

Distinct molecular genetics of chronic lymphocytic leukemia in Taiwan: clinical and pathogenetic implications

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ABSTRACT

Differences in chronic lymphocytic leukemia between the Asian and the Western population are widely known. To further clarify these ethnic differences, we profiled the molecular genetics in a cohort of 83 newly diagnosed patients from Taiwan. In detail, we assessed: (i) the usage and the mutational status of the clonotypic immunoglobulin heavy-chain variable region (*IgHV*) genes, (ii) the presence of VH CDR3 stereotypes, and (iii) *TP53*, *NOTCH1*, *SF3B1*, *BIRC3*, and *MYD88* mutations. The *IgHV* gene repertoire was biased and distinct from that observed in the West with the most common *IgHV* genes being *IgHV3-23*, *IgHV3-7*, and *IgHV3-48*. In terms of *IgHV* gene mutational status, 63.8% of patients carried mutated rearrangements, whereas 22.4% of patients were assigned to stereotyped subsets (6.9% to major subsets and 15.5% to minor ones). The frequencies of *NOTCH1*, *SF3B1*, *BIRC3* and *MYD88* mutations were 9.6%, 7.2%, 1.2%, and 2.4%, respectively; however, the frequency of *TP53* mutations was significantly higher (20.5%). Patients with *TP53* mutations or *del(17p)*, *SF3B1* mutations and unmutated *IgHV* had a worse outcome compared to the other patients. In conclusion, the differences observed in *IgHV* properties suggest different pathogenetic factors implicated in the development of chronic lymphocytic leukemia, while the high frequency of *TP53* mutations could in part explain the dismal outcome of these patients in Taiwan

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent adult leukemia in the Western world, accounting for 5-11% of non-Hodgkin lymphomas (NHL).¹ CLL belongs to indolent NHL, yet its clinical course varies widely.² In the past 3 decades, many clinical and molecular features have been identified as predictors of outcome or response to therapy. Chromosome aberrations are important prognostic markers. With traditional cytotoxic agents and rituximab as the backbone of therapies, patients harboring 17p13 deletions have a very short survival with a median of less than 3 years, whereas those with 13q14 deletions, trisomy 12, or normal karyotype have much better outcomes, with a median survival up to 10 years or more, and those carrying 11q22-q23 deletions have a survival time in between the aforementioned.³ In addition, the mutational status of the immunoglobulin (*IG*) gene is one of the most robust prognostic factors; mutated *IG* is associated with better outcomes, compared to unmutated *IG*.⁴ With the advance of next-generation sequencing technology, some recurrent genetic mutations are found to possess prognostic significance, such as the negative survival impacts of *TP53*, *NOTCH1*, *SF3B1* or

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BIRC3 mutations and the favorable survival impact of *MYD88* mutations.^{5,6} These molecular and clinical markers of CLL greatly assist in our understanding of disease biology and, perhaps most importantly, in the optimization of individual patient's management options.

CLL in Asia differs in terms not only of a lower prevalence^{7,8} but also of different treatment outcomes.⁹ Up to now, CLL has mostly been viewed as a "Western disease", and the majority of knowledge is derived from studies in Western populations. This is especially true in terms of novel prognostic factors, which have not been thoroughly studied among Asian patients. Such a gap in knowledge becomes even more compelling since studies concerning the clinical course and the epidemiology of CLL have reported different outcomes in Taiwan and Japan compared to the West.^{9,10} Thus, any differences in the biological characteristics that may underlie the disparities in prognosis of CLL in Asian populations would be of great interest and value. The study herein was aimed at characterizing and validating the clinical implications of CLL immunogenetic and genetic features in a cohort of Taiwanese CLL patients in order to highlight any potential geographical differences that may underlie the outcome disparities. This knowledge could lead to the refinement of management strategies for Taiwanese CLL patients.

