL	6	93	WGS	Complete genomics	80	MLL2 (50%), NOTCH2 (25%)	2012	69
SMZL	8	109	WES	HiSeq	111	NOTCH2 (21%), NOTCH1 (5%), SPEN (5%),DTX1 (2%)	2012	68
WM	30	54	WGS	Complete genomics	66	MYD88 L265P (91%)	2012	26

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; BL, Burkitt's lymphoma; ChIPSeq, chromatin immunoprecipitation sequencing; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; ETP ALL, early T-cell precursor acute lymphoblastic leukemia; FL, follicular lymphoma; HCL, hairy cell leukemia; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; MethylSeq, methylation sequencing; MM, multiple myeloma; MPN, myeloproliferative neoplasms; mRNASeq, messenger RNA sequencing; NHL, non-hodgkins lymphoma; NR not reported; PCNSL, primary central nervous system lymphoma; SMZL, splenic marginal zone lymphoma; WM, Waldenströms macroglobulinemia. Platforms: GALL, GALLx, HiScanSQ and HiSeq2000 are from Illumina, Genome Sequencer FLX from 454 Sequencing Roche and SOLiD from Life Technologies. ^aIndicates significant pathway enrichment.

without increased sideroblasts to 85% of cases with increased sideroblasts.³³ The mutations were mutually exclusive in disease subtypes,^{33–35} suggesting a key role of the spliceosome mutations in the pathogenesis of myeloproliferative disorders. On the other hand, mutations affecting the spliceosome were significantly lower in *de novo* AML and myeloproliferative neoplasms.^{33,34}

SF3B1 was the most commonly mutated of these genes, and it was significantly enriched in the group of MDS with increased ring sideroblasts, refractory anemia with ring sideroblasts, and refractory cytopenia with multilineage dysplasia and ring sideroblasts ($P < 0.001$).³⁴ Clinically, *SF3B1* mutations were associated with fewer cytopenias and longer event-free survival.^{34,35} The high prevalence of *SF3B1* mutations in diseases with ring sideroblasts and the confirmation that the mutation can be identified in peripheral blood suggest that *SF3B1* could potentially be used as a biomarker.

SF3B1 mutation was also one of the most significant discoveries in CLL, found in 10–15% of cases.^{36,37} Mutations in *SF3B1* were associated with deletion 11q ($P = 0.004$).³⁶ Moreover, *SF3B1* mutations and/or deletion 11q were predictive markers of an earlier need for treatment ($P < 0.0001$).³⁶ Altogether, these results indicate that mutations of the spliceosome are involved in hematological malignancies and offer a novel therapeutic avenue for MDS and CLL.

MUTATIONS MODULATING TRANSCRIPTION AND TRANSLATION

One of the most interesting themes arising from the study of hematological malignancies is that alterations of genes modulating transcription and expression are a recurrent finding. Sequencing studies in DLBCL and follicular lymphomas reported genes involved in the histone modification process.^{28,29,38,39} *MLL2*, a histone methyltransferase specific to the H3K4 residue, was the most commonly mutated gene in follicular lymphoma, affecting almost 90% of cases.²⁸ The vast majority of mutations had an inactivating effect and included missense and frameshift mutations affecting or truncating the C-terminal domains, including the SET domain.^{28,29} These findings place *MLL2* collectively with the t(14;18)(q32;q21), as the two most common abnormalities in follicular lymphoma.

In addition, genes involved in histone modification were collectively identified in ~20–40% of DLBCL^{29,39} and early T-cell precursor acute lymphoblastic leukemia (ETP ALL).⁴⁰ Furthermore, *EZH2*, which is involved in histone methylation, is mutated in

DLBCL and follicular lymphoma,^{28,29} with the mutations occurring in a critical SET domain.⁴¹ Preclinical studies in DLBCL have found the inhibition of *EZH2* an effective therapeutic approach for tumors containing activating mutations, thus presenting a novel therapeutic target for the treatment of DLBCL.⁴¹

