ORIGINAL ARTICLE

Cytogenomic Profiling of Chronic Lymphocytic Leukaemia Patients Using DNA Microarray

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ABSTRACT

Introduction: Chronic lymphocytic leukaemia (CLL) is the most frequent adult leukaemia in the Western world. The clinical presentation varies greatly, from very indolent cases to those with aggressive and fast advancing disease. This variation has significant implications for clinical approaches, therapeutic tactics, and, ultimately, survival durations from diagnosis. Acquired chromosomal aberrations play a key role in CLL aetiology. Due to difficulty to obtain abnormal metaphases for analysis, few methods such as fluorescence *in-situ* hybridization (FISH) and multiplex ligation-dependent probe assay (MLPA) were employed to detect chromosomal aberration however the methods are limited to specific locus only. Thus, this study is aimed to detect the chromosomal aberrations using DNA microarray platform. **Methods:** In this retrospective study, DNA archive obtained from 7 CLL patients which collected at diagnosis and subjected to Affymetrix CytoScan® 750K single nucleotide polymorphism (SNP) array following the manufacture procedure. The raw data obtained were analysed using the Chromosome Analysis Suite (ChAS) software (Affymetrix) using annotations of genome version GRCh38 (hg38). **Result:** Out of 7 patients, 4 of them showing deletion of 13q while 3 of them showing deletion of 14q in various region . Some of the deleted loci were too small (0.42-0.6Mb) to be detected by conventional cytogenetic analysis (CCA). There was also the presence of additional chromosomal aberrations that could be missed by CCA, FISH, or MLPA due to cryptic deletion or duplication that was as small as 0.4MB in size. **Conclusion:** The present study showed that low resolution chromosomal aberration was able to be detected using DNA microarray platform in comparison to CCA, FISH and MLPA.
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INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is a lymphoproliferative disorder characterised by the proliferation of neoplastic mature B lymphocytes in the blood, bone marrow, lymph nodes, and spleen (1, 2). CLL is the most common leukaemia in adults in developed nations, accounting for 5%–11% of lymphoproliferative disorders. CLL affects approximately 4 to 6 persons per 100,000 population each year. This condition is most prevalent in persons in their sixties and seventies, with 70% of patients being over the age of 65. According to the America National Cancer Institute, roughly 3930 people died of CLL in 2019, accounting for 0.6% of all cancers. Males are more prone to CLL than females (M: F ratio of 1.5 - 2.1:1)(3).

In comparison, this disease is uncommon in Asia, especially the Far East, and is perpetuated by migrants and their descendants (4). CLL accounted for between 1% to 3% of lymphoproliferative diseases in Asian countries. According to the Malaysian National Cancer Registry's 2007-2011 report, the total number of patients diagnosed with chronic lymphocytic leukaemia was 124 for that five-year period, meaning that Malaysia has an average of 24.8 newly diagnosed CLL cases per year (5).

Newly diagnosed CLL patients had a wide variety of clinical presentations, ranging from a relatively indolent disease with a near-average life expectancy to a rapidly developing disease with early mortality(6). The majority of CLL cases present with no symptoms and were typically watched until signs such as cytopenia, lymphadenopathy, and splenomegaly manifest. Some patients may transformed into an aggressive form of B lymphocyte cancer, such as diffuse large B cell lymphoma (DLBCL) or, less frequently, Hodgkin lymphoma or other aggressive lymphoma (1).

CLL was diagnosed using a complete blood count with lymphocytes count greater than $5x10^9$/ L, peripheral

blood/bone marrow/trephine biopsy morphology and immunophenotyping with peripheral blood/marrow cytogenetics, as well as clinical examination to determine nodal involvement (1, 2, 7). Clinicians primarily employ established risk stratification for CLL at the time of diagnosis in the form of modified Rai classifications and/or the Binet classification using haematological markers such as total lymphocytes count, haemoglobin level and platelet count as well as number of affected nodal area. The modified Rai classifications placed an emphasis on lymphocyte count, nodal involvement, and organ involvement, whereas the Binet classification placed an emphasis on haemoglobin level, platelet count, and the number of affected nodal areas. However, the information gleaned from these categories following a patient's CLL diagnosis cannot be used to foretell disease development in any individuals.

Numerous factors contributing to CLL heterogeneity have been identified through studies conducted throughout the years (8). CLL development can be viewed as a cooperative effort between risk factors and genetic abnormalities. Genetic aberration has a significant impact on the prognosis and course of CLL in patients (9). Multiple studies have been undertaken in an attempt to identify risk factors for CLL development, however no single acquired risk factor for CLL development has been identified yet. There is substantial evidence that CLL can be inherited (10). A family history of haematological malignancy (CLL and/or non-Hodgkin lymphoma (NHL)) is also considered as a host factor. According to Slager et al., relatives of CLL patients have a two- to eightfold increased risk of having CLL and a twofold increased risk of developing NHL (11). Goldin et al. complement this conclusion by claiming that familial CLL is more frequently identified at a younger age than random CLL (12). Additionally, there are case reports involving familial CLL, which occurs when two or more members of the same family are affected by the disease.

