Distinct immunoglobulin heavy chain variable region gene repertoire and lower frequency of del(11q) in Taiwanese patients with chronic lymphocytic leukaemia

Ying-Jung Huang,1 Ming-Chung Kuo,1,2 Hung Chang,1,2 Po-Nan Wang,1 Jin-Hou Wu,1 Yen-Min Huang,3 Ming-Chun Ma,4 Tsung-Chih Tang,1 Ching-Yuan Kuo4 and Lee-Yung Shih1,2
1Division of Haematology-Oncology, Chang Gung Memorial Hospital at Linkou, 2Chang Gung University, Taoyuan, 3Division of Haematology-Oncology, Chang Gung Memorial Hospital at Keelung, Keelung and 4Division of Haematology-Oncology, Chang Gung Memorial Hospital at Kaohsiung, Kaohsiung, Taiwan

Received 19 February 2019; accepted for publication 29 April 2019

Correspondence: Dr. Lee-Yung Shih, Division of Haematology-Oncology, Chang Gung Memorial Hospital at Linkou, 5, Fuxing Street, Guishan District, Taoyuan City 333, Taiwan.
E-mail: sly7012@adm.cgmh.org.tw

Summary

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in Western countries but very rare in Asia. Peripheral blood or bone marrow mononuclear cells obtained at initial diagnosis from 194 patients with CLL were analysed to determine the ethnic difference in genetic abnormalities. Mutated IGHV was detected in 71-2% of Taiwanese CLL and IGHV3-23 was the most frequently used gene. Stereotyped BCR was present in 18-3% with subset 8 being the most frequent. All cases with subset 8 belonged to IGHV 4-39 and were exclusively associated with un-mutated IGHV and poor outcome. Mutation frequencies of SF3B1 (9-7%), NOTCH1 (8-6%), BIRC3 (1-1%), ATM (16-9%) or TP53 (8-1%), and frequencies of cytogenetic abnormalities including trisomy 12 (18-6%), del(17p) (10-4%), del (13q) (43-7%) and IGH translocation (10-1%) were comparable to those reported from Western countries, except del(11q) (6-9%) which was lower in our patients. Patients with un-mutated IGHV, subset 8, disrupted TP53, trisomy 12, and SF3B1 mutations had a worse outcome compared to patients without these mutations. In conclusion, IGHV3-23 usage, stereotyped subset 8 and lower frequency of del(11q) show an ethnicity-dependent association in Taiwanese CLL patients.

Keywords: chronic lymphocytic leukaemia, IGHV, ATM, TP53, BCR stereotype.

Chronic lymphocytic leukaemia (CLL) is a disease characterized by the proliferation and accumulation of morphologically mature lymphocytes expressing CD5, CD20 and CD23, together with low expression of surface IgM (Rozman & Montserrat, 1995). CLL is the most common leukaemia in Western countries (Rozman & Montserrat, 1995; Morton et al, 2006) but its incidence is very low in Asia (Weiss, 1979). In CLL, mutational status of the immunoglobulin heavy-chain variable (IGHV) gene, which is widely accepted as one of the most reliable predictors of clinical outcome, can be divided into two subgroups with prognostic relevance: mutated IGHV associated with indolent clinical course and un-mutated IGHV, with a progressive disease course even in the patients with early stage disease (Damle et al, 1999; Hamblin et al, 1999; Oscier et al, 2002).

The reported frequencies of mutated IGHV ranged from 42-4% to 64-2% in the West (Duke et al, 2003; Tobin et al, 2004; Agathangelidis et al, 2012) compared to 60-78-8% in Oriental patients with CLL (Nakahashi et al, 2009; Xia et al, 2015; Marinelli et al, 2016). In addition to IGHV mutational status, IGHV repertoire analysis showed that IGHV families or IGHV gene usage had geographically biased predispositions. In the West, the most predominant IGHV family was IGHV3 followed by IGHV1 (Hamblin et al, 1999; Duke et al, 2003; Tobin et al, 2004). In contrast, Asian cohorts were dominated by IGHV3 followed by IGHV4 (Chen et al, 2008; Hojjat-Farsangi et al, 2009; Nakahashi et al, 2009; Marinelli et al, 2016) except in one recent small series study from Taiwan, which described a very low frequency of IGHV4 (Wu et al, 2017).

Another important characteristic of the IGHV repertoire is the expression of stereotyped B-cell receptors (BCRs). Homologous stereotyped Complementarity-Determining region 3 (CDR3) within BCRs were identified in 20–30% of CLL cases, which has been suggested to be involved in the pathogenesis of CLL and could be subdivided into 19 major
subsets and other minor subsets (Stamatopoulos et al, 2007; Agathangelidis et al, 2012; Rani et al, 2016). Notably, the frequency of the usage of BCR subsets showed great variation among different geographic areas (Marinelli et al, 2016).

In addition to IGHV mutational status, chromosomal aberrations and gene mutations are other important prognostic markers of CLL. Patients with 17p deletion [del(17p)] had inferior survival whereas those with del(13q) or trisomy 12 had better outcomes, and patients carrying del(11q) had an intermediate survival (Dohner et al, 2000). CLL patients with TP53, NOTCH1, SF3B1, ATM or BIRC3 mutations had poor prognosis (Foà et al, 2013; Puiggrós et al, 2014). A substantial difference in the frequencies of gene mutations was observed between Caucasian and Asian patients (Xia et al, 2015). In the present study, we sought to investigate the untreated diagnostic samples of a larger cohort of CLL patients in Taiwan for the frequencies of IGHV mutational status, IGHV usage, BCR stereotype, chromosomal aberrations and gene mutations, as well as correlate the genetic abnormalities with their outcomes.