Methods

Patients, treatments, and survival

In total, 83 consecutive patients with newly diagnosed CLL at the National Taiwan University Hospital (NTUH) between February 1994 and December 2006 constituted the study cohort. The clinical characteristics and cytogenetic profiles in 80 of them have been reported previously.¹¹ The diagnosis was made through the findings of blood cell counts, classification, morphology, and immunophenotyping, all of which were carried out at the Specialized Hematology Laboratory in NTUH, according to the World Health Organization (WHO) classification.¹² The patients' demographics is summarized in the *Online Supplementary Table S1*. The study was approved by the Institutional Review Board of the National Taiwan University Hospital; written informed consent in accordance with the Declaration of Helsinki was obtained from all participants.

SF3B1, *NOTCH1*, *MYD88*, *BIRC3* and *TP53* mutations

Gene mutations were determined by polymerase chain reaction (PCR) of genomic DNA extracted from bone marrow samples, followed by direct Sanger sequencing in 83 patients. The PCR protocols of gene mutation analyses for *MYD88* (exon 5),¹³ *NOTCH1* (exon 34),¹⁴ *SF3B1* (exons 14, 15, and 16),¹⁵ and *TP53* (exons 3 to exon 9)¹⁶ were adapted from prior reports, and that for *BIRC3* (exons 7, 8, and 9) was designed in-house. The primer sequences are summarized in the *Online Supplementary Table S2*. The results were compared with the data derived from Western cohorts in plots to delineate the differences and similarities between the populations.^{5,6}

Amplification and analysis of Immunoglobulin (*IG*) gene rearrangements

Analysis of Immunoglobulin (*IG*) gene rearrangements was successfully performed in 58 patients. The methods for the amplification of *IgHV-IgHD-IgHJ* rearrangements sequences were adapted from Chia *et al.*¹⁷ and Hamblin *et al.*⁴ Nucleotide sequences were aligned to the IMGT/V-QUEST database in order to determine the

IG heavy-chain genes usage and somatic hypermutation status. The clusters of sequences with common heavy-chain complementarity determining region 3 (HCDR3) motifs, described as "stereotyped" B-cell receptors, were identified by the methods described by Bystry, Agathangelidis and Bikos *et al.*¹⁸ The results were compared with the data derived from Western cohorts in plots to delineate the differences and similarities between the populations.^{4,19,20}

Conventional cytogenetic analysis and fluorescence *in situ* hybridization (FISH)

Chromosomal and FISH analyses were performed in 80 patients as previously described.^{21,22} The FISH panels, including the probes for the centromere of chromosome 12 (CEP12), 13q14.3 (LSI D13S319), 17p13 (p53), and 11q22.3 (ATM), were all from Vysis Inc. (Downers Grove, IL, USA).

Statistical analysis

χ^2 or Fisher's exact tests were used for the between-group comparison of discrete variables. Two-sample *t*-test was used for the between-group comparison of the means. Kaplan-Meier survival curves were used for the estimation of overall survival (OS). Log-rank test was used to test for the differences in the OS between groups. All the directional P-values were two-tailed and a P-value of <0.05 was considered as significant. All analyses were performed by using the PASW 18 software (SPSS, Inc., Chicago, IL, USA).

Results

CLL *IGH* gene rearrangement analysis

Using 2% difference from the closest germline gene as the cutoff, *IG* heavy-chain rearrangements were classified as mutated in 37 of the 58 subjects (63.8%). The median difference from the closest *IgHV* germline gene was 7.7% (range 2.1–11.9%). The *IgHV* gene repertoire in Taiwanese patients is summarized in Figure 1A: *IgHV3-23*, *IgHV3-7*, and *IgHV3-48* genes were those most frequently expressed (17.2%, 15.5%, and 13.8%, respectively). All 10 *IgHV3-23* gene rearrangements were mutated, whereas *IgHV1-69* and *IgHV3-15* rearrangements were unmutated (Figure 1A). *IgHV3-23* and *IgHV3-7* genes were more common in Taiwan as compared to the West (Figure 1B), whereas *IgHV4* genes, especially *IgHV4-34*, one of the most overexpressed *IgHV* genes in Western CLL patients,^{4,19} and *IgHV1-69*, the most common *IgHV* gene in European patients,²⁰ were underrepresented in the current study. The frequency of *IgHV3-21* was 3.4%, similar to that reported in Chia *et al.*²⁰