While CLL is more prevalent in Western countries than in Asian countries, Asian patients have a more rapid disease development and a shorter time to first treatment (TTFT) (13). This phenomenon was hypothesised to arise as a result of the Asian population's presence of different biomarkers and susceptibility factors. Numerous case reports established that Asian CLL patients have a variety of distinct chromosomal abnormalities compared to Western CLL patients (4). The most common copy number variants in Western and Asian CLL are deletion of long arm of chromosome 13 (del(13q)) at 13q14 region, deletion of short arm of chromosome 17 (del(17p)), deletions of long arm of chromosome 11 (del(11q)) and trisomy 12 (15). Additionally, Kawamata et al. discovered that Asian CLL patients were more likely to have trisomy 3q or trisomy 18/dup 18q, but none of these chromosomal aberrations were identified in Western CLL patients. Wu and colleagues also discovered that Asian CLL patients had a greater rate of TP53 mutations than Western CLL patients (15).

In comparison to the earlier modified Rai and Binet risk classifications, the most recent CLL risk stratification (CLL-IPI) proposed by the International CLL IPI working group in 2016 takes genetic abnormalities into account (7). This inclusion highlighted the importance of early detection of chromosomal aberrations in order to improve patient monitoring and treatment.

Up to 80% of CLL patients have chromosomal abnormalities (16). Among them, it has been demonstrated that del(11q), del(13q), del(17p), and trisomy 12 are predictive and play a critical role in CLL pathogenesis and evolution, influencing patient outcome and treatment modalities. In comparison to acute leukaemia or myelodysplastic syndromes, only a few studies have examined conventional cytogenetic analysis in CLL, owing to the disease's limited in vitro proliferation activity even in the presence of B-cell mitogens. This insufficient proliferative activity of mature neoplastic B cells resulted in either complete inhibition of metaphase production or the detection of clonal aberrations in 40–70% of cases due to poor quality metaphases or because normal haematopoietic cells proliferated in vitro but CLL cells did not (9).

For the last decade, both fluorescence in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) have been used to diagnose CLL in addition to CCA. Despite their excellent sensitivity, both approaches are limited to well-characterized genomic areas (17, 18). Cytogenomic profiling of CLL patients using a microarray platform is gaining popularity as a diagnostic technique due to the array's high resolution for examining cryptic abnormalities in copy number variants (18).

The purpose of this study was to investigate chromosomal aberrations in newly diagnosed CLL patients and to stratify them according to their chromosomal aberrations and initial clinical presentation. As there is a dearth of data on chromosomal aberration in CLL patients in Malaysia due to the disease's lower prevalence than in Western counterparts and the majority of patients being asymptomatic, this study will serve as a pilot study to collect data on chromosomal aberration in CLL patients using a DNA microarray platform in Malaysia to assist clinicians in providing better treatment and counselling to patients and their relatives in explaining the disease and its risk on the relatives. This platform also helps us to detect additional cytogenetic aberrations in normal karyotype CLL patients by CCAs.

MATERIALS AND METHODS

Samples

This is a retrospective study involving 7 patients who were newly diagnosed with CLL from January 2012

to June 2012 using their archive DNA materials from previous study. All the patients recruited fulfilled the diagnostic criteria for chronic lymphocytic leukaemia by International Workshop of Chronic Lymphocytic Leukemia (iwCLL) 2008. Detailed information was extracted from patients' cytogenetic request form and medical records including age of diagnosis, sociodemographic data, clinical presentations and relevant laboratory results.

DNA extraction

Archive genetic materials (DNA material) was extracted from peripheral blood using commercialized QIAGEN QIAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), following manufacturer's protocol previous study (USM Short Term grant title: Analysis of Hypermethylation Status of Tumor Suppressor Genes p16INK4a, p15INK4b, ADAM12 and PCDHGB7 in Chronic Lymphocytic Leukemia Patients and Normal Individuals)(JEPeM code: USMKK/PPP/JEPeM[234.3(07)).The QIAmp Blood Mini Kit is designed to extract the total genomic DNA without any prior separation of leukocytes from 200µl fresh whole blood which had been treated with EDTA. After extraction, the pure genomic DNA was stored at -20°C until needed.

Qualitative and quantitative estimation of DNA

The quantity and quality of archived DNA materials were measured using NanoQuant Plate™ (Tecan Austria), Invitrogen™ Qubit™ Fluorometer (Fisher Scientific USA) and followed by gel electrophoresis. The minimum concentration needed for DNA microarray is 50ng/ µL and DNA purity ranging from 1.8-2.0.

Cytogenomic profiling data using Cytoscan 750K Array

Out of 7 samples, 2 samples were outsourced to a private laboratory, GenomixLAB® for analysis while the rest were processed in Human Genome Centre, Universiti Sains Malaysia. However, during this research period, some parts of the microarray system were broken down. Further technical process i.e hybridization, washing and chip scanning was performed in collaboration with Research Instruments Sdn Bhd.

