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Whole genome sequencing identified new somatic mutations for chronic myelomonocytic leukemia

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Abstract. – OBJECTIVE: We aimed to gain new insight into the molecular alterations of Chronic Myelomonocytic Leukemia (CMML).

PATIENTS AND METHODS: We performed whole-genome sequencing (WGS) and subsequent Sanger sequencing validation analysis in three individuals with CMML. Genomic DNA samples from bone marrow and matching buccal mucosa samples were sequenced.

RESULTS: For all six samples, a total of 806.43 Gb data were generated, achieving a minimum mean depth of 30.76. A total of 22 somatic variants were found to be protein-altering, including 1 exonic frame shift indel, 18 missense SNVs, 2 stop gain SNVs, and 1 stop loss SNV. We focused on the five novel variants which have not been reported in known databases and successfully validated three missense SNVs in AKAP4, COL2A1, and MAML1, respectively.

CONCLUSIONS: WGS analyzes provided us a new insight into the molecular events governing the pathogenesis of CMML. The somatic variants we reported here may provide new targets for further therapeutic studies.

Key Words:
Chronic myelomonocytic leukemia, Whole-genome sequencing, Somatic mutation, Validation.

Introduction

Chronic myelomonocytic leukemia (CMML) is a type of leukemia characterized by overlapping features of both myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPNs)[1]. The incidence of CMML was estimated to be less than 1 per 100,000 persons per year and CMML has a 20-30% chance of transformation to AML[2]. Currently, allogeneic stem cell transplant remains the only curative option for CMML[3].

Cancer is considered as a genetic disease that arises from a single clone of cells expanding in an unregulated fashion due to genomic instability and somatic mutations[4,5]. Identification of 'driver' somatic mutations may provide potential therapeutic targets in clinical practice. Next-generation sequencing (NGS) technologies provide an efficient tool to identify the somatic mutations in a cancer[6]. Several reports[7-9] involving whole-genome and/or whole-exome sequencing on CMML were recently published, and provide a catalog of somatic mutations in CMML, including mutations in genes involving epigenetic regulation (*TET2*, *DNMT3A*, *IDH1*, and *IDH2*), chromatin regulation (ASXL1 and *EZH2*), splicing machinery (*SF3B1*, *SRSF2*, *SF3A1*, *U2AF1*, *ZRSR2*, *PRPF30B*, and *SF1*), DNA damage response (*TP53* and *PFH6*), signal transduction and cellular/receptor tyrosine kinase pathways (*JAK2*, *KRAS*, *NRAS*, *CBL*, *FLT3*, and *RUNX1*). However, these genetic changes occur only in a subset of CMML cases.

To gain new insight into the molecular alterations of CMML, we performed whole-genome sequencing (WGS) and subsequent Sanger sequencing validation analysis in three individuals with CMML.

Patients and Methods

Ethics Statement

This study was approved by the Ethics Committees of Guangzhou First People's Hospital. Written informed consent was obtained from all subjects.

Patients

As shown in Table I, three patients aged from 63 to 82 were diagnosed as CMML in Guangzhou

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Table I. Clinical characteristics of the patients in sequencing.

Samples	Sex	Age	% BM leukemia cells at diagnosis	White cells count (×10⁹/L)	Karyotype
S1	F	63	16%	90.16	46,XY
S2	M	66	6.63%	18.05	46,XX,add (11) (q23) [7]/46,XX[9]
S3	F	82	8%	20.63	46,XY

First People's Hospital. Genomic DNA samples for whole genome sequencing were obtained from bone marrow samples and matching buccal mucosa at primary diagnosis. DNA was extracted through traditional phenol-chloroform method.

Sequencing

Genomic DNA libraries were prepared following Illumina's (Illumina, San Diego, CA, USA) protocol. Briefly, for each sample, DNA was firstly sheared into fragments of about 350 bp with the Covaris system. The fragments were then end-repaired, A-tailed, ligated to paired-end adaptors and PCR amplified for library construction. The resulting DNA libraries were subjected to 150 bp pair-end sequencing on the Illumina HiSeq X platform (Illumina, San Diego, CA, USA). The library preparation and massively parallel sequencing were performed in Wuxi AppTec (Wuxi AppTec, Shanghai, China).