Among the analyzed *IG* gene rearrangements, 13 (22.4%) were assigned to stereotyped subsets based on a robust bioinformatics algorithm.^{23,24} Nine cases (15.5%) were assigned to minor subsets, and 4 (6.9%) to major subsets: 2 to subset #1, 1 to subset #8 and 1 to subset #77. The assignment to major subsets was further confirmed through the use of AssignSubsets, a novel major subset assignment tool.¹⁸ The clinical course and molecular profile of patients assigned to major subsets are summarized in Table 1. Similar to the report regarding prognostic impact,¹⁸ patients in aggressive subsets #1 and #8 had concurrent poor prognostic profiles and much shorter survival, whereas the patient in indolent subset #77 had a more favorable prognostic profile and longer survival.

Gene mutations in CLL patients

The profiles of gene mutations are summarized in Figure 2A and the *Online Supplementary Table S3*. Mutations in *TP53*, *NOTCH1*, *MYD88*, *SF3B1* or *BIRC3* were identified in 30 of the 83 patients (36.1%): 17 patients (20.5%) with *TP53* mutations, 8 (9.6%) with *NOTCH1* mutations, 6 (7.2%) with *SF3B1* mutations, 2 (2.4%) with *MYD88* mutations, and 1 (1.2%) with a *BIRC3* mutation. Three patients had concurrent mutations in *TP53* and *NOTCH1*, and 1 patient in *TP53* and *SF3B1*. The summary of the gene mutations and cytogenetic changes is shown in Figure 2A. Compared with the data derived from Western populations,⁵ the frequency of *TP53* mutation among newly diagnosed patients was dramatically higher in our cohort, whereas those of other mutations were quite similar (Figure 2B). In addition, a novel *TP53* mutation, V31I, was noted in 3 cases in the current cohort. Cytogenetic changes detected by either conventional cytogenetics or FISH were available in 80 patients. Correlations of gene mutations with cytogenetic abnormalities demonstrated that *TP53* mutations were closely associated with del(17p); 5 (71.4%) of the 7 patients with del(17p) had *TP53* mutations while 12 (16.4%) of the 73 patients without del(17p) had *TP53* mutations ($P=0.004$). On the other hand, *SF3B1* mutations were correlated with del(11q); 5 (55.6%) of the 9 patients with del(11q) had *SF3B1* mutations while only 1 (1.4%) of the 71 patients without del(11q) had *SF3B1* mutations ($P<0.001$) (*Online Supplementary Table S4*).

Associations between *IgHV* mutational status and genetic alterations

The mutational status of the rearranged *IgHV* gene correlated closely with the genetic profiles (Table 2). In more detail, patients with unmutated *IgHV* genes were more likely to have concurrent *TP53*, *NOTCH1*, or *SF3B1* mutations, compared to those with mutated *IgHV* genes (45.0% vs. 5.3%, $P=0.001$; 20.0% vs. 0%, $P=0.011$; and 20.0% vs. 2.6%, $P=0.044$, respectively). Furthermore, the unmutated *IgHV* gene was correlated with del(17p) and del(11q) (26.3% vs. 5.6%, $P=0.041$; and 26.3% vs. 2.8%, $P=0.015$, respectively). Lastly, patients with mutated *IgHV* genes were diagnosed with early disease stage in comparison with unmutated cases (63.2% vs. 25.0% at Binet stage A, $P=0.017$).

The prognostic impact of gene mutations

Novel gene mutations correlated with the patients OS: patients with either del(17p) or *TP53* mutations had significantly shorter OS compared to those with neither aberrations (41.8 months vs. 86.8 months, $P=0.048$, Figure 3A). *SF3B1* mutations were also associated with shorter OS (11.1 months vs. 86.8 months, $P<0.001$, Figure 3B), whereas patients with mutated *IgHV* genes had longer OS (89.2 months vs. 40.0 months, $P=0.001$, Figure 3C).