Archive DNA (genomic DNA) were analysed using Affymetrix CytoScan® 750K single nucleotide polymorphism (SNP) array. Initially, the genomic DNA was digested by Nsp1 and amplified using a ligation mediated PCR with adapters covalently linked to the restrictive fragments.

PCR products obtained on the previous day were purified using magnetic beads, fragmented using DNase1, labelled with biotin and were hybridized overnight for 17 hours to a 49-format array.

After completion of hybridization, the array was washed and stained with streptavidin using GeneChip Fluidic Station 450. Finally, arrays were scanned by GeneChip Scanner 3000, using GeneChip Command Console Software (Thermo Fisher Scientific) to generate the CEL files that included the intensity probe signal.

Data Analysis

CEL files were analysed using the Chromosome Analysis Suite (ChAS) software package (Affymetrix) and converted to CYCHP files containing information on copy number, loss of heterozygosity (LOH) and mosaicism cells and reported using UCSC Human Genome Build 38 (NCBI build 38, Dec 2013). The genes involved in the deleted region were analysed against Online Mendelian Inheritance in Man (OMIM), ClinVar-NCBI and Catalogue of Somatic Mutations In Cancer (COSMIC) to find its clinical significance.

Ethical clearance

This study was approved by Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/20060302).

RESULTS

Among 7 patients included in this study, five (71.4%) were males while two (28.6%) were female patients with their age ranging from 53 – 78 years old. Mean age at the diagnosis was 65.1 years old ± 8.15. All the patients recruited in this present study were of Malay ethnicity. Most of them (71.4%) were diagnosed accidentally during annual medical check-up and the rest were diagnosed as they seek treatment for their lymphadenopathy. Six of them (85.7%) were classified as low risk group while 1 patient was classified as intermediate risk group according to modified RAI and Binet score (Table I).

At presentation, all patients exhibited lymphocytosis, ranging from 30-50x10^9/L However, out of 7 patients, only 2 (28.6%) of them had concurrent lymphadenopathy and hepatomegaly, 1 (14.3%) had concurrent lymphadenopathy and splenomegaly while another (14.3%) had concurrent lymphadenopathy only (Table I). Interestingly, none of patients were having anaemia and thrombocytopaenia at initial presentation during diagnosis.

Out of the 7 patients, only 4 (57.1%) were proceeded with conventional cytogenetic analysis, the remaining three were not able to be tested due to unavoidable issue such as insufficient bone marrow particle for culture or patient refusal to do bone marrow (Table I). However, in view of CLL is made up of mature B cell clones that were difficult to grow in conventional bone marrow media, all the patients did not have adequate and analysable metaphases for diagnosis. For CLL culture, stimulators such as interleukin-2 (IL-2) and Cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODN) should be added to the conventional bone marrow culture to create suitable environment for mature B cells in CLL to grow. It had to be noted that in year 2012, the

Table 1: Clinical presentation of CLL patients

	P1	P2	P3	P4	P5	P6	P7
Age	70	59	65	69	53	78	62
Race	Malay	Malay	Malay	Malay	Malay	Malay	Malay
Gender	M	M	M	M	F	M	F
Clinical Presentation							
Lymphocytosis (>5x10^9/L)	Present	Present	Present	Present	Present	Present	Present
Lymphadenopathy							
< 3 area involvement	Present	Present	Nil	Nil	Nil	Present	Present
>3 area involvement	Yes	Yes				Yes	Yes
Splenomegaly							
Hepatomegaly	Nil	Nil	Nil	Nil	Nil	Nil	Present
Anaemia (<11g/dl)	Present	Present	Nil	Nil	Nil	Nil	Nil
Thrombocytopaenia	Nil	Nil	Nil	Nil	Nil	Nil	Nil
(<100x10^9/L)	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Immunological Markers							
Type of light chain	κ	κ	κ	κ	κ	κ	λ
CD5 expression	Present	Present	Present	Present	Present	Present	Present
CD23 expression	Present	Present	Present	Present	Present	Present	Present
Dim20 expression	Present	Present	Present	Present	Present	Present	Present
Dim79b expression	Present	Present	Present	Present	Present	Present	Present
Low sIg	Present	Present	Present	Present	Present	Present	Present
Disease stage	RAI I/ BINET A	RAI I /BINET A	RAI 0 /BINET A	RAI 0 /BINET A	RAI 0 /BINET A	RAI I /BINET A	RAI II /BINET B
Cytogenetic result	N/A	NMS	N/A	NMS	NMS	NMS	N/A

*F=female, κ = Kappa light chain, λ= lambda light chain, M=Male, N/A=Not available, NMS=no metaphase spread, sIg= surface immunoglobulin

stimulants were unavailable in most centres in Malaysia. Diagnosis of this disease was done using the bone marrow sample, peripheral blood and/or lymph node biopsy. All the samples were done immunophenotyping to determine the type of lymphoproliferative disorders. All patients in this study exhibited low surface immunoglobulin, low CD20 and CD79b signals, expression of CD5 and CD23 (except in 2 patients) and monoclonal light chain expression (Table I). These immunological findings are consistent with the diagnosis of CLL as recommended by the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) guidelines.