Variant Calling

The reference genome was downloaded from the UCSC (University of California, Santa Cruz, CA, USA) database (http://genome.ucsc.edu). Low-quality reads were discarded and the resulting clean data were aligned to the human reference sequence (hg19) with bwa (v0.7.8-r455)[10]. Duplicate reads were removed using Picard (http://sourceforge.net/projects/picard/). The aligned reads were sorted with SAMtools[11] and proceeded with the Genome Analysis Toolkit[12,13]. Somatic single nucleotide variations (SNVs) and indels were identified using two different software packages: muTect (v1.1.4)[14] and Strelka (v1.0.13)[15]. All variants were annotated with ANNOVAR[16].

Validation of Somatic Variants

Selected mutations were subjected to validation using PCR and Sanger sequencing. Primers for all variants were designed using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA, USA). PCR was performed with the following procedure: 95°C for 2 min, 35 cycles of 95°C for 15 sec, 60°C for 20 sec and 72°C for 30 sec, followed by 72°C for 2 min. Sanger sequencing for purified PCR products was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The resulting sequence files were manually analyzed.

Results

To identify somatic mutations related to CMML, we performed the WGS for the three pairs of bone marrow and matched buccal mucosa samples. As shown in Table II, for all six samples, a total of 806.43 Gb data were generated, achieving a minimum mean depth of 30.76.

Table II. Data summary of the whole genome sequencing.

Samples	S1		S2		S3	
	Bone marrow	Buccal mucosa	Bone marrow	Buccal mucosa	Bone marrow	Buccal mucosa
Raw reads	699,175,390	1,127,183,236	764,616,300	824,574,656	994,721,416	966,777,320
Raw data (Gb)	104.88	169.08	114.69	123.69	149.13	144.96
Clean reads	695,771,260	1,098,425,638	744,869,938	805,001,056	936,487,366	962,160,522
Mapped reads	692,867,025	832,615,604	731,557,315	708,706,115	935,098,716	877,792,694
PE mapped reads	690,906,100	831,529,998	730,346,554	707,341,346	934,669,508	877,060,730
Mean depth (×)	31.10	30.76	33.14	31.97	35.73	33.72
Coverage (%)	99.73%	99.44%	99.12%	99.27%	99.53%	98.86%

The coverage of the whole genome for each sample was all over 98%. All properly pair-end mapped sequences were used for subsequent variants detection.

As listed in Table III, a total of 2184, 5039, and 1945 somatic SNVs were identified for the three samples, respectively. The number of SNVs in the coding DNA sequence region was 10, 31 and 8, respectively (Table IV). A total of 35, 71, and 27 somatic indels were identified for S1, S2, and S3 respectively. We focused on the variants that might affect the translation of amino acid, and according to the annotation results, a total of 22 variants were found to be protein-altering, including 1 exonic frame shift indel, 18 missense SNVs, 2 stop gain SNVs, and 1 stop loss SNV (Table V).

In the validation experiments, we focused on the mutations which have not been reported by dbSNP (version 144) or the catalog of the somatic mutations in cancer (COSMIC) database (version 75, http://cancer.sanger.ac.uk/cosmic) before. After filtering these variants, five SNVs were remained and subjected to PCR and Sanger sequencing. Three SNVs in gene *AKAP4*, *COL2A1*, and *MAML1*were validated successfully (Figure 1).

Discussion

We performed whole genome sequencing of bone marrow samples and corresponding buccal mucosa from three Chinese CMML patients to identify key somatic mutations contributing to the progression of this disease.

We detected 10, 31 and 8 somatic SNVs or indels in CDS of the three samples, respectively. And the number of protein-altering somatic SNVs or indels in the three samples was 6, 9, and 6, respectively. These numbers are similar to the previous studies sequencing studies on blood cancer, including CMML, AML and MDS[8,17,18], but less than the number of other solid tumors, such as liver cancer[18] and breast cancer[19], reflecting a common character of a small number of somatic mutations in blood cancer.

