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

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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

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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 (Foa *et al*, 2013; Puiggros *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 $\geqq 5 \times 10^9$/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 germline 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/arrest/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 AmpliSeq™ 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 pyroseqencing 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-

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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.

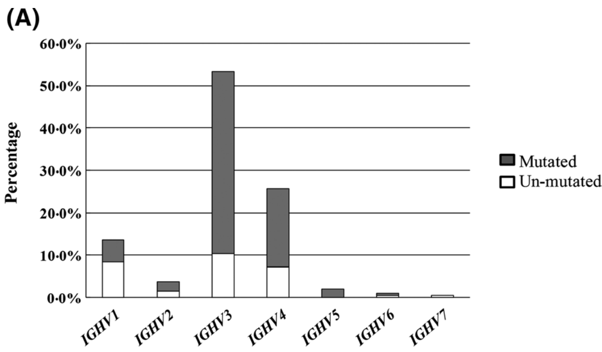
Results

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%), *IGHV3-74* (*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 78·6%). *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), 13 (*n* = 1) and 98 (*n* = 1), and the remaining seven clusters, which could not be allocated to the known subsets, were numbered as Clusters 1–7 with two each for the seven clusters.



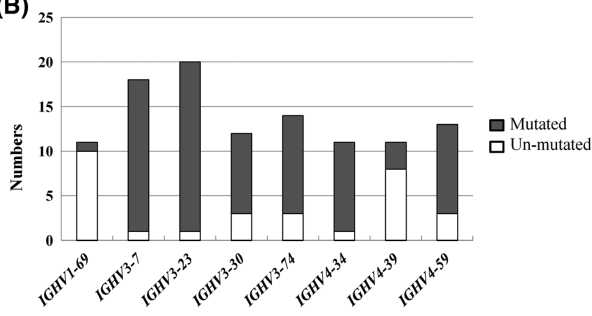


Fig 1. (A) Distribution of *IGHV* usage in 191 chronic lymphocytic leukaemia patients; (B) Number of mutated *IGHV* in the most frequent subtypes.

Table I shows the subsets or clusters of the 35 patients carrying stereotyped BCR, their *IGHV* subtypes and mutational status, CDR3 length, and amino acid sequence. Un-mutated *IGHV* gene showed strong association with stereotyped BCR compared with non-stereotyped BCR group (21 of 35 vs. 34 of 156; *P* < 0·0001). Clusters 1, 2, 3, 6 and subset 2 were composed mainly of mutated *IGHV* sequences whereas subsets 1, 7 and 8, and Clusters 5 and 7 were composed of un-mutated sequences. For *IGHV* CDR3 length, the median amino acid (aa) length of the 191 CLL patients was 14 aa (range: 6–37). Un-mutated *IGHV*-CLL patients had a significantly longer *IGHV* CDR3 (median aa: 19, range: 9–37) than mutated *IGHV*-CLL patients (median aa: 13, range: 6–27) (*P* < 0·0001).

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

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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	53	*IGHV7-4*	UM	13	**AREQWLVLPYFDY**
1	95	*IGHV1-3*	UM	13	**AREQWLVRVYFDY**
1	211	*IGHV1-8*	UM	13	**ARVQWLVLDYFDY**
2	131	*IGHV3-30*	M	9	**ARDSYGMDV**
2	202	*IGHV3-21*	M	9	**ASDRNGMDV**
5	312	*IGHV1-69*	UM	21	ARVKARGVITSLYYYYYYMDV
8	23	*IGHV4-39*	UM	19	**ARRDGYSSSWYQRENWFDP**
8	79	*IGHV4-39*	UM	19	**ARRVGYSSSWYSHDNWFDP**
8	93	*IGHV4-39*	UM	19	**ARTAGYSSSWYSSYNWFDP**
8	130	*IGHV4-39*	UM	19	**ARRVGYSSSWYSTHNWFDP**
8	151	*IGHV4-39*	UM	19	ARLVGYSSSWYGPYNWFDP
8	230	*IGHV4-39*	UM	19	**ARGLGYSSSWYGVYNWFDP**
8	241	*IGHV4-39*	UM	18	ASLNGYSSSWHSNNWFDP
8	249	*IGHV4-39*	UM	18	AKASGYSSSWYGSNWFDP
12	214	*IGHV1-46*	UM	19	**ARDSYYYDSSGYYSGFFDY**
14	336	*IGHV4-34*	M	10	**ARGGLRRADP**
77	326	*IGHV4-34*	M	14	**ARGADTTGWNAFDY**
7	178	*IGHV4-59*	UM	24	ARSWARDYDFWSGHRPAYYYYMDV
7	343	*IGHV4-59*	UM	23	ARATTYYDFWSGYSPYYYYYMDV
13	288	*IGHV4-59*	M	18	ARDYYCSGGTCFDWFSDL
98	59	*IGHV3-30*	UM	25	ATSVPTYYDFWSGLGDYYYYYGMDV
Cluster					
1	275	*IGHV3-30*	M	12	ANSADYGDRFDY
1	284	*IGHV3-74*	M	12	ASAGDYGDYADY
2	159	*IGHV3-7*	M	11	ARDQHRQAYNY
2	182	*IGHV3-7*	M	11	ARDQHRQAYNY
3	238	*IGHV5-10*	M	16	ARQRYYFGSGSSPMDV
3	308	*IGHV5-51*	M	16	ARQRYNFGSLLSQVDF
4	36	*IGHV3-7*	M	11	AKDGTKYSFDY
4	180	*IGHV3-43*	UM	12	AKDGSSGYLVDY
5	133	*IGHV3-74*	UM	20	ARDSGGYSYGIYYYYYGMDV
5	298	*IGHV3-30*	UM	22	ARDSTYYYDSSGYYYYYYGMDV
6	165	*IGHV3-74*	M	11	AGGEGGQCLDS
6	306	*IGHV3-74*	M	11	ARDEGGQCLDY
7	104	*IGHV2-5*	UM	18	AHSPAETLIAAPVGYFDY
7	164	*IGHV2-5*	UM	18	AHSPAETLIAAPVGYFDY