In this present study, microarray analysis showed del(13q) in 4 patients (57.1%) and 3 patients (42.9%) showed del(14q) in variable sizes. Most of patients had additional chromosome abnormalities as listed in the table II except for 3 patients who had sole abnormality of either del(13q) only (Patient 3) or del(14q) only (Patient 2 and Patient 4) . All the genes involved in the deleted regions were analysed against OMIM, ClinVar-NCBI and COSMIC to detect the clinical significance to CLL patients. In patients with del(13q), only Patient 7 (Fig 4A) showed a deletion of 4.7Mb of chromosome 13 at 13q14 region. Del(13q) at 13q14.3 region which also can be detected with high resolution CCA, involving deletion of *LRCH1, ESD, HTR2A, SUCLA2, NUDT15, MED4, ITM2B, RB1, LPAR6, RCBTB2, CYSLTR2, FNDC3A, MLNR, CAB39L, SETDB2, PHF11, RCBTB1, ARL11, EBPL, KPNA3, SPRYD7, DLEU2, TRIM13, KCNRG , MIR16-1, MIR15A, DLEU1, DLEU7, RNASEH2B, GUCY1B2*. The other 3 patients (Patient 1 (Fig 1A),3 (Fig 2A) and 6 (Fig 3B)) were having interstitial deletion of chromosome 13q14.2-q14.3 region with deletion size ranging 1.07Mb to 2.14Mb which can be considered as a cryptic deletion of long arm of chromosome 13 and most of times will be missed by CCA due to poor resolution. The genes involved in this deleted region include *SPRYD7, DLEU2, TRIM13, KCNRG, MIR16-1, MIR-15A, DLEU1, DLEU7* and *RNASEH2B*.

For patients with del(14q), 2 of them (Patient 4 (Fig 2B) and 5 (Fig 3A)) were having cryptic interstitial deletion of chromosome 14q32.33 region ranging 0.42Mb and 0.45Mb. While in Patient 2 (Fig 1B), a deletion of 39.97Mb was detected on the long arm of chromosome 14 at 14q23.3-14q32.33 region involving deletion of genes; *LRCH1, ESD, HTR2A, SUCLA2, NUDT15, MED4, ITM2B, RB1, LPAR6, RCBTB2, CYSLTR2, FNDC3A, MLNR, CAB39L, SETDB2, PHF11, RCBTB1, ARL11, EBPL, KPNA3, SPRYD7, DLEU2, TRIM13, KCNRG, MIR16-1, MIR-15A, DLEU1, DLEU7, RNASEH2B, GUCY1B2, MEG3, RTL1, MIR-431, MIR-433, MIR-127, MIR-136, MEG8, SNORD11 2, MIR-370, SNORD11 3-1, SNORD11 4-1, MIR-379, MIR-380, MIR-494, MIR-495, MIR-376C, MIR-376A2, MIR-376B, MIR-376A1, MIR-487B, MIR-134, MIR-485, MIR-409, MIR-369, MIR-410, MIR-656, DIO3OS, DIO3* and *PPP2R5C*. This large deletion of long arm of chromosome 14 usually will be detected by CCA as CCA usually is able to detect any chromosomal abnormalities more than 5Mb in resolution.

Additional chromosomal abnormalities detected included trisomy 3 (Fig 1A), deletion of chromosome Xp22.33 (0.65MB) (Fig 3A) involving deletion of *PLCXD1, GTPBP6, PPP2R3B* and *SHOX* genes and deletion of chromosome 16p11.2 (1.96Mb) (Fig 4A) involving deletion of *TP53TG3*. In two separate patients, detection of abnormal gain 0.6Mb of chromosome 14q32.33 (Fig