Materials and methods

Patients and samples

Between July 1991 and December 2017, 194 patients with CLL were consecutively diagnosed and were regularly followed-up at a single tertiary referral centre in Taiwan. Diagnosis of CLL was based on the International Workshop of CLL-National Cancer Institute (IWCLL-NCI) criteria (Hallek et al, 2008). All patients met the criteria of ≥ 5 x 10^9/l monoclonal B cells in the peripheral blood with expression of CD5, CD20 and CD23 by flow cytometry. Peripheral blood or bone marrow mononuclear cells (MNC) were obtained from the diagnostic sample of each patient by Ficoll-Hypaque density gradient centrifugation and freshly frozen at −80°C until testing. The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (105-1282C).

gDNA extraction, cDNA synthesis, PCR amplification, and sequencing

gDNA and total RNA were extracted from peripheral blood or bone marrow MNC and complementary DNA (cDNA) was synthesized. To determine IGHV usage, clonal IGH rearrangements were amplified from cDNA using two primer pairs: (i) seven leader primers and an IgM/IgG primer (Fais et al, 1998); (ii) seven leader primers and a JH primer (McCarthy et al, 2003). In three cases with no cDNA as template, targets were amplified from gDNA. PCR products were purified and sequenced in both directions, and then aligned to the closest matched germine gene by using the IMGT/V-QUEST analysis software (IMGT; http://www.imgt.org/, Montpellier, France). Sequences with a germline identity of 98% or higher were considered un-mutated IGHV, and those with less than 98% identity as mutated IGHV (Hamblin et al, 1999). The IGHV CDR3 of each sequence was also analysed by IMGT analysis software. For the clustering analysis, sequences were applied to ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and then successive filtering was carried out on the basis of previously proposed criteria (Darzentas et al, 2010). All IGHV sequences were also evaluated using the online tool ARReST/AssignSubsets (http://tools.bat.infspire.org/arra/assignsubsets) (Bystry et al, 2015). Novel clusters identified in this study but not defined (Agathangelidis et al, 2012) were assigned a number preceded by the word ‘Cluster’.

Fluorescence in situ hybridisation in CLL

Locus-specific probes including TP53 (17p13)/MPO (17q22) (Kreatech, Amsterdam, Netherlands) for del(17p), D13S319/13q34 (Vysis, Des Plaines, IL, USA) for del(13q), ATM (11q22)/GLI1 (12q13) (Kreatech) for del(11q)/trisomy 12 and IGH breakapart (Vysis) for IGH translocation were used.

Analysis of ZAP70 expression by flow cytometry

Fluorescently labelled antibodies to CD5-peridinin chlorophyll protein (PerCP)-cyanine (Cy) 5.5 and CD19-allophycocyanin (APC) were obtained from Becton Dickinson (San Jose, CA, USA). ZAP70-Alexa Fluor 488 and Mouse IgG1 Alexa Fluor 488 antibodies were purchased from CalTag (Buckingham, UK). Frozen or fresh MNC were stained by adding CD5-PerCP-Cy5.5 and CD19-APC, permeabilised with 8E reagent, which was kindly provided by Prof. Dario Campana at National Singapore University, and followed by ZAP70-Alexa Fluor 488 staining. Cells were then analysed with a BD FACS Aria III flow cytometer and FACS Diva Software (Becton Dickinson, San Jose, CA, USA). The percentage of B cells positive for ZAP70 was determined by gating the CD19/CD5 population. The threshold was set at 20%, as described previously (Crespo et al, 2003; Richardson et al, 2006).

Mutational analysis using next generation sequencing

Ion AmpliSeq primer pools for TP53 (exons 2–11), NOTCH1 (exons 28–34), SF3B1 (exons 11–16), BIRC3 (exons 3–9) and ATM (whole coding exons) were used to amplify the targets. Library was constructed by using Ion AmpliSeqTM Library kit (Life Technologies, Carlsbad, CA, USA) and sequenced on the Ion Torrent PGM (Life Technologies) machine. Mutations were then analysed with the Variant Caller software offered by the Torrent Server. Sanger sequencing or pyrosequencing was used to validate the mutations.

Statistical analysis

Patients were followed until initiation of CLL-specific treatment or death or end of follow-up, defined as treatment-
free survival (TFS), and until death or end of follow-up, defined as overall survival (OS). All statistical analyses were carried out using the SigmaPlot statistical package (Systat Software Inc., San Jose, CA, USA). Categorical variables were compared using Fisher exact test. Multivariate analysis was done by Cox proportional hazard regression. Survival curves were constructed by Kaplan–Meier estimate and differences were evaluated by log rank test. Two-tailed P values less than 0.05 were considered as statistically significant.

Results

IGHV usage

Three of 194 patients with no reliable or reproducible clonal IGHV were excluded from IGHV analysis. Based on a cut-off of 2% deviation from the germline sequence, 136 out of 191 patients (71.2%) had IGHV mutated gene sequences, and the remaining 55 had sequences (28.8%) that belonged to the un-mutated subgroup. The most frequently expressed IGHV family was IGHV3 (n = 102, 53.4%), followed by IGHV4 (n = 49, 25.7%), IGHV1 (n = 26, 13.6%), IGHV2 (n = 7, 3.7%), IGHV5 (n = 4, 2.1%), IGHV6 (n = 2, 1.0%) and IGHV7 (n = 1, 0.5%) (Fig 1A). For mutational status of IGHV region among the most common IGHV families, the vast majority of IGHV1 expressing-cases showed more un-mutated (16/26; 61.5%) compared to IGHV3 (20/102; 19.6%; P < 0.0001) or IGHV4 families (14/49; 28.6%, P < 0.0071) (Fig 1A). The IGHV mutational status in the most frequent IGHV usage subtypes is shown in Fig 1B. IGHV3-23 (n = 20, 10.5%) was the most used, followed by IGHV3-7 (n = 18, 9.4%), IGHV3-7 (n = 14, 7.3%), IGHV4-59 (n = 13, 6.8%), IGHV3-30 (n = 12, 6.3%), IGHV4-34 (n = 11, 5.8%), IGHV4-39 (n = 11, 5.8%) and IGHV1-69 (n = 11, 5.8%). IGHV3-23 (19.6%), IGHV3-7 (17.6%) and IGHV3-74 (13.7%) constituted 51.0% of all IGHV3 cases, which were highly associated with mutated IGHV status (95.0%, 94.4% and 76.6%). IGHV4-59 (26.5%), IGHV4-34 (22.4%) and IGHV4-39 (22.4%) constituted 71.4% of all IGHV4-expressing cases, in which mutated cases were 76.9%, 90.9%, and 27.3%, respectively. In the IGHV1 family, IGHV1-69 (42.3%) was the most frequent IGHV1-expressing subtype with a mutated frequency of 9.1%.