Discussion

It is well known that the incidence and prevalence of cases of CLL are much lower in Eastern countries.^{7,8} In addition to this disparity, quite a few studies have demonstrated that the cytogenetic profile and outcome of CLL in this area are different from that observed in the West.^{7,9,11} As for the molecular genetics of CLL, ethnical/geographical differences between populations have also been reported.^{8,25} In the study herein, we studied a series of immunogenetic and genetic features which are commonly evaluated at CLL diagnosis in the West, in a cohort of Taiwanese patients. The study results demonstrated that

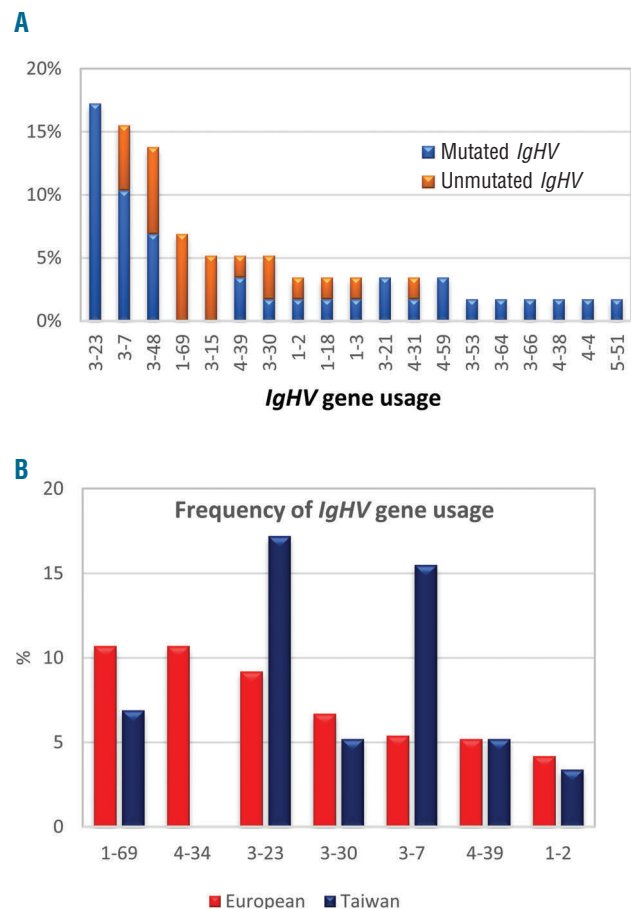


Figure 1. *IgHV* gene usage. (A) Frequency of the *IgHV* subgroups and somatic hypermutation status in Taiwanese CLL patients. (B) Comparison between Western and Taiwanese CLL patients regarding the frequency of *IgHV* gene usage. The data of these common *IgHV* genes, derived in the West for references, are from the study by Ghia et al.²⁰

Table 1. Brief clinical profiles of patients in major CDR3 stereotype subsets.

| Subset | UPN | <i>IgHV</i> | Gene mutations | Cytogenetics | Binet | Course |
|--------|-----|-----------------------------|-------------------------|--------------|-------|----------------------|
| #1 | 026 | <i>IgHV1-3</i> , unmutated | <i>TP53</i> mutation(+) | 17p deletion | 3 | Dead; OS 3 months |
| #1 | 079 | <i>IgHV1-18</i> , unmutated | <i>TP53</i> mutation(+) | 17p deletion | 2 | Dead; OS 31 months |
| #8 | 012 | <i>IgHV4-39</i> , unmutated | None | Trisomy 12 | 1 | Dead; OS 64 months |
| #77 | 017 | <i>IgHV4-59</i> , mutated | None | None | 1 | Alive; OS 148 months |

OS: overall survival; UPN: unique patient number.