Table II: Details of microarray-based genomic profiling

ID	Region involved	Chromosomal aberration	Size (MB)	ISCN (2016)
P1	3p26.3-3q29 13q14.2-13q14.3 14q32.33	Trisomy 3 Deletion 13q14.2-13q14.3 Duplication 14q32.33	106.2Mb 2.14Mb 0.6Mb	arr[GRCh38] 3p26.3p25.3(20213_9593787)x3, 3p25.3p25.2(10173103_12360924)x3, 3p25.2q11.2(12658109_98110407)x3, 3q11.2q12.2(98189832_100404275)x3, 3q12.2q21.2(100794120_125603408)x3, 3q21.2q22.3(125954527_136448485) x3, 3q22.3q25.1(136889225_151776103)x3, 3q25. 2q25.31(152885161_155893222)x3, 3q25.31q26.2(155970194_169514638)x3, 3q26.2q26.32(169591410_179189610) x3, 3q26.33q29(179680828_196050232)x3, 3q29(197099539_197799157)x3, 13q14.2(47078817_48312627)x1, 13q14.2q14.3(49899420_50809130)x1, 14q32.33(105659007_106266356)x3
P2	14q23.3-14q32.33	Deletion 14q23.3-14q32.33	39.97Mb	arr[GRCh38] 14q23.3q32.2(65677061_100727766)x1, 14q32. 2q32.33(100747047_105666418)x1
P3	13q14.2-13q14.3	Deletion 13q14.2-13q14.3	1.78Mb	arr[GRCh38] 13q14.2q14.3(49824769_50925678)x1, 14q32.33(106042381_106716799)x1
P4	14q32.33	Deletion 14q32.33	0.45Mb	arr[GRCh38] 14q32.33(106320702_106769691)x1
P5	14q32.33 Xp22.33	Deletion 14q32.33 Deletion Xp22.33	0.42Mb 0.65Mb	arr[GRCh38] 14q32.33(106260745_106685105)x1, Xp22.33(251887_901725)x1
P6	13q14.2-q14.3 22q11.2	Deletion 13q14.2-13q14.3 Duplication 22q11.2	1.07Mb 0.42Mb	arr[GRCh38] 13q14.2q14.3(49899421_50971829)x1, 22q11.22(22426974_22848394)x3
P7	13q14.13-13q14.3 16p11.2	Deletion 13q14.13-13q14.3 Deletion 16p11.2	4.7Mb 1.96 Mb	arr[GRCh38] 13q14.13q14.3(46338221_51049259)x1, 16p11.2(32013068_33979395)x1

Figure 1: (A) Whole genome view of P1. Presence of extra copy of chromosome 3 (trisomy 3), cryptic deletion of chromosome 13q14.2-13q14.3 and duplication of 14q32.33. (B) Whole genome view of P2. Presence of deletion of chromosome 14q23.3-14q32.33

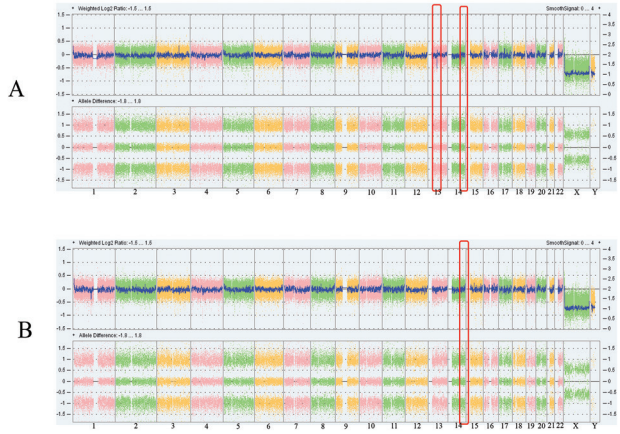

Figure 2: (A) Whole genome view of P3. Presence of cryptic deletion of chromosome 13q14.2-13q14.3. (B)Whole genome view of P4. Presence of cryptic deletion of chromosome 14q32.33.

1A) and 0.42Mb of chromosome 22q11.2 (Fig 3B) were observed. The gain in chromosome 22q11.2 involved *PRAME*, *GGTLC2* and *MIR-650*.

DISCUSSION

In the present study, there was a male preponderance accounting for 71.4% of the study subjects compared to female CLL patients. This finding is in concordance with the Cancer Statistic 2020, published by America Cancer Society which reported that the incidence of CLL among males is slightly higher compared to females (3). The population of Malaysia especially in the Kelantan state, is predominantly of Malay ethnicity and accounts for approximately 69.6% of the overall population including indigenous group (19). This explains why all the recruited patients in the current study were of Malay ethnicity.

Patients who were newly diagnosed with CLL exhibit a wide variety of clinical manifestations. Each patient's CLL status was different, ranging from a reasonably slow-growing cancer with a near-average life expectancy to a fast-progressing disease with an early mortality (1, 9). It is worth noting that in this present study, most patients presented asymptomatic and were identified with lymphocytosis during their annual medical check-up. Only a few of them sought therapy for enlarging lymph

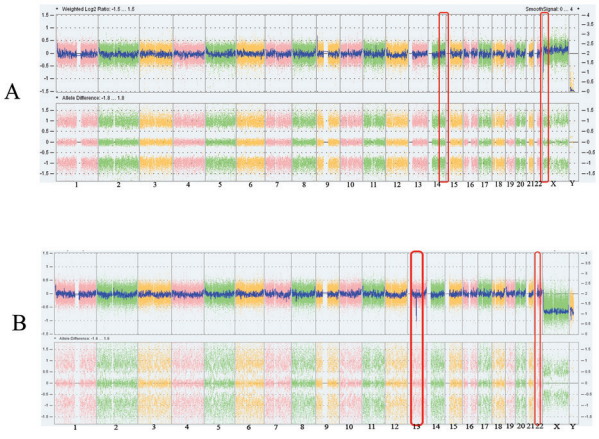

Figure 3: (A) Whole genome view of P5. Presence of cryptic deletion of chromosome 14q32.33 and chromosome Xp22.33. (B) Whole genome view of P6. Presence of cryptic deletion of chromosome 13q14.2 – 13q14.3 and duplication of chromosome 22q11.2.