Among the 22 somatic protein-altering point mutations, 6 SNVs and 1 frameshift Indel have been included in the COSMIC database. Specifically, rs121434596 in *NRAS* has been confirmed as a somatic mutation related to T-cell precursor AML[20]. The Indel in *ASXL1* was reported to be related to CMML[21] and the progression of MDS[22] while rs150637282 in *DSPP* was considered to be associated with multiple myeloma[23]. Our results confirmed their involvement in the pathogenesis of blood cancer.

We focused on the novel variants and successfully validated three missense SNVs in *AKAP4*, *COL2A1*, and *MAML1*, respectively. The protein encoded by *AKAP4* is a member of the A-kinase anchor protein (AKAP) family. Previous studies have demonstrated the association betweenAKAP4 expression and cervical cancer[24], breast cancer[25]. It was also reported as a potential therapeutic target for colorectal cancer[26] and ovarian serous carcinoma[27]. In addition, it is a cancer testis antigen for multiple myeloma[28]. Therefore, although the relationship between this gene and CMML has not been reported before, it might also contribute to the pathogenesis of this disease. The protein encoded by *COL2A1* is the alpha-1 chain of type II collagen, which is mainly found in cartilage and the vitreous humor of the eye. The mutations of this gene

Table III. Summary of detected somatic SNPs in all sequencing samples.

Sample	S1	S2	S3
CDS	10	31	8
Synonymous_SNP	4	22	2
Missense_SNP	6	7	5
Stop gain	0	1	1
Stop loss	0	1	0
intronic	664	1600	549
UTR3	8	31	5
UTR5	0	7	2
Splicing	0	3	1
ncRNA_exonic	9	14	6
ncRNA_intronic	143	353	149
ncRNA_UTR3	1	0	1
ncRNA_UTR5	0	1	0
ncRNA_splicing	0	0	0
Upstream	22	31	5
Downstream	11	25	16
Intergenic	1316	2943	1203
Total	2184	5039	1945

Table IV. Summary of detected somatic INDELs in all sequencing samples.

Sample	S1	S2	S3
CDS	0	1	0
Frameshift_insertion	0	1	0
Intronic	4	22	2
ncRNA_intronic	0	5	0
Downstream	1	0	1
Intergenic	30	43	24
Total	35	71	27

Table V. Identified protein-altering somatic variations in sequencing samples.

Chromosome	Position	Ref	Alt	Mutation type	AA change	Gene	Transcript	dbsnp144	Cosmic ID
chr1	115258744	C	T	Missense SNV	p.G13D	NRAS	NM_002524	rs121434596	COSM573
chr1	153066067	C	T	Missense SNV	p.C54Y	SPRR2E	NM_001024209	rs75229137	COSM225968
chr1	201180652	A	G	Missense SNV	p.K2211E	IGFN1	NM_001164586	rs201816054	–
chr12	48391409	G	C	Missense SNV	p.G102R	COL2A1	NM_033150	–	–
chr19	22574898	C	T	Missense SNV	p.C380Y	ZNF98	NM_001098626	rs1835996	–
chr19	22940232	G	T	Missense SNV	p.Q827K	ZNF99	NM_001080409	–	–
chr19	56029340	A	C	Missense SNV	p.T1233P	SSC5D	NM_001144950	–	–
chr19	56499279	G	C	Stop loss SNV	p.X1049Y	NLRP8	NM_176811	rs306457	–
chr20	31022441	A	AG	Frameshift INDEL	p.G642fs	ASXL1	NM_015338	-	COSM1738035
chr3	195513443	T	G	Missense SNV	p.T1670P	MUC4	NM_018406	rs201054227	–
chr4	88536899	A	G	Missense SNV	p.N1029D	DSPP	NM_014208	rs150637282	COSM1235561
chr5	41061715	C	T	Stop gain SNV	p.W191X	MROH2B	NM_173489	rs1023840	–
chr5	179201783	G	A	Missense SNV	p.V986M	MAML1	NM_014757	–	–
chr6	31324143	G	T	Stop gain SNV	p.Y140X	HLA-B	NM_005514	rs12697944	–
chr6	36297893	C	T	Missense SNV	p.R192H	C6orf222	NM_001010903	rs41272160	–
chr7	75124676	G	A	Missense SNV	p.G81D	SPDYE5	NM_001099435	rs28422159	–
chr9	43915558	G	C	Missense SNV	p.R1214P	CNTNAP3B	NM_001201380	–	COSM4593893
chr9	141107539	A	G	Missense SNV	p.Q74R	FAM157B	NM_001145249	rs4470981	–
chrX	6451886	A	G	Missense SNV	p.L154P	VCX3A	NM_016379	rs143802324	COSM3694686
chrX	24382453	G	C	Missense SNV	p.A526P	SUPT20HL1	NM_001136234	rs112697166	–
chrX	37028375	G	A	Missense SNV	p.R631H	FAM47C	NM_001013736	–	COSM457461
chrX	49958916	G	A	Missense SNV	p.A150T	AKAP4	NM_003886	–	–