CLL in Taiwan is highly distinct compared to Western CLL in terms of molecular genetic profiles.

With regard to immunogenetics, 63.8% of patients in the current cohort had mutated *IG* rearrangements, a rate similar to that in the West where 50–60% of patients show somatic hypermutations.^{4,26} Associations between *IgHV* gene usage and somatic hypermutation status reported in the West were also evident in Taiwanese patients, with *IgHV1-69* gene rearrangements belonging to the unmutated CLL fraction and *IgHV3-23* to the mutated one (Figure 1A). On the contrary, the *IgHV* gene usage distribution in Taiwanese CLL patients was quite different from that in the West, characterized by the underrepresentation of the *IgHV1-69* gene and the absence of *IgHV4-34* gene rearrangements. A similar profile of *IgHV* subgroup gene usage was observed between our cohort and mainland Chinese patients, characterized by a higher frequency of the *IgHV3* subgroup and a lower frequency of the *IgHV4* subgroup compared to the West. However, the distribution of individual *IgHV* genes in Taiwanese patients was different from that in Western as well as Chinese patients, with the most characteristic example being that of the *IgHV4-34* gene, which was the most common gene in Chinese patients,²⁷ yet it was completely absent in our cohort. Since people from mainland China and Taiwan are expected to be similar in terms of genetic background, such differences in *IgHV* gene usage support the notion that environmental variables with population-specific antigenic selection

may play a role in the development of CLL. Although *IgHV3-7*, *IgHV3-26*, and *IgHV4-34* have been correlated with antibodies against influenza A, influenza B, and I

Table 2. The correlations of *IgHV* mutation status and other molecular genetics, cytogenetics, and clinical stages.

| | <i>IgHV</i> mutation(-) | <i>IgHV</i> mutation(+) | <i>P</i> value |
|----------------------------|--------------------------------|---------------------------------|----------------|
| Molecular genetics | (n=20) | (n=38) | |
| <i>TP53</i> mutation (+) | 9 (45.0%) | 2 (5.3%) | 0.001 |
| <i>NOTCH1</i> mutation (+) | 4 (20.0%) | 0 (0%) | 0.011 |
| <i>SF3B1</i> mutation (+) | 4 (20.0%) | 1 (2.6%) | 0.044 |
| <i>MYD88</i> mutation (+) | 0 (0%) | 1 (2.6%) | N.S. |
| <i>BIRC3</i> mutation (+) | 0 (0%) | 1 (2.6%) | N.S. |
| FISH | (n=19) | (n=36) | |
| 17p deletion | 5 (26.3%) | 2 (5.6%) | 0.041 |
| 11q deletion | 5 (26.3%) | 1 (2.8%) | 0.015 |
| 13q deletion | 6 (31.6%) | 19 (52.8%) | N.S. |
| Trisomy 12 | 5 (26.3%) | 5 (13.9%) | N.S. |
| Normal | 2 (10.5%) | 14 (38.9%) | 0.033 |
| Binet stage | (n=20) | (n=38) | |
| A:B:C | 5(25.0%):9(45.0%): 6(30.0%) | 24(63.2%): 7(18.4%):7(18.4%) | 0.017 |

N.S.: not significant; FISH: fluorescence *in situ* hybridization.