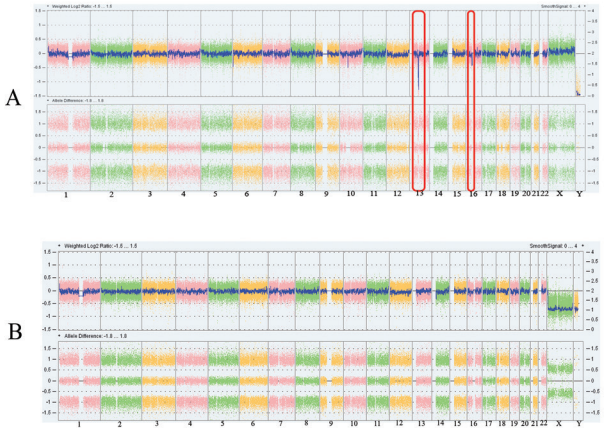

Figure 4: (A) Whole genome view of P7. Presence of cryptic deletion of chromosome 13q14.13-13q14.3 and cryptic deletion of chromosome 16p11.2. (B) Whole genome of normal male control

nodes. Several of them developed splenomegaly or hepatomegaly in addition to lymphadenopathy detected during clinical examination. With the exception of one patient who was classified as Rai Stage II and Binet B, the majority of patients were classified as Rai Stage 0-1 and Binet A. This observation is consistent with the patient's clinical stage of chronic lymphocytic leukaemia as determined by the modified Rai and Binet scores. Using CLL risk classification, all patients were categorized as low risk, except for one who is classified as intermediate risk. However, it is necessary to keep in mind that the modified Rai and Binet scoring systems are ineffective at predicting CLL disease progression in the early stages.

At the time of initial clinical presentation in this study, none of the patients had anaemia. This finding was favourable for the patient. Anaemia plays a critical role in promoting the angiogenic processes involved in the progression of CLL, as altered tissue oxygenation caused by anaemia results in an increased production of angiogenic cytokines (vascular endothelial growth factor, basic fibroblast growth factor) by malignant B cell clones or bone marrow microenvironment cells (20). Numerous investigations have revealed that an increase in bone marrow microvessels, as well as increased circulating vascular endothelial growth factor levels, correspond with biological characteristics associated with poor prognosis. Clinically, anaemia and thrombocytopaenia had detrimental effects on the quality of life of patients with CLL, increases the likelihood of having blood transfusions, and eventually has a detrimental effect on overall survival (21).

CLL was diagnosed by utilising immunophenotyping of blood, bone marrow, and lymph node biopsies in conjunction with a complete blood count. Typically, a complete blood count reveals a predominance of lymphocytosis of at least 5 x 10^9/L. All of the patients in this study displayed CD5+, CD23+, dim CD79b, dim CD20, and low surface immunoglobulin (sIg) (Table I). All of these markers are diagnostic of CLL and help to distinguish it from other mature B cell lymphomas. Clonal malignant B cells in CLL were expressed either kappa or lambda immunoglobulin light chains only.

CLL is defined by a spectrum of genomic instability manifested by cytogenomic aberrations (CA) ranging from numerical aberrations to genetic alterations. CA is present in 80% of CLL cases worldwide. The common CA in CLL consists of del(13q), del(11q), del(17p) and trisomy 12 (1, 9). All the common chromosomal aberrations can be detected using conventional cytogenetic analysis (CCA), fluorescence in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA). FISH/interphase FISH is gold standard in diagnosing CLL CAs due to limitation of CCA to yield good analysable metaphases. However, FISH and MLPA is limited to their own specific loci detection and unable to detect other CAs that could present in the patients. Genomic profiling using DNA microarray platform is gaining popularity as a diagnostic technique due to the array's high resolution which can detect as low as 25-50kb copy number changes for cryptic abnormalities examination in copy number variants in cancer cases including CLL, compared to CCA which can only detect chromosomal aberrations of more than 5Mb.

The microarray data from this study demonstrated that the CAs pattern in CLL patients might be exceedingly heterogeneous. The majority of these low-risk CLL group, which was determined by modified Rai and Binet score, had a single chromosomal abnormality, whereas those in the intermediate risk group had two or more as shown in table II. In this study, deletion of long arm of chromosome 13 (del(13q)) was detected in 4 patients (57.1%) and 3 patients had deletion of long arm of chromosome 14 (del(14q)) (42.9%) in variable size, detected by Affymetrix CytoScan 750K array. It

also detected several CAs such as trisomy 3 and cryptic CAs such as microduplication of 14q32.33 and 22q11.2 and microdeletion of 16p11.2 and Xp22.33 as shown in Table II. This finding proved high sensitivity of this microarray platform to detect cryptic CA as small as 0.42Mb in size compared to CCA which can only detect CA at 5 to 10Mb in resolution (22).