Stereotyped BCR

Thirty-five of 191 patients (18.3%) had homologous IGHV CDR3 (stereotyped BCR), 17 of them (8.9%) were assigned to the seven defined major subsets, i.e. subset 8 (n = 8), subset 1 (n = 3), subset 2 (n = 2), and one each for subset 5, subset 12, subset 14 and subset 77. Thirteen of 17 cases could be assigned to a previously identified subset using ARResT/AssignSubsets tool (Bystry et al., 2015). Four sequences belonged to three minor subsets, including subsets 7 (n = 2),

Cytogenetic and genetic lesions

Cytogenetic abnormalities were detected in 80 of 183 (43.7%) for del(13q), 35 of 188 (18.6%) for trisomy 12, 19 of 183 (10.4%) for del(17p), 13 of 188 (6.9%) for del(11q) and 18 of 179 (10.1%) for IGH translocation. The mutational status of TP53, NOTCH1 and SF3B1 was analysed in 186 patients with a frequency of 8.1%, 8.6% and 9.7%, respectively; 31 of 183 patients (16.9%) had ATM mutations, and 2 of 183 patients (1.1%) had BIRC3 mutations. The
results of gene mutations in CLL patients are summarized in Fig 2. Of the 183 patients with mutational status available for all 5 genes, 12 patients had 2 concurrent mutations: 5 co-existing SF3B1 and ATM mutations; 2 each co-existing SF3B1 and NOTCH1, and ATM and TP53 mutations; one each co-existing NOTCH1 and ATM, NOTCH1 and BIRC3, and SF3B1 and TP53 mutations. NOTCH1 and TP53 mutations were mutually exclusive. The remaining patients had only one mutation. Correlations of gene mutations with cytogenetic abnormalities demonstrated that TP53 mutations were closely associated with del(17p) compared with no del(17p) (42.1% vs. 4.4%; $P < 0.0001$), and ATM mutations correlated with del(11q) compared with no del(11q) (53.8% vs. 14.4%; $P = 0.0019$).

Among the gene mutations analysed, SF3B1 mutations were significantly associated with un-mutated IGHV (10 of 52 vs. 7 of 131; $P = 0.0083$) and absent for stereotyped BCR subset 8. There was no correlation between mutated genes and ZAP70 > 20% but a higher frequency of un-mutated IGHV in patients with ZAP70 > 20% ($P = 0.0064$) was observed: 25 (39.7%) of the 63 patients with ZAP70 > 20% had un-mutated IGHV compared with 3 (10.7%) of the 28 patients with ZAP70 ≤ 20% had un-mutated IGHV. Although no correlation of SF3B1, NOTCH1 or TP53 mutations with stereotyped BCR subsets was observed, we found TP53-mutated cases had a higher frequency of IGHV1-69 usage compared with TP53-unmutated cases (3 of 15 vs. 8 of 168; $P = 0.0495$).

Table I. IGHV CDR3 cluster distribution in 35 CLL patients with stereotyped BCR.

| Subset or cluster | Case no. | IGHV gene | Mutational status | CDR3 length | Amino acid sequence |
|-------------------|----------|-----------|-------------------|-------------|---------------------|
| Subset            | 1        | IGHV7-4   | UM                | 13          | AREQWLVLPYFDY       |
|                   | 1        | IGHV1-3   | UM                | 13          | AREWLVRRYFDY        |
|                   | 1        | IGHV1-8   | UM                | 13          | ARVQWLVLYFDY        |
|                   | 2        | IGHV3-30  | M                 | 9           | ARDSYGSMDV          |
|                   | 2        | IGHV3-21  | M                 | 9           | ASDRNGMDV           |
|                   | 5        | IGHV1-69  | UM                | 21          | ARVKGARTLSSLYYYYYMDV |
|                   | 8        | IGHV4-39  | UM                | 19          | ARRGYSSWYQRENWFDP   |
|                   | 8        | IGHV4-39  | UM                | 19          | ARVGGSSWYSHDNWFDP   |
|                   | 8        | IGHV4-39  | UM                | 19          | ARTAGYSSWYSSYNWFDP  |
|                   | 8        | IGHV4-39  | UM                | 19          | ARVGGSSWYSTHNWFDP   |
|                   | 8        | IGHV4-39  | UM                | 19          | ARVGGSSWYSTHNWFDP   |
|                   | 8        | IGHV4-39  | UM                | 19          | ARVGGSSWYSTHNWFDP   |
|                   | 1        | IGHV4-39  | UM                | 19          | ARVGGSSWYSTHNWFDP   |
|                   | 1        | IGHV4-39  | UM                | 19          | ARVGGSSWYSTHNWFDP   |
|                   | 1        | IGHV3-7   | M                 | 14          | ARDQHQLAQNY         |
|                   | 7        | IGHV4-59  | M                 | 23          | ARRYYCSSGTCDFWDFSL  |
|                   | 7        | IGHV4-59  | M                 | 23          | ARRYYCSSGTCDFWDFSL  |
|                   | 7        | IGHV4-59  | M                 | 23          | ARRYYCSSGTCDFWDFSL  |
|                   | 8        | IGHV3-30  | UM                | 25          | ATSVPTYDFWGLYGDDYYYYMDV |