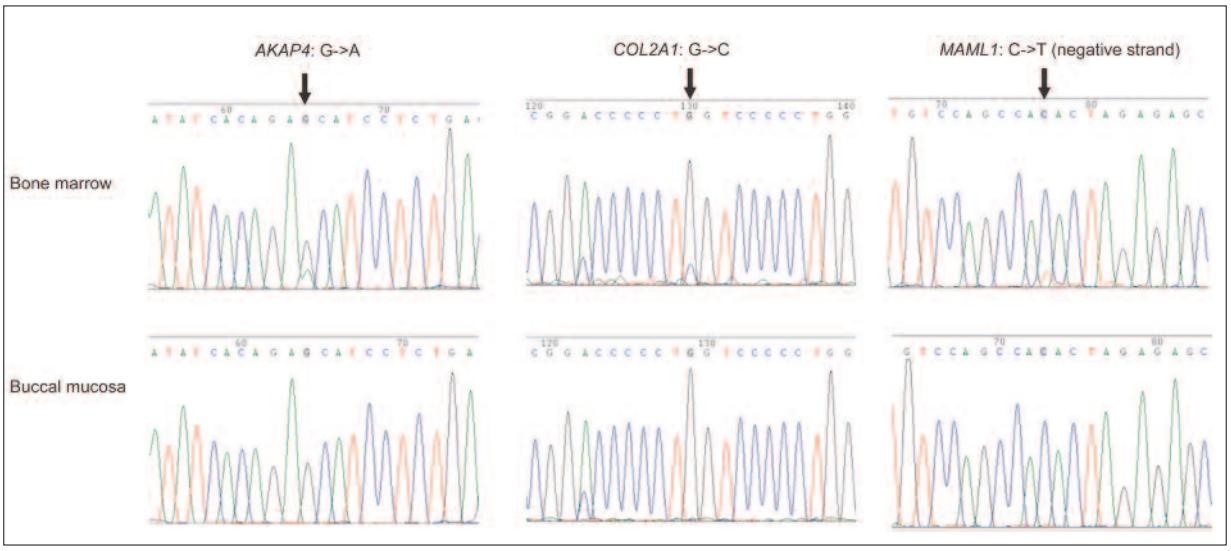

Figure 1. Sanger sequencing results for the three new identified variants. The arrows indicate the positions of the missense SNVs.

have been associated with chondrosarcoma[29], and its expression predicts tumor recurrence in high-grade ovarian cancer[30]. Our results suggested that somatic mutation of this gene may be related to CMML. *MAML1* is one of the Notch signaling pathway genes and mutations of this gene have been related to myeloid progenitor differentiation in CMML[31]. Here we reported a new missense mutation of *MAML1*. This is the only protein-altering mutation we detected in this gene. These new missense mutations we identified here may serve as a potential target for future investigations.

Conclusions

WGS of three CMML patients and further Sanger sequencing validation were carried out to identify new variants which might contribute to the CMML pathogenesis. We successfully validated three novel missense SNVs in *AKAP4*, *COL2A1*, and *MAML1*, respectively. Therefore, WGS analyzes provided us a new insight into the molecular events governing the pathogenesis of CMML. The somatic variants that we reported here may provide new targets for further therapeutic studies.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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