Del(13q) was observed in 4 patients in variable break point deletions involving region 13q14.13 to 13q14.3. Del(13q) is the most frequent cytogenetic abnormality and is regarded to be a favourable prognostic factor in CLL as sole aberration (23, 24) and is frequently associated with indolent disease course compared to other CAs (25). However, only one patient had sole abnormality of deletion 13q while the other three patients had additional chromosomal abnormalities such as trisomy 3, duplication of 14q, deletion of 16p and duplication of 22q as mentioned in Table II. The implication of having these CAs will be discussed later. Del(13q) has been implicated in the acceleration of B cell proliferation in CLL. *DLEU1* and *DLEU2*, which are situated in the minimal deleted region (MDR) of chromosome 13, as well as the miR15a/mir16-1 microRNA cluster, were associated with CLL disease progression. The deletion of the *DLEU2/miR15A/miR16-1* locus, which encodes negative regulators of BCL2 expression, results in an inability to regulate the gene expression governing cell cycle progression (23). Parker et al. discovered that two breakpoint cluster regions (BCRs), the proximal BCR, which targets *C13orf1, TRIM13, KCNRG*, and exons 7–11 of the DLEU2 gene, and the distal BCR, which targets *NASEH2B* and *GUCY1B2*, are strongly linked to CLL progression (25).

Further classification of del(13q) is possible. Class I deletions are restricted to a 2-Mb region where breakpoints frequently occur in the two BCRs found, which include *FLJ31945, FAM10A4, BCMS*, and *DLEU7* in addition to the genes within the MDR. While in class II deletions, del(13q) went beyond this region in either a centromeric or telomeric orientation, they included a large number of additional genes, including *SETDB2, PHF11*, and *RCBTB1*. Class II deletions raised the likelihood of disease progression considerably and were more prevalent in individuals who had a partial or no response to treatment (26). While it is frequently included in the FISH-CLL panel, the effect of treatment selection upon the detection of aberrant del(13q) in CLL patients is unknown.

Del(14q) has also been observed in this present study, reported to be rare in CLL and have an incidence rate of 1.7% compared to other mature B cell lymphomas (27). Out of 3 patients with del(14q), one of them showed deletion from 14q23.3-14q32.33 region while the other 2 patients showed cryptic deletion at 14q32.33 region. Reindl et al. found that del(14q) can be variable in size. A centromeric breakpoint cluster was detected in 14q24.1 between BAC PR11-35D12 and BAC RP11-226F19, whereas the most frequent telomeric breakpoint was identified in the IGH locus (14q32.3) between the IGH 3′-flanking probe and the IGHV probe (27). A similar finding was also supported by studies done by Cosson et al. and Nguyen-Khac et al.(28,29) .They also found that recurrent interstitial deletion of 14q24.1-14q32.3 region was usually associated with prognostic factors for prognosis in CLL such as mutated *NOTCH1*, and unmutated IGHV status, with an overrepresentation of the V1-69 gene, causing shorter time of survival compared to other CAs but better than deletion of 17p.

Numerous leukaemia and lymphomas are genetically associated with the immunoglobulin heavy chain locus (IGHV), which is located on chromosome 14 at 14q32 region. 14q32 abnormalities, notably deletions within the area, can occur in CLL and are difficult to detect with karyotypic analysis. Deletion of 14q32 may involve deletion of *FAM30A, ADAM6, LINC00226*, and *LINC00221* that are encoded within the region. *FAM30A* is a long non-coding RNA associated with the immune system that is significantly expressed in B cells and differentially expressed in oral diseases. ADAM6 (ADAM metallopeptidase domain 6, pseudogene) has been recently suggested as a potential marker for the miRNA-lncRNA-mRNA interaction network and as a potential therapeutic target in lung cancer. It plays a role in cell adhesion, migration, proteolysis, and cell signalling and has been linked to colorectal (30) and melanoma cancers (31).

Long non-coding RNAs (lncRNAs) have been shown to express differentially during the genesis and progression of cancer. Within the 14q32 deletion areas, two lncRNAs, *LINC00226* and *LINC00221* are mapped. Study done by Harris et al. revealed the possibility of *LINC00221* to play a role in CLL pathogenesis (32). This finding was supported by Huang et al. in which *LINC00221* expression was decreased in acute myeloid leukaemia (AML) and acute lymphocytic leukaemia (ALL), and it exhibited anti-proliferative and pro-apoptotic activity in ALL (33). CLL cases with a 14q32 deletion have an intermediate time to first treatment (TTFT) that is shorter than that of CLL cases with sole 13q14 deletion but longer than that of CLL cases with an 11q22.3 (*ATM*) or 17p13 deletion (*TP53*) (32). Duplication of 14q32.33 was also detected in one patient (Fig 1A). Duplication of 14q32.33 reported in 66.7% of DLBCL patients. Even though it was reported, it did not affect the clinical outcome or relapse of disease (34). It reported to be associated with poor clinical outcome in melanoma patients (31).