Cluster

| Case no. | IGHV3-30 | M     | 12          | ANSADYGDFDY |
|----------|----------|-------|-------------|-------------|
| 2        | IGHV3-7  | M     | 12          | ASAGYGDYADY |
| 7        | IGHV3-7  | M     | 11          | ARDQHQLAQNY |
| 2        | IGHV3-7  | M     | 11          | ARDQHQLAQNY |
| 2        | IGHV5-10 | M     | 16          | ARQRYFLGSGPMDV |
| 3        | IGHV5-51 | M     | 16          | ARQRYFLGSSLQVDF |
| 4        | IGHV3-7  | M     | 11          | AKDGTLYFDY  |
| 4        | IGHV3-43 | UM    | 12          | AKDGGGQLVDY |
| 5        | IGHV3-74 | UM    | 20          | ARDSSGYSGYYGMDV |
| 5        | IGHV3-74 | UM    | 22          | ARDSTYDDSGYYYYGMDV |
| 6        | IGHV3-74 | M     | 11          | AEGGQQQLDS |
| 6        | IGHV3-74 | M     | 11          | AEGGQQQLDS |
| 7        | IGHV2-5  | UM    | 18          | AHSPAETLIAAPVGFDY |
| 7        | IGHV2-5  | UM    | 18          | AHSPAETLIAAPVGFDY |

The sequences in bold were identified by ARResT/AssignSubsets (Bystry et al., 2015).

BCR, B-cell receptor; CDR3, complementarity-determining region 3; CLL, chronic lymphocytic leukaemia; M, mutated; UM, un-mutated.
Prognostic relevance of cytogenetic/genetic abnormalities

With a median follow-up of 49.6 months, the median TFS and OS was 24.4 months [95% confidence interval (CI): 13.6–35.2] and 110.0 months (95% CI: 82.3–136.7), respectively. The impact of cytogenetic abnormalities on outcomes was analysed. Patients with cytogenetic lesions had a comparable TFS among all groups (P = 0.215) or between any two different groups except that patients with trisomy 12 had a shorter TFS than those with del(13q) (median, 19.4 months vs. 30.0 months, P = 0.028). In contrast, there was a significant difference in OS among different cytogenetic groups (P = 0.001). The median OS of patients with del(13q), del (11q), trisomy 12, del(17p) and normal karyotypes was 154.6 months, 88.5 months, 80.5 months, 44.3 months and 95.8 months, respectively. Patients with trisomy 12 had an inferior OS compared to those with del(13q) (P = 0.0001), or those with normal karyotypes (P = 0.037).

Prognostic impact of the genetic abnormalities in CLL is shown in Table II. By univariate analysis, TFS was significantly shorter in patients with un-mutated *IGHV* (median, 7.7 months vs. 36.2 months; P = 0.001), subset 8 (median, 5.1 months vs. 28.0 months; P = 0.001), trisomy12 (median, 19.4 months vs. 27.7 months; P = 0.044), TP53 disruption (TP53 mutations and/or 17p deletions) (median, 10.6 months vs. 30.0 months, P = 0.038), SF3B1 mutations (median, 1.7 months vs. 32.7 months, P = 0.001), and ZAP70 > 20% (median, 13.1 months vs. 84.5 months, P = 0.009). OS was significantly worse in patients with un-mutated *IGHV* (median, 63.0 months vs. 144.2 months; P < 0.0001), subset 8 (median, 41.1 months vs. 114.4 months; P = 0.001), trisomy 12 (median, 80.5 months vs. 153 months; P = 0.001), negative for del(13q) (median, 88.1 months vs. 154.6 months, P = 0.016), TP53 disruption (median, 44.3 months vs. 123.0 months, P = 0.003) and SF3B1 mutations (median, 58.0 months vs. 126.3 months, P = 0.0001). In multivariate analysis (Table III), the independent predictors for inferior TFS included SF3B1 mutations [Hazard ratio (HR) = 2.942, 95% CI: 1.217–7.114; P = 0.017], trisomy12 (HR = 1.997, 95% CI: 1.009–3.951; P = 0.047), and a borderline level for TP53 disruption (HR = 1.876, 95% CI: 0.902–3.904; P = 0.092) and ZAP70 > 20% (HR = 2.070, 95% CI: 0.984–4.356; P = 0.055). Independent predictors for inferior OS included un-mutated *IGHV* (mutated *IGHV*: HR = 0.487, 95% CI: 0.262–0.907; P = 0.023), trisomy12 (HR = 2.301, 95% CI: 1.150–3.951; P = 0.007), TP53 disruption (HR = 3.667, 95% CI: 2.025–6.639; P < 0.0001), SF3B1 mutations (HR = 2.786, 95% CI: 1.239–6.267; P = 0.013) and at a borderline significant level for BCR subset 8 (HR = 2.307, 95% CI: 0.896–5.938; P = 0.083) (Table III).

We divided the patients into four genetic groups based on TP53 disruption and IGHV mutational status. Patients with wild-type TP53 and mutated IGHV had a longer TFS compared to those with TP53 disruption and un-mutated IGHV, those with wild-type TP53 and un-mutated IGHV, and those with TP53 disruption and mutated IGHV (P = 0.001) (Fig 3A). As shown in Fig 3B, patients with wild-type TP53 and mutated IGHV had a superior OS compared with those with wild-type TP53 and un-mutated IGHV (P < 0.0001). OS of
Table II. Risk factors that influence the outcome of 194 Taiwanese CLL patients.