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.

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; $P = 0·0495$).

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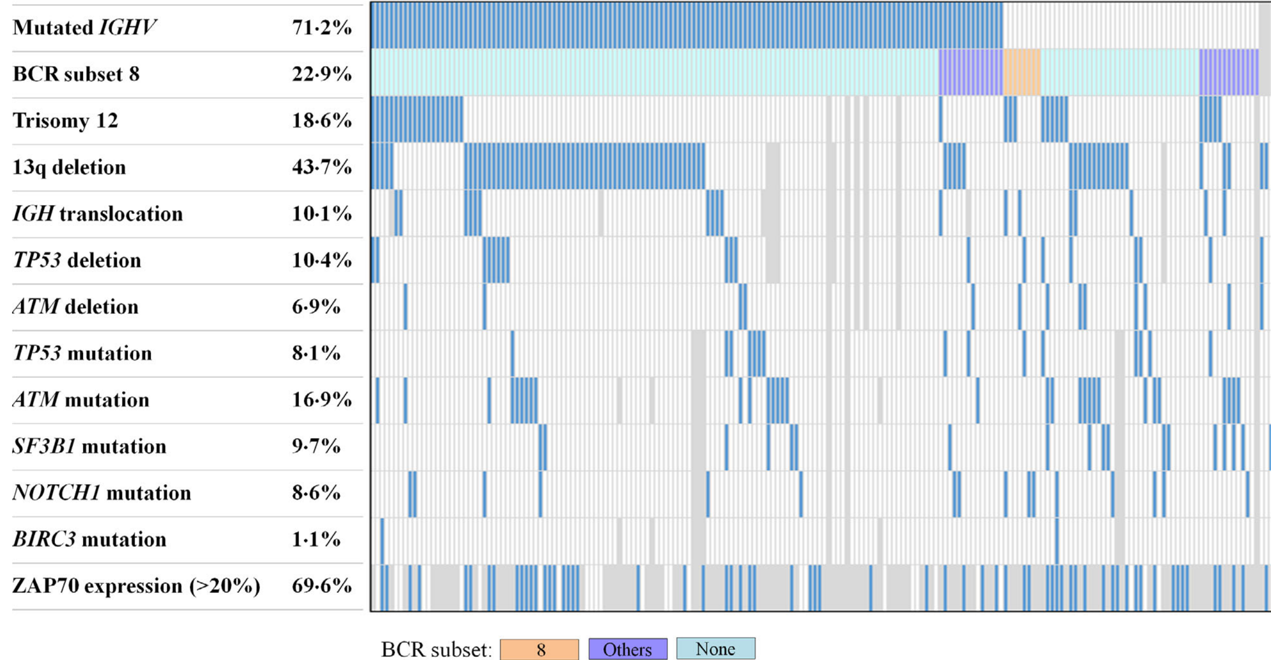

Fig 2. Correlation among the genetic lesions in 194 patients with chronic lymphocytic leukaemia. Areas not examined are indicated in grey. BCR, B-cell receptor. [Colour figure can be viewed at wileyonlinelibrary.com]

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 ($P = 0{\cdot}215$) 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, $P = 0{\cdot}028$). In contrast, there was a significant difference in OS among different cytogenetic groups ($P = 0{\cdot}001$). 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) ($P = 0{\cdot}0001$), or those with normal karyotypes ($P = 0{\cdot}037$).