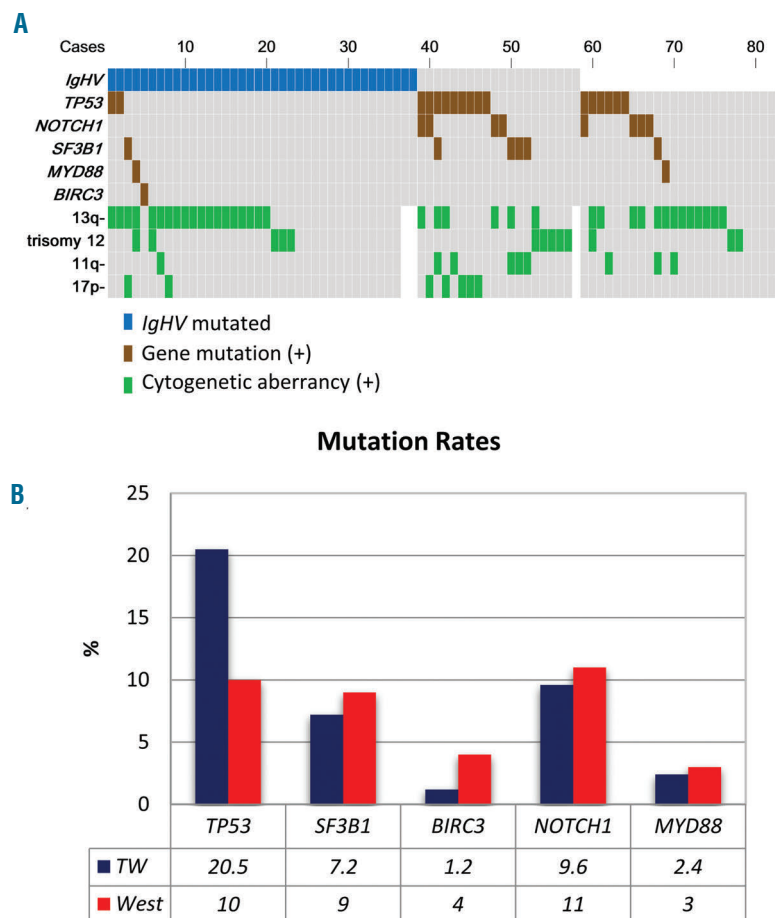


Figure 2. Gene mutation and cytogenetic status. (A) The mutation landscape including *IgHV* mutation, gene mutation, and cytogenetic aberrancy in the current study. Each column represents an untreated CLL case. (B) The frequencies of common gene mutations in Taiwanese CLL patients and Western CLL patients. The Western data for references are derived from the review by Foà *et al.*⁵ and Martínez-Trillos *et al.*⁶

antigen in cold agglutinin disease,²⁸⁻³² respectively, no apparent racial differences in these diseases have been reported to support the possible associations between chronic stimulation resulting from these antigens or pathogens and CLL development in this region. Regarding stereotyped VH CDR3, although only a few patients were assigned to major subsets, the associations between stereotypes and clinical characteristics held true in the current cohort (Table 1) with subset #1 and #8 patients exhibiting aggressive disease behavior in contrast to the subset #77 patient who showed an indolent disease course.

Frequencies of *SF3B1*, *NOTCH1*, *MYD88*, and *BIRC3* mutations in the current cohort were similar to those reported in the West. The only exception was the *TP53* gene whose mutation rate at diagnosis was twice as high compared to the West (20.5% vs. 5~10%, Figure 2B),⁵ which is in line with previous studies in Korea.³³ In this context, the fact that *TP53* mutations frequently coincide with del(17p) together with the finding that del(17p) is also more common in Taiwan and other Asian cohorts^{11,34-36} corroborate our findings. Since *TP53* mutations can be acquired during disease progression, one might question that this high frequency of *TP53* mutations could result from diagnosis delay. However, the mutation analysis was performed on samples obtained at the time of diagnosis of all patients, and the mutation rate in the current Taiwanese cohort was even higher than that of patients after first treatment in the West (8~11%).⁵ In addition, *SF3B1* mutations, which are also frequently acquired during disease progression,⁵ were not more common in Taiwan. With these observations, delayed diagnosis *per se* does not appear to be able to explain such a huge disparity of *TP53* mutation rates. Since *TP53* mutation/del(17p) is one of the strongest poor prognostic factors in CLL,⁵ the higher mutation rate might partially explain the dismal outcomes of CLL patients in Taiwan. A surprising observation was that the V31I mutation in exon 3 of *TP53*, which was recurrently identified in our cohort, has not been reported in Western CLL populations. According to the literature, this mutation could be of significance given the fact that it has been found in other hematological malignancies and solid cancers.^{16,37-42} In addition, findings from a functional study suggested that this mutation correlated with lower transcriptional activity and lower cell proliferation suppressing activity compared to wild-type *TP53*.⁴³ The mechanisms and effects of this mutation in CLL pathogenesis need to be further clarified. Of note, it has been reported in the literature that around 3% to 12% of cases without del(17p) harbored *TP53* mutations,^{5,44-46} but the mutation rate was higher, up to 16.4% (12 of 73 cases, *Online Supplementary Table S4*) in the current study. Such a high mutation rate in this population suggests that *TP53* mutation analysis is of great importance in assessing the risk and prognosis in patients without del(17p) in Taiwan.