| Feature | Treatment-free survival (months) | Overall survival (months) |
|---------|---------------------------------|---------------------------|
|         | Positive                         | Negative                  | Positive                                    | Negative                  |
|         | N | Median (95% CI)   | P     | N | Median (95% CI)   | P     | N | Median (95% CI)   | P     | N | Median (95% CI)   | P     |
| Mutated \(\text{IGHV}\) | 129 | 36.2 (21.0–51.4) | 0.001 | 136 | 144.2 (112.6–175.8) | <0.0001 | 55 | 63.0 (44.4–81.6) | <0.0001 |
| BCR subset 8 | 7 | 5.1 (0.0–15.6) | 0.001 | 8 | 41.1 (22.4–59.8) | 0.001 | 186 | 114.4 (84.9–143.7) | 0.001 |
| Trisomy 12 | 32 | 19.4 (5.6–33.2) | 0.044 | 35 | 80.5 (35.4–125.6) | 0.001 | 153 | 144.2 (86.4–202.0) | 0.001 |
| 13q deletion | 75 | 30.0 (14.5–45.5) | 0.193 | 80 | 154.6 (120.8–188.4) | 0.016 | 103 | 88.1 (57.4–118.8) | 0.016 |
| \(\text{IGH}\) translocation | 15 | 22.8 (0.0–58.1) | 0.795 | 18 | 175.3 | 0.430 | 161 | 110.0 (69.6–150.4) | 0.430 |
| \(\text{TP53}\) disruption | 26 | 10.6 (3.0–18.2) | 0.038 | 26 | 44.3 (8.8–79.8) | 0.003 | 152 | 123.0 (76.8–169.2) | 0.003 |
| ATM disruption | 45 | 16.7 (5.8–27.6) | 0.254 | 47 | 95.8 (57.8–133.8) | 0.961 | 143 | 114.4 (81.5–147.3) | 0.961 |
| \(\text{SF3BI}\) mutation | 17 | 1.7 (0.0–6.8) | 0.001 | 18 | 58.0 (32.4–83.6) | 0.0001 | 168 | 126.3 (80.6–172.0) | 0.0001 |
| \(\text{NOTCH1}\) mutation | 14 | 13.6 (11.2–16.0) | 0.092 | 16 | 57.7 (37.7–77.7) | 0.340 | 170 | 110.0 (80.1–139.9) | 0.340 |
| \(\text{BIRC3}\) mutation | 2 | 0.3 | 0.144 | 2 | 4.3 | 0.070 | 181 | 110.0 (82.1–137.8) | 0.070 |
| ZAP70 expression (>20%) | 62 | 13.1 (6.7–19.5) | 0.009 | 64 | 96.8 (45.2–148.4) | 0.009 | 28 | 173.6 | 0.009 |

BCR, B-cell receptor; CI, confidence interval; CLL, chronic lymphocytic leukaemia; N; number of cases.

Table III. Survival by multivariate analysis of risk factors in Taiwanese CLL patients.

| Feature                 | Treatment-free survival | Overall survival |
|-------------------------|-------------------------|------------------|
|                         | Univariate              | Multivariate     | Univariate              | Multivariate     |
|                         | HR 95% CI | P value | HR 95% CI | P value | HR 95% CI | P value | HR 95% CI | P value |
| Mutated \(\text{IGHV}\) | 0.517 | 0.347–0.769 | 0.001 | 0.870 | 0.433–1.747 | 0.695 | 0.386 | 0.242–0.617 | <0.0001 |
| BCR subset 8            | 3.386 | 1.556–7.367 | 0.002 | 1.444 | 0.394–5.300 | 0.579 | 3.643 | 1.645–8.065 | 0.001 |
| Trisomy 12              | 1.613 | 1.005–2.587 | 0.048 | 1.997 | 1.009–3.951 | 0.047 | 2.339 | 1.370–3.992 | 0.002 |
| 13q deletion            | 0.776 | 0.528–1.140 | 0.196 | 1.876 | 0.902–3.904 | 0.092 | 0.561 | 0.348–0.904 | 0.018 |
| \(\text{TP53}\) disruption | 1.689 | 1.021–2.794 | 0.041 | 1.876 | 0.902–3.904 | 0.092 | 2.313 | 1.320–4.054 | 0.003 |
| \(\text{SF3BI}\) mutation | 2.715 | 1.504–4.904 | 0.001 | 2.942 | 1.217–7.114 | 0.017 | 3.148 | 1.695–5.846 | 0.0003 |
| ZAP70 expression (>20%) | 2.421 | 1.219–4.809 | 0.012 | 2.070 | 0.984–4.356 | 0.055 | 2.185 | 0.752–6.353 | 0.151 |

BCR, B-cell receptor; CI, confidence interval; CLL, chronic lymphocytic leukaemia; HR, hazard ratio.
patients with TP53 disruption and mutated IGHV was also significantly different from patients with TP53 disruption and un-mutated IGHV ($P = 0.040$). A significant difference was also observed between mutated IGHV patients with and without TP53 disruption ($P = 0.002$), and between un-mutated IGHV patients with and without TP53 disruption ($P = 0.005$). Patients with wild-type TP53 and mutated IGHV had the longest OS among the four groups ($P < 0.0001$).

**Discussion**

A frequency of 71.2% mutated IGHV genes in our Taiwanese patients with CLL was comparable with a Japanese study (78.8%) (Nakahashi et al, 2009) and a study from Tianjin, China (75.3%) (Marinelli et al, 2016). As shown in Table SI, the frequency of IGHV hypermutation was higher in Asian countries. The distribution of IGHV family usage in our cohort followed an order of IGHV3>IGHV4>IGHV1, which was similar to that observed in other Asian cohorts (Nakahashi et al, 2009; Marinelli et al, 2016) but different from IGHV3>IGHV1>IGHV4 hierarchy in the West (Agathangelidis et al, 2012; Marinelli et al, 2016). Our findings confirmed that IGHV gene repertoire in CLL is geographically heterogeneous. The present study showed that the frequency of mutated IGHV was higher in IGHV3 and IGHV4 families than that of un-mutated IGHV whereas IGHV1 family carried predominantly un-mutated IGHV, which was in line with the studies of Caucasians (Duke et al, 2003; Karan-Djurasevic et al, 2012; Rani et al, 2016). Notably, IGHV3-23 was most frequently expressed in the present study and another Taiwanese study (Wu et al, 2017), which were different from all other Asian countries in which IGHV4-34 was the most frequently expressed gene in Japan and China (Nakahashi et al, 2009; Marinelli et al, 2016). On the contrary, the most frequently used gene in the Western studies was IGHV1-69 (Agathangelidis et al, 2012; Marinelli et al, 2016) (Table SI).