Chromatin modifiers are also recurrently affected in MM. *MMSET*, a histone methyltransferase transcriptional repressor, is over-expressed in ~15% of MM as a consequence of the t(4;14)(p16;q32).⁴² Sequencing studies show that other chromatin modifiers are mutated in a significant subset of MM, including *KDM6A* and *HOXA9*.¹⁹ In addition, in the analysis of MM there was an enrichment of mutations within genes involved in protein translation. Thus, 42% of MM cases had mutations in this pathway, mainly affecting *FAM46C* (13%), *DIS3* (11%) and *LRRK2* (8%).¹⁹

A major finding in MDS and AML was the identification of mutations in a set of genes associated with DNA methylation. *DNMT3A*, a methyltransferase, is the most commonly mutated gene in AML found in around 20–30% of AML cases. Interestingly, no mutations were found in the related genes *DNMT1*, *DNMT3B* or *DNMT3L*.³ *DNMT3A* mutations were associated with poor survival ($P < 0.001$). In addition, mutations have been identified in *U2AF1* in MDS patients, and those harboring *U2AF1* mutations were more likely to progress to secondary AML.⁴³

OTHER BREAKTHROUGH DISCOVERIES BY NGS: *IDH1* AND *IDH2* MUTATIONS IN AML

Another major discovery in AML was the identification of mutations in *IDH1*, which encodes isocitrate dehydrogenase 1, and the related *IDH2* gene. *IDH1* mutations have been observed in DLBCL,³⁹ cartilaginous tumors,⁴⁴ astrocytoma^{45,46} and glioblastoma,⁴⁷ whereas *IDH2* mutations have been reported in astrocytoma.⁴⁵ Interestingly, patients with grade II astrocytoma who have *IDH1* mutations show significantly shorter progression-free survival than tumors with wild-type *IDH1*.⁴⁶ Studies in AML have reported mutations in 10–15% of cases, preferentially found in the intermediate-risk cytogenetic group, and their association with worse prognosis in a subset of AML patients that have been confirmed.^{48,49} Mutations in *IDH1* and *IDH2* are mutually exclusive and primarily affect *IDH1* at codon R132 and *IDH2* at codons R140 or R172.⁵⁰ Mutations in *IDH1* were enriched in cases possessing *DNMT3A* mutations.³ Conversely, *IDH2* mutations are rarely found together with other known recurrent mutations.^{48,49} In addition, mutations in *IDH1* and *IDH2* seem to be mutually exclusive with *TET2* mutations.^{51,52} A recent study reported that mutations in *IDH1*

or *IDH2* disrupted TET2 function and led to a hypermethylation phenotype with impaired hematopoietic differentiation.⁵³

It becomes clear then that the morphological and clinicopathological classification of AML is now challenged by these new genetic findings. How many subcategories of AML exist? How does this heterogeneity exist, or not, in the better defined entities at the chromosome level (for example, M3)? In short, the various new perspectives to classify AML may ultimately lead more toward a molecular and pathway approach, but in some cases they might still have very significant resemblance to older cytogenetic classification.

OTHER BREAKTHROUGH DISCOVERIES BY NGS: NOTCH MUTATIONS

Aberrant NOTCH1 signaling has been identified in both solid and hematological tumors, and is a therapeutic target of interest currently in preclinical and clinical trials.^{54,55} *NOTCH1* encodes a transcription factor that transduces extracellular signals into expression changes in targets genes, including *MYC*⁵⁶ and PI3K-AKT signaling pathways.⁵⁷ NOTCH receptors are involved in cell fate determination, having a critical role in T-cell development. In fact, impaired NOTCH1 results in a block at the earliest stages of T-cell lymphopoiesis.⁵⁸ Mutations in *NOTCH1* lead to an active protein isoform lacking the C-terminal domain, and have been identified in over 50% of T-cell ALL and, to a lesser extent, in CLL, MCL and Burkitt's lymphoma.^{59–62} These mutations mainly target the PEST domain, which is required for NOTCH1 interaction with FBW7, and subsequent NOTCH1 targeting for proteosomal degradation.