In summary, the distinct usage of *IgHV* genes in Taiwanese CLL patients suggests different pathogenetic mechanisms with distinct antigenic elements being implicated in the development of the disease. In addition, we demonstrated herein a significantly higher *TP53* mutation frequency, a finding compatible with the overall dismal outcome of CLL patients in Taiwan. Further studies are needed to better characterize and understand

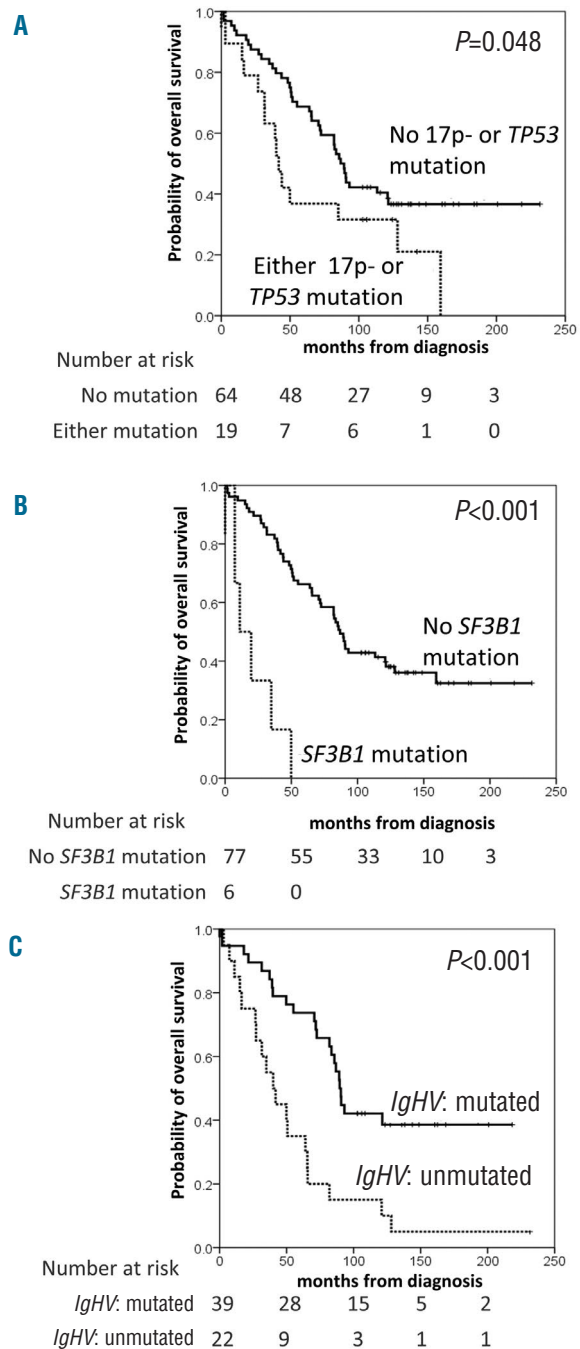


Figure 3. The impact of molecular genetics on CLL patients overall survival in Taiwan. (A) The OS for patients with or without 17p deletion or *TP53* mutation. (B) The OS for patients with or without *SF3B1* mutation. (C) The OS for patients with or without *IgHV* gene mutation.

the actual impact of these differences in the development and progression of CLL among different ethnic and geographical groups.

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