Based on the stringent criteria for the analysis of stereotypy in IGHV CDR3 amino acid sequences (Darzentas et al, 2010), the expression frequency of stereotyped BCR was lower than those observed in Western studies (Stamatopoulos et al, 2007; Agathangelidis et al, 2012; Marinelli et al, 2016) (18.3% vs. 25–30%). The length of IGHV CDR3 in the mutated CLL patients was significantly smaller than that in the un-mutated CLL cases in the present study, as well as in other Asian or Western countries (Bianchi et al, 2010; Agathangelidis et al, 2012; Marinelli et al, 2016; Rani et al, 2016), suggesting that mutated IGHV and smaller CDR3 length might work together to affect the antibody-binding pocket for antigen. According to the studies listed in Table SI, there was a reverse correlation of the frequency of patients with stereotyped BCR and mutated IGHV. The higher frequency of mutated IGHV might be attributed to the lower frequency of stereotyped BCR in our Taiwanese cohort.

We also observed that the frequency of major subsets of stereotyped BCR was lower in Taiwan and China compared with that in the West. Among the reported 19 major subsets, subset 8 was the most frequent subset in our patients, which was the least frequent subset in the Western cohort (Agathangelidis et al, 2012; Marinelli et al, 2016). These also supported an ethnicity-dependent association of IGHV usage. In addition, previous studies showed that stereotyped BCRs were of prognostic relevance: subset 1 or 2 was associated with poor outcome in the Western studies (Maura et al, 2011; Strefford et al, 2013; Bialiakas et al, 2014; Del Giudice et al, 2014), whereas patients with subset 4 might not require treatment (Rani et al, 2016). Both the present study and that reported by Marinelli et al. (2016) observed that patients with subset 8 had an unfavourable survival.

The cytogenetic and mutational analyses were performed at initial diagnosis (untreated samples) in all our patients. The frequencies of cytogenetic abnormalities in the present...
study were in line with those reported from European
descendants with CLL at initial diagnosis, except for del
(11q). The incidence of del(11q) (6-9%) was slightly lower in
our series than that in Western studies (10-25%) (Dohner
et al, 2000; Amare et al, 2013). A lower frequency of del
(11q) was also observed in a Chinese study (9-5%) (Xu et al,
2008), a Korean study (12-5%) (Yoon et al, 2014) and
another Taiwanese study (11%) (Wu et al, 2017). These data
suggested that del(11q) was relatively lower in Asia compared
with that in the West.

The mutation rate of TP53 (8-1%) in the present study
was comparable to 8% of 166 diagnostic CLL samples while
the mutation frequency was 15% in 307 CLL patients of all
stages in a report from China (Xia et al, 2015) and Western
studies (Takahashi et al, 2018; Leeksma et al, 2019). The
frequency of 8-1% for TP53 mutations was lower than 20-5% of
a small Taiwanese cohort (Wu et al, 2017). It is plausible
that different times of sample collection from untreated
patients was one of the major reasons to explain these
discrepancies. The relationship among molecular alterations
in our study was mostly consistent with the reported data,
including that NOTCH1 mutations occurred exclusively
without TP53 mutations (Rossi et al, 2012) and un-mutated
IGHV was frequently associated with SF3B1 mutations (Xia
et al, 2015). However, more IGHV-mutated patients in our
cohort had ZAP70 expression compared with that of
reported data (Wiestner et al, 2003). The higher frequency of
more than 70% of IGHV hypermutation in our CLL patients
might partly explain the lower correlation between un-muta-
ted IGHV and ZAP70 expression in our cohort. In addition,
we also failed to find associations of un-mutated IGHV
with TP53 or NOTCH1 mutations (Xia et al, 2015) and the
correlation of stereotyped BCR subsets with any gene muta-
tions (Sutton et al, 2016). Notably, the present result showed
that TP53 mutations were especially common in cases using
IGHV1-69, suggesting that IGHV usage rather than subsets
of stereotyped BCR correlated with gene mutations.

Our patients were consecutively diagnosed and were regu-
larly followed-up in a single institution, a tertiary referral cen-
tre; however, in our health care system, every patient could
visit the tertiary centre directly without following the referral
procedure. As the prevalence of CLL is very low and no stan-
dard protocol or guideline was available in the earlier years,
it was possible that physicians might give oral alkylating agent
(chlorambucil) to asymptomatic patients, making the TFS
shorter. We believe that OS, rather than the TFS, is more rep-
resentative of outcome in our CLL cohort. The survival of our
patients was inferior to that reported by previous studies from
Western countries (Hamblin et al, 1999; Dohner et al, 2000),
to which we have compared our series with regard to the dis-
tribution of clinical stages; no differences were observed. The
worse survival and earlier progression of Asian CLL patients
were also previously reported from South Asia (India, Pak-
istan or Bangladesh), China and Taiwan (Gunawardana et al,
2008; Wu et al, 2013; Marinelli et al, 2016), suggesting that
the inferior outcome of the present series compared with that
of Western patients might be also attributed to ethnic differ-
ences. The underlying mechanisms require further investiga-
tion. In agreement with the previous studies, our results
showed that patients with del(13q) had the most favourable
OS and those with del(17p) had the most unfavourable OS
(Dohner et al, 2000; Rossi et al, 2013; Fischer et al, 2016).
However, the results of the impact of del(11q) or trisomy 12
on outcome were conflicting among different series (Rossi
et al, 2013; Hernandez et al, 2015; Fischer et al, 2016; Gonzalez-Gascon et al, 2016). Our results showed that there was no
effect on TFS in patients with trisomy 12 or del(11q) but a
shorter OS was found in patients with trisomy 12 compared
with that of del(11q) patients, and a shorter OS compared
with patients without four types of cytogenetic lesions. Three
(8-6%) of the 35 patients with trisomy 12 carried a poor risk
factor of BCR subset 8 compared with 5 (3-3%) of the 150
patients without trisomy 12 carrying BCR subset 8, which
might partly explain one of the reasons for the poor outcome
of patients with trisomy 12. However, it will be necessary to
confirm this association by enrolling more cases with BCR
subset 8. In addition, we showed that patients without TP53
disruption and un-mutated IGHV had the longest TFS and
OS compared with patients with other groups of different
combination of the two unfavourable factors of outcome,
which was also reported in other studies (Xia et al, 2015; Fis-
cher et al, 2016).