Data suggest that *NOTCH1* mutations are a progressive event in CLL, increasing in prevalence from newly diagnosed CLL to chemorefractory CLL to CLL patients with Richter syndrome that underwent transformation to DLBCL.⁶³ *NOTCH1* mutations were associated with trisomy 12 ($P = 0.009$) and with *IGHV*-unmutated status.^{36,59,64} In addition to the association with more advanced stages of the disease and with transformation to DLBCL, *NOTCH1* mutations were associated with adverse biological course and worse overall survival in CLL ($P = 0.03$)^{59,63} and MCL ($P = 0.003$).⁶⁰ In T-cell ALL, *NOTCH1* mutations were associated with improved response to glucocorticoid therapy; however, the association of NOTCH activation and clinical outcome seems to be therapy dependent.^{65–67}

On the other hand, recurrent *NOTCH2*-activating mutations were identified in 21–25% splenic marginal zone lymphomas, but only rarely in nonsplenic MZLs and other low-grade B-cell lymphomas and leukemias.^{68,69} Although these studies evaluated the association of *NOTCH2* mutations and clinical outcomes, the findings are conflicting and additional work is necessary to clarify the potential clinical impact of mutations in *NOTCH2*.^{68,69} Small molecule pan-NOTCH inhibitors have not shown significant effects as single agents targeting T-cell ALL, but there is an improved antileukemia effect when used in combination with inhibitors of PI3K-AKT-mTOR pathway or CDK inhibitors.^{70,71}

NGS AS A TOOL FOR DISCRIMINATION OF RELATED DISEASES

Besides the importance of identifying pathognomonic mutations and pathways, sequencing is also a powerful tool to differentiate related entities. Overall, 67% of ETP ALL had mutations in the RAS signaling pathway (*BRAF*, *JAK1*, *JAK3*, *KRAS*, *NRAS*) or cytokine receptors (*IL7R*), which was significantly higher than that in non-ETP ALL (19%; $P < 0.0001$).⁴⁰ Furthermore, genes involved in hematopoiesis and lymphoid development (*RUNX1*, *IKZF1*, *ETV6*, *GATA3* and *EP300*) were also more frequently mutated in ETP ALL (58%) than that in non-ETP ALL (17%; $P < 0.0001$). Altogether, 81% of ETP ALL cases have mutations in either of these pathways compared with 31% of non-ETP ALL cases ($P < 0.0001$). A similar enrichment was identified in

genes involved in histone modification (*EED*, *EZH2* and *SUZ12*), which were more commonly mutated in ETP ALL (42%) compared with non-ETP ALL (12%; $P = 0.0001$).

Mutations in genes affecting the RAS pathway, cytokine receptor and epigenetic modification are common in AML, but are rare in B- and T-cell neoplasias.^{40,72} These findings together with previous data demonstrate that ETP ALL has a gene expression signature closer to leukemic stem cells and granulocyte precursors, suggesting that ETP ALL is a distinct entity from non-ETP ALL with a less mature phenotype that retains the potential to become a myeloid cell.

GENOMIC SEQUENCING IN THE ANALYSIS OF CLONAL ARCHITECTURE AND CLONAL EVOLUTION

Genomic sequencing performed in high-coverage depth is a useful tool for characterizing the clonal architecture and analyzing the clonal evolution in disease progression and in response to therapy. Ding *et al.*⁷³ have provided a good example of the power of genomic sequencing in sequential analysis. WGS was performed in eight AML cases utilizing normal skin biopsies paired with tumor samples collected at diagnosis and after relapse. Candidate somatic events were analyzed by deep sequencing with a median of 590X coverage. In five out of eight cases, the primary sample was characterized by up to four mutation clusters, thus indicating the existence of multiple (sub)clones. Two major patterns of clonal evolution were identified when comparing primary versus relapse samples. Either the original clone in the primary tumor sample acquired additional mutations and evolved into the relapse clone, or most of the (sub)clones were eradicated by therapy leaving one clone. This clone is usually observed at a low frequency in the primary sample, it then survives the initial therapy, gains additional mutations and expands, becoming the predominant clone at relapse. Another interesting finding was obtained by comparing the transition with transversion mutation rate between primary and relapse samples. The data obtained strongly suggest that the chemotherapy regimen used (cytarabine and anthracycline for induction and additional cytotoxic chemotherapy for consolidation) have had a significant effect in the origin of novel mutations in the AML relapse sample.