Trisomy 3 in CLL is a rare chromosomal aberration in CLL. Kawamata et al. revealed that trisomy 3 or duplication of chromosome 3q are frequently found in Asian CLL but not found in Western CLL. Trisomy 3 is more commonly found in other types of mature B cell lymphoma such

as non-Hodgkin lymphoma (NHL) and marginal zone B cell lymphoma (MZBCL). BCL6 which is located at 3q27 region acts as proto-oncogenes. Normally, BCL6 protects germinal centre of B cells from premature activation and differentiation and maintains an environment that is tolerant of DNA breaks caused by immunoglobulin gene remodelling mechanisms involved in the creation of high-affinity antibodies of various isotypes (35). In trisomy 3, deregulated expression of *BCL6* postulate to contribute to lymphomagenesis. *BCL6* antagonized function of *TP53* as tumour suppressor gene by binding to two specific DNA sites within *TP53* promoter region and alters DNA damage-induced apoptotic responses in germinal-centre B cells. Deregulation of *BCL6* enables germinal-centre B lymphocytes to endure the physiological DNA breaks required for immunoglobulin class switch recombination and somatic hypermutation without generating an apoptotic response mediated by *TP53* (36).

Deletion 16p11.2 is commonly associated with autism spectrum disorder (37). However, it has also been reported to be involved in childhood AML, adult ALL and AML (38, 39) and in neuroblastoma (40). Deletion of *TP53TG3* which is encoded in chromosome 16 at segment 16p11.2 is also reported to be involved in cervical cancer pathogenesis (41). However, up until this point, there is no specific study that revealed the significance of deletion 16p11.2 in CLL or other haematological malignancies.

In this present study, cryptic deletion involving Xp22.33 region also been observed. There are two tumour suppressor gene encoded in this region namely *PPP2R3B* and *SHOX*. *PPP2R3B* function is to encode one of the subunits of protein phosphatase 2A (*PP2A*). PP2A functions as serine/threonine phosphatase that modulates a variety of intracellular signalling pathways. It is widely considered to be a tumour suppressor. *PP2A* deletion, mutation, or inactivation are widely reported in a variety of cancer types such as colorectal carcinoma, hepatocellular carcinoma and endometrial carcinoma (42). Downregulation of *PP2A* due to phosphorylation by LYN proto-oncogenes (Src family tyrosine kinase) causing overexpression of oncoprotein known as SET which prevent *PP2A* to switch off the survival signal network in CLL (43, 44). Overall, suppressing the PPP2R3B gene dosage via deletion or mutation may result in lower overall *PP2A* activity, which could promote proliferation and confer leukemic cells a survival advantage.

Short stature homeobox gene (SHOX) is one of the main growth genes in humans which is important for bone growth and maturation regulation. It can be found in the pseudoautosomal region (PAR1) of the short arm of the X and Y chromosomes. *SHOX* haploinsufficiency has since been discovered in people with a variety of phenotypes, ranging from idiopathic short stature (ISS) to Lėri-Weill dyschondrosteosis (LWD) (45). Lėri-Weill dyschondrosteosis has been identified to be associated with Hodgkin's lymphoma (46) and acute lymphocytic leukaemia (47). However, *SHOX* deletion or haploinsufficiency in CLL is still undetermined until this date.

The 22q11.2 microduplication syndrome is a very diverse disorder with phenotypes ranging from severe intellectual disability to facial dysmorphism, cardiac problems, and urogenital abnormalities. It is commonly linked to intellectual disability and developmental delay in children, as well as a variety of psychiatric and behavioural abnormalities such as attention deficit hyperactivity disorder (ADHD), autism, and other social and behavioural issues (48). Haematological disorder with duplication 22q11.2 is rarely reported. In 2011, Chang et al. reported a case of pre-B ALL with cystinuria linked with microduplication at the 22q11.21 locus (49). They postulated that overexpression of genes involved in the cell cycle and duplication in the duplicated region may have contributed to the creation of the leukemic clone in the bone marrow. Similar finding is also found in case report by Vaisvilas et al. but with a larger size of duplication (50).

Based on this cytogenomic result, the majority of patients at the time of diagnosis had a single chromosomal abnormality and are classified as Binet A/RAI 0-I, which is regarded to be low risk. They typically exhibit lymphocytosis, hepatomegaly, splenomegaly, or lymphadenopathy. These clinical manifestations are frequent observations at the time of CLL diagnosis. The study conducted by Hallek et al. supports this conclusion (7).

CONCLUSION

In summary, it is imperative for us to detect the genomic aberrations in CLL patients in early diagnosis especially involving the microdeletion and microduplication of chromosomes which cannot be detected by CCA or FISH. This study proves the importance of using DNA microarray platform as supplementary method in diagnosing CAs especially in "normal karyotype" CLL patients. It will help the clinician to make precise risk stratification for each patient according to disease outcome and to cater for individualized treatment. From this study, we also propose *LINC00221* and *PPP2R3B* as potential candidate genes for CLL together with common chromosomal aberrations in CLL such as del(13q), del(17p), trisomy 12 and del(11q).

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