To summarise, in a relatively large Taiwanese cohort of
CLL, we showed a high frequency of mutated IGHV
(71-2%), low frequency of stereotyped BCR (18-3%), the
most frequent usage of IGHV3-23 gene, lower frequency of
del(11q) (6-9%) and the most frequent stereotyped BCR sub-
set 8. Our results showed that IGHV features and occurrence
of del(11q) are ethnicity-dependent associated. In addition,
un-mutated IGHV, TP53 disruption, trisomy 12, and SF3B1
mutations were independent predictors for inferior OS.

Acknowledgements
This work was supported by Chang Gung Memorial Hospital
(CMRPG3E0271, CMRPG3E0272, and CMRPG3E0273). We
thank Mr. Tung-Huei Lin for statistical analysis and Ms.
Ting-Yu Huang for secretarial assistance.

Authorship contributions
LY designed and supervised the study; MC, H, PN, JH, YM,
MC, TC, CY, and LY provided patients’ samples and their
clinical data; LY and YJ developed the methodology, LY and
YJ analysed and interpreted the data, YJ and LY wrote the
manuscript.

Disclosure of conflict of interest
The authors have no conflict interest.
References

Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.J., Davis, Z., van Gavel-Mol, E.J., Tresoldi, C., Chu, C.C., Cahill, N., Giudicelli, V., Tichy, B., Pedersen, L.B., Foroni, L., Bonello, L., Janus, A., Smedby, K., Anagnostopoulos, A., Merle-Beral, H., Lau-taris, N., Juliuuson, G., di Celle, P.F., Pospisilova, S., Jurlander, J., Geisler, C., Tsafaris, A., Lefranc, M.P., Langerak, A.W., D.G.S., Chiorazzi, N., Belesi, C., Davi, F., Rosenquist, R., Ghia, P. & Stamatopoulos, K. (2012) Stereotyped B-cell receptors in one-third of chronic lymphocytic leukaemia: a molecular classification with implications for targeted therapies. Blood, 119, 4467–4475.

Amare, P.S., Gadage, V., Jain, H., Nikalje, S., Manju, S., Mittal, N., Gujral, S. & Nair, R. (2013) Clinico-pathological impact of cytogentic subgroups in B-cell chronic lymphocytic leukaemia: experience from India. Indian Journal of Cancer, 50, 261–267.

Baliakas, P., Hadzidimitriou, A., Sutton, L.A., Minga, E., Agathangelidis, A., Nichellati, M., Tsanouna, A., Scarfo, L., Davis, Z., Yan, X.J., Shanafelt, T., Plevova, K., Sandberg, Y., Vojde-man, F.J., Boudjahra, M., Trenou, T., Chatzouli, M., Chu, C.C., Veronese, S., Gardiner, A., Mansouri, L., Smedby, K.E., Pedersen, L.B., van Lom, K., Giudicelli, V., Francova, H.S., Nguyen-Khac, F., Panagiotidis, P., Juliuusson, G., Angelis, L., Anagnostopoulos, A., Lefranc, M.P., Facco, M., Trentin, L., Catherwood, M., Montillo, M., Geisler, C.H., Langerak, A.W., Pospisilova, S., Chiorazzi, N., Oescri, D., Jelinek, D.F., Darzen-tas, N., Belesi, C., Davi, F., Rosenquist, R., Ghia, P. & Stamatopoulos, K. (2014) Clinical effect of stereotyped B-cell receptor immunoglobulins in chronic lymphocytic leukaemia: a retrospective multicentre study. The Lancet Haematology, 1, e74–e84.

Bianchi, S., Moreno, P., Landoni, A.L., Naya, H., Oppezzo, P., Dighiero, G., Gabus, R. & Pritsch, O. (2010) Immunoglobulin heavy chain V-D-J gene rearrangement and mutational status in Uruguayan patients with chronic lymphocytic leukaemia. Leukaemia & Lymphoma, 51, 2070–2078.

Bisuty, V., Agathangelidis, A., Bikos, V., Sutton, L.A., Baliakas, P., Hadzidimitriou, A., Stam- atopoulos, K. & Darzentas, N. (2015) ARResT/AssignSubsets: a novel application for robust subclassification of chronic lymphocytic leukaemia based on B cell receptor IG stereotypy. Bioinformatics, 31, 3844–3846.

Chen, L., Zhang, Y., Zheng, W., Wu, Y., Qiao, C., Fan, L., Xu, W. & Li, J. (2008) Distinctive IgVH gene segments usage and mutation status in Chinese patients with chronic lymphocytic leukaemia. Leukemia Research, 32, 1491–1498.

Crespo, M., Bosch, F., Villamor, N., Bellosillo, B., Colomer, D., Rozman, M., Marce, S., Lopez-Guillermo, A., Campo, E. & Montserrat, E. (2003) ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. New England Journal of Medicine, 348, 1764–1775.

Damlé, R.N., Wasi1, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S.L., Buchbinder, A., Budman, D., Dittmar, K., Kohlt, J., Lichtman, S.M., Schultmann, F., Vinciguerra, V.P., Rai, K.R., Ferrarini, M. & Chiorazzi, N. (1999) Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukaemia. Blood, 94, 1840–1847.

Darzentas, N., Hadzidimitriou, A., Murray, F., Hatzi, K., Josefsson, P., Lauatris, N., Moreno, C., Anagnostopoulos, A., Jurlander, J., Tsafaris, A., Chiorazzi, N., Belesi, C., Ghia, P., Rosenquist, R., Davi, F. & Stamatopoulos, K. (2010) A different ontogenesis for chronic lymphocytic leukaemia cases carrying stereotyped antigen receptors: molecular and computational evidence. Leukemia, 24, 125–132.