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; $P = 0{\cdot}001$), subset 8 (median, 5·1 months vs. 28·0 months; $P = 0{\cdot}001$), trisomy12 (median, 19·4 months vs. 27·7 months; $P = 0{\cdot}044$), *TP53* disruption (*TP53* mutations and/or 17p deletions) (median, 10·6 months vs. 30·0 months, $P = 0{\cdot}038$), *SF3B1* mutations (median, 1·7 months vs. 32·7 months, $P = 0{\cdot}001$), and ZAP70 > 20% (median, 13·1 months vs. 84·5 months, $P = 0{\cdot}009$). OS was significantly worse in patients with un-mutated *IGHV* (median, 63·0 months vs. 144·2 months; $P < 0{\cdot}0001$), subset 8 (median, 41·1 months vs. 114·4 months; $P = 0{\cdot}001$), trisomy 12 (median, 80·5 months vs. 153 months; $P = 0{\cdot}001$), negative for del(13q) (median, 88·1 months vs. 154·6 months, $P = 0{\cdot}016$), *TP53* disruption (median, 44·3 months vs. 123·0 months, $P = 0{\cdot}003$) and *SF3B1* mutations (median, 58·0 months vs. 126·3 months, $P = 0{\cdot}0001$). In multivariate analysis (Table III), the independent predicators for inferior TFS included *SF3B1* mutations [Hazard ratio (HR) = 2·942, 95% CI: 1·217–7·114; $P = 0{\cdot}017$], trisomy12 (HR = 1·997, 95% CI: 1·009–3·951; $P = 0{\cdot}047$), and a borderline level for *TP53* disruption (HR = 1·876, 95% CI: 0·902–3·904; $P = 0{\cdot}092$) and ZAP70 > 20% (HR = 2·070, 95% CI: 0·984–4·356; $P = 0{\cdot}055$). Independent predicators for inferior OS included un-mutated *IGHV* (mutated *IGHV*: HR = 0·487, 95% CI: 0·262–0·907; $P = 0{\cdot}023$), trisomy 12 (HR = 2·301, 95% CI: 1·150–3·951; $P = 0{\cdot}007$), *TP53* disruption (HR = 3·667, 95% CI: 2·025–6·639; $P < 0{\cdot}0001$), *SF3B1* mutations (HR = 2·786, 95% CI: 1·239–6·267; $P = 0{\cdot}013$) and at a borderline significant level for BCR subset 8 (HR = 2·307, 95% CI: 0·896–5·938; $P = 0{\cdot}083$) (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* ($P = 0{\cdot}001$) (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* ($P < 0{\cdot}0001$). OS of

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Table II. Risk factors that influence the outcome of 194 Taiwanese CLL patients.

	Treatment-free survival (months)					Overall survival (months)				
	Positive		Negative			Positive		Negative		
Feature	*N*	Median (95% CI)	*N*	Median (95% CI)	*P*	*N*	Median (95% CI)	*N*	Median (95% CI)	*P*
Mutated *IGHV*	129	36·2 (21·0–51·4)	51	7·7 (0·8–14·6)	0·001	136	144·2 (112·6–175·8)	55	63·0 (44·4–81·6)	<0·0001
BCR subset 8	7	5·1 (0·0–15·6)	176	28·0 (15·9–40·1)	0·001	8	41·1 (22·4–59·8)	186	114·4 (84·9–143·7)	0·001
Trisomy 12	32	19·4 (5·6–33·2)	145	27·7 (12·7–42·7)	0·044	35	80·5 (35·4–125·6)	153	144·2 (86·4–202·0)	0·001
13q deletion	75	30·0 (14·5–45·5)	98	14·3 (7·2–21·4)	0·193	80	154·6 (120·8–188·4)	103	88·1 (57·4–118·8)	0·016
IGH translocation	15	22·8 (0·0–58·1)	155	33·7 (14·1–33·3)	0·795	18	175·3	161	110·0 (69·6–150·4)	0·430
TP53 disruption	26	10·6 (3·0–18·2)	142	30·0 (14·3–45·7)	0·038	26	44·3 (8·8–79·8)	152	123·0 (76·8–169·2)	0·003
ATM disruption	45	16·7 (5·8–27·6)	134	28·6 (10·0–46·7)	0·254	47	95·8 (57·8–133·8)	143	114·4 (81·5–147·3)	0·961
SF3B1 mutation	17	1·7 (0·0–6·8)	158	32·7 (18·8–46·6)	0·001	18	58·0 (32·4–83·6)	168	126·3 (80·6–172·0)	0·0001
NOTCH1 mutation	14	13·6 (11·2–16·0)	161	30·0 (15·5–44·5)	0·092	16	57·7 (37·7–77·7)	170	110 (80·1–139·9)	0·340
BIRC3 mutation	2	0·3	170	27·7 (14·4–41·0)	0·144	2	4·3	181	110·0 (82.-137·8)	0·070
ZAP70 expression (>20%)	62	13·1 (6·7–19·5)	28	84·5	0·009	64	96·8 (45·2–148·4)	28	173·6	0·141