In our sequencing analysis performed in MM, we have observed the presence of single nucleotide variants waxing and waning over the course of several longitudinally collected samples from a single patient.⁷⁴ This shift in the presence of single nucleotide variants suggests the presence of multiple clones rising and falling in dominance over time. In the work by Walker *et al.*,⁷⁵ a single MM patient, from whom they had WES data, identified three populations containing mutations in four genes: *ATM*, *FSIP2*, *CLTC* and *GLMN*. When they then evaluated which mutations were shared in a single cell, they identified one population with only an *ATM* mutation, a second with *ATM* and *FSIP2* and a third with *ATM*, *CLTC* and *GLMN* mutations.⁷⁵ The observed presence of these different clones suggests that if these patients were to be followed longitudinally, clonal dominance would likely shift as the tumor evolves with time and treatment.

The study of clonal complexity and clonal evolution is an old field that has been reinvigorated since the introduction of NGS. We believe NGS will help us to elucidate several unanswered questions such as what are the driver-initiating mutations in the different hematological malignancies? What are the specific mutations associated with disease progression in the different hematological malignancies? What mutations are the primary contributors to chemoresistance? Does clonal heterogeneity need to be considered in the context of determining therapeutic options? Do all the clones need to be targeted?

Some of these questions have been at least partially answered in a recent study.⁷⁶ The authors analyzed 149 CLL cases, including 18 that were analyzed at two time points, using WES and SNP arrays. Data obtained from this study confirm previous findings

showing the existence of linear and multibranching clonal evolution in CLL.^{77,78} Furthermore, the authors were able to infer the order of genetic changes occurring in CLL pathogenesis. Thus, it was suggested that the clonal driver mutations, which are proposed to be initiating events, mainly affect genes that selectively affect B cells, such as *MYD88* and *del13q*, whereas subclonal driver mutations associated with disease progression affect genes more ubiquitously involved in carcinogenesis such as *TP53* and *ATM*. The number of subclonal mutations increases in treated compared with untreated cases; thus, the therapy would be a trigger for natural selection leading to the emergence of more aggressive subclones. Furthermore, the study shows the importance of subclonal driver mutations as an independent risk factor for rapid disease progression and poor outcome. Thus, this study suggests that dissecting the clonal architecture of CLL is crucial not only for developing novel risk-stratification algorithms but also for designing novel therapeutic approaches, considering the presence of driver mutations as well as the genomic landscape.

We expect to see similar efforts in several other hematological malignancies, which will ultimately help to elucidate the clonal complexity and its importance in each particular disease.

HOW ARE NEW DISCOVERIES TRANSLATING INTO NOVEL THERAPEUTIC APPROACHES?

Several novel somatic mutations, such as *SF3B1*, *IDH1*, *IDH2*, *DNMT3A*, *MYD88* and *MLL2* have been identified as a consequence of NGS efforts, leading to the discovery of previously unrecognized genes and molecular processes/pathways with pathogenic effects. The genomic profiling of each individual cancer will potentially have a key role clinically assisting in early disease diagnosis, risk stratification, longitudinal analyses of tumor evolution and selection of the most favorable and personalized therapeutic intervention.

One of the most emblematic examples is AML. The unprecedented characterization of the AML cancer genome may substantially affect the clinical management and the therapeutic decisions. The prior characterization of mutations in *FLT3*, *NPM1*, *RUNX1* and *CEBPA* together with the recent identification of mutations in *IDH1*, *IDH2*, *DNMT3A* and *TET2* encourage the incorporation of genomic studies as part of routine clinical tests and may enable optimization of therapeutic plans based on this patient-specific genomic background.