Dohner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Krober, A., Bullinger, L., Dohner, K., Bentz, M. & Lichter, P. (2000) Genomic aberrations and survival in chronic lymphocytic leukaemia. New England Journal of Medicine, 343, 1910–1916.

Duke, V.M., Gandini, D., Sherrington, P.D., Lin, C., Heelan, B., Amlot, P., Mehta, A.B., Hoff-brand, A.V. & Foroni, L. (2003) V(H) gene usage differs in germline and mutated B-cell chronic lymphocytic leukaemia. Haematologica, 88, 1259–1271.

Fais, F., Ghiotto, F., Hashimoto, S., Sellars, B., Valetto, A., Allen, S., Schulman, P., Vinciguerra, V.P., Rai, K., Rassenti, L.Z., Kipps, T.J., Dighiero, G., Schroeder, H.W.J., Ferrari, M. & Chiorazzi, N. (1998) Chronic lymphocytic leukaemia B cells express restricted sets of mutated and unmutated antigen receptors. Journal of Clinical Investigation, 102, 1515–1527.

Fischer, K., Balho, J., Fink, A.M., Goede, V., Her- ling, C.D., Cramer, P., Langerheins, P., von Tresckow, J., Engelke, A., Maurer, C., Kovacs, G., Herling, H., Tausch, E., Kreuzer, K.A., Eich-horst, B., Bottcher, S., Seymour, J.F., Ghia, P., Marlon, P., Kneba, M., Wendtner, C.M., Dohner, H., Stilgenbauer, S. & Hallek, M. (2016) Long-term remissions after FCR chemiomunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. Blood, 127, 208–215.

Foq, R., Del Giudice, I., Guarini, A., Rossi, D. & Gaidano, G. (2013) Clinical implications of the molecular genetics of chronic lymphocytic leukemia. Haematologica, 98, 675–685.

Del Giudice, I., Chiaretti, S., Santangelo, S., Tavo-laro, S., Peragine, N., Marinelli, M., Ilari, C., Raponi, S., Messina, M., Nanni, M., Mauro, F.R., Picciocchi, A., Bontempi, K., Rossi, D., Gai-dano, G., Guarini, A. & Foq, R. (2014) Stereotyped subset 81 chronic lymphocytic leukemia: a direct link between B-cell receptor structure, function, and patients’ prognosis. American Journal of Hematology, 89, 74–82.

Gonzalez-Gascon, Y.M.J., Hernandez-Sanchez, M., Rodriguez-Vicente, A.E., Sanzo, C., Aventin, A., Puiggoros, A., Collado, R., Heras, C., Munoz, C., Delgado, L., Ortega, M., Gonzalez, M.T., Maru- gan, I., de la Fuente, I., Recio, I., Bosch, F., Espi-net, B., Gonzalez, M., Hernandez-Rivas, J.M. & Hernandez, J.A. (2016) A high proportion of cells carrying trisomy 12 is associated with a worse outcome in patients with chronic lymphocytic leukaemia. Hematological Oncology, 34, 84–92.

Gunawardana, C., Austen, B., Powell, J.E., Fegan, C., Wandroo, F., Jacobs, A., Pratt, G. & Moss, P. (2008) South Asian chronic lymphocytic pati- nets have more rapid disease progression in comparison to White patients. British Journal of Haematology, 142, 606–609.

Hallek, M., Cheson, B.D., Catovsky, D., Caligaris-Cappio, F., Dighiero, G., Dohner, H., Hillmen, P., Kreating, M.J., Montserrat, E., Rai, K.R. & Kipps, T.J. (2008) Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukaemia updating the National Cancer Institute-Worl Group 1996 guidelines. Blood, 111, 5464–5456.

Hamblin, T.J., Davis, Z., Gardiner, A., Oscar, D.G. & Stevenson, F.K. (1999) Unmutated Ig V (H) genes are associated with a more aggressive form of chronic lymphocytic leukaemia. Blood, 94, 1848–1854.

Hernandez, J.A., Hernandez-Sanchez, M., Rodri-guez-Vicente, A.E., Grossmann, V., Collado, R., Heras, C., Puiggoros, A., Martin, A.A., Puig, N., Benito, R., Robledo, C., Delgado, L., Gonzalez, T., Quezian, I.A., Galende, J., de la Fuente, I., Martin-Nunez, G., Alonso, J.M., Abrigarta, P., Luno, E., Marugan, L., Gonzalez-Gascon, I., Bosch, F., Kohlmann, A., Gonzalez, M., Espinet, B. & Hernandez-Rivas, J.M. (2015) A low fre- quency of losses in 11q chromosome is associ- ated with better outcome and lower rate of genomic mutations in patients with chronic lymphocytic leukaemia. PLoS ONE, 10, e0143073.

Table SI. Comparative analysis of IGHV features of Tai-wanese CLL patients with other studies.
Xia, Y., Fan, L., Wang, L., Gale, R.P., Wang, M., Tian, T., Wu, W., Yu, L., Chen, Y.Y., Xu, W. & Li, J.Y. (2015) Frequencies of SF3B1, NOTCH1, MYD88, BIRC3 and IGHV mutations and TP53 disruptions in Chinese with chronic lymphocytic leukemia: disparities with Europeans. Oncotarget, 6, 5426–5434.

Xu, W., Li, J.Y., Wu, Y.J., Yu, H., Shen, Q.D., Li, L., Fan, L. & Qiu, H.X. (2008) Prognostic significance of ATM and TP53 deletions in Chinese patients with chronic lymphocytic leukemia. Leukemia Research, 32, 1071–1077.

Yoon, J.H., Kim, Y., Yahng, S.A., Shin, S.H., Lee, S.E., Cho, B.S., Eom, K.S., Kim, Y.J., Lee, S., Kim, H.J., Min, C.K., Kim, D.W., Lee, J.W., Min, W.S., Park, C.W., Lim, J., Kim, Y., Han, K., Kim, M. & Cho, S.G. (2014) Validation of Western common recurrent chromosomal aberrations in Korean chronic lymphocytic leukaemia patients with very low incidence. Hematological Oncology, 32, 169–177.