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.

	Treatment-free survival						Overall survival					
	Univariate			Multivariate			Univariate			Multivariate		
Feature	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Mutated *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	0·487	0·262–0·907	0·023
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	2·307	0·896–5·938	0·083
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	2·301	1·150–3·951	0·007
13q deletion	0·776	0·528–1·140	0·196				0·561	0·348–0·904	0·018	0·801	0·469–1·369	0·417
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	3·667	2·025–6·639	<0·0001
SF3B1 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	2·786	1·239–6·267	0·013
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.

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patients with *TP53* disruption and mutated *IGHV* was also significantly different from patients with *TP53* disruption and un-mutated *IGHV* ($P = 0{\cdot}040$). A significant difference was also observed between mutated *IGHV* patients with and without *TP53* disruption ($P = 0{\cdot}002$), and between un-mutated *IGHV* patients with and without *TP53* disruption ($P = 0{\cdot}005$). Patients with wild-type *TP53* and mutated *IGHV* had the longest OS among the four groups ($P < 0{\cdot}0001$).

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 stereotype 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; Baliakas *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

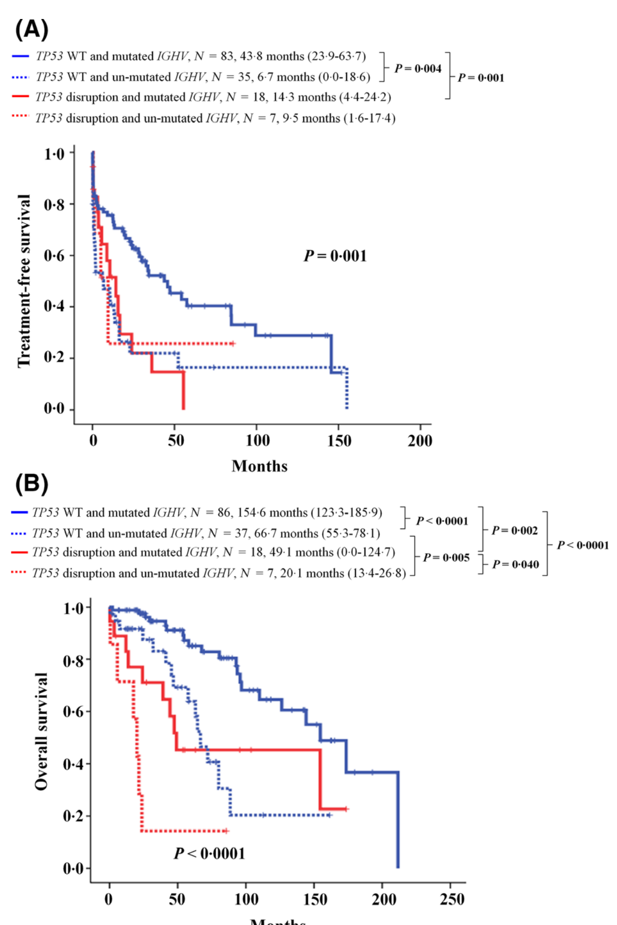

Fig 3. Kaplan–Meier estimates of (A) treatment-free survival and (B) overall survival according to *TP53* disruption and *IGHV* mutational status in Taiwanese chronic lymphocytic leukaemia patients. [Colour figure can be viewed at wileyonlinelibrary.com]

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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-mutated *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 mutations (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 regularly followed-up in a single institution, a tertiary referral centre; 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 standard 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 representative 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 distribution 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, Pakistan 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 differences. The underlying mechanisms require further investigation. 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; Fischer *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 subset 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.

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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.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table SI. Comparative analysis of *IGHV* features of Taiwanese CLL patients with other studies.

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