However, the genetic characterization of AML will not improve patient survival *per se*, unless it is synchronized with the development of alternative therapeutic approaches. One of the major limitations in the treatment of AML is the intrinsic drug resistance of the tumor cells. Standard induction chemotherapy regimens, consisting of cytarabine and anthracycline combinations, have remained largely unchanged in the treatment of AML over decades. Thus, the major challenge is to provide the AML patients with alternative drug combinations targeting novel genes/pathways discovered in chemoresistant cases.

The discovery of novel genes/pathways not only increases our understanding of the pathogenesis of the disease but also opens new therapeutic avenues. The existence of potential 'Achilles' heels to be exploited for generating a unifying targeted therapy for all patients is very provocative and opens an exciting era for translational research. Exploiting this knowledge is critical in hematological malignancies when considering that most of them are still incurable and more effective therapies are urgently needed.

So far, we have discovered a different range of genetic heterogeneity across tumor types. We have learnt that some malignancies have a mutated gene or pathway that affect most or all cases. An excellent example is provided by the *BRAF* V600E, common to all HCL patients,¹⁸ or *MYD88* L265P, found in most

WM.^{26,28,79} For example, V600E *BRAF* can be targeted with *BRAF* inhibitors^{22,23} alone or in combination with MEK or ERK inhibitors.

Conversely, the majority of hematological malignancies are characterized by considerable tumor heterogeneity, making the search for therapeutic targets more difficult. One of the biggest challenges is to reduce the complexity of the generated data by first, distinguishing the driver over the passenger mutations and clones, and second, generating systematic and more sophisticated approaches for data analysis integration, thus unifying the vast genomic heterogeneity of these cancers into more homogeneous groupings based on cellular pathways rather than on single genes. As in the case of single gene mutations, the disruption of specific pathways may be exploited therapeutically.

With the dawn of the \$1000 genome drawing close, we anticipate an ever-increasing role of genomic sequencing in the diagnosis and treatment of patients. As this technology moves ever closer to widespread clinical application, there are several challenges that must be addressed. First, the management of the data obtained from sequencing must be addressed. Not only does the physical storage of the data present a challenge but how the information obtained is reported to the patient, retained over time and/or destroyed, are important issues that also must be discussed. Second, incidental findings of mutations in genes unrelated to the medical reason, a patient is seeking genome sequencing can result in legal and ethical dilemmas for the care providers. Furthermore, knowledge about genomics and disease is rapidly expanding, thus consideration of whether a patient's genome should be re-evaluated at a later time must be considered.

As our focus shifts from large population-based studies with large cohorts to the 'N of one' with individualized genomic medicine, it is imperative that the recommendations made to patients be based on evidence from well-designed functional studies. Translating this genetic data into the clinic is challenging and a significant amount of functional work is still required to better understand the biological significance of these hits using both *in vitro* and *in vivo* models. In the near future, we anticipate a standard of care for personalized medicine that involves sending samples for sequencing at the time of biopsy. A variant report will be generated for the physician who will then base treatment decisions on the findings from sequencing in addition to pathology and clinical diagnostics.

The ultimate goal in the post-genomic era will be to extend to other hematological malignancies the successful transition from gene discovery to therapeutic intervention observed in the paradigmatic BCR-ABL CML cases treated with imatinib.

CONFLICT OF INTEREST

RF has received a patent for the prognostication of MM based on genetic categorization of the disease. He has received consulting fees from Medtronic, Otsuka, Celgene, Genzyme, BMS, Lilly, Millenium and AMGEN. He also has sponsored research from Cylene and Onyx. EB is a recipient of the Marriott Specialized Workforce Development awards in Individualized Medicine, The Henry Predolin Foundation Career Development award and the George Haub Family Career Development award fund in Cancer Research. JBE is a recipient of the Multiple Myeloma Research Foundation Research Fellow award. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

EB, JBE, RF, AKS wrote the manuscript. All authors reviewed and gave final approval of the manuscript.

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