Hematological Oncology
Hematol Oncol 2013; **31** (Suppl. 1): 84–88
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/hon.2073

Supplement Article

XV. Malignant lymphoma as a consequence of clonal evolution

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Keywords: **Clonal evolution; lymphomagenesis; genome profile**

Introduction

B-cell and T-cell malignancies have been identified as a monoclonal disease since the introduction of Immunoglobulin (*IG*) and T-cell receptor genes as clonal markers, respectively. Characteristic chromosome translocations are associated with specific disease entities and are known to play a pivotal role in lymphoma development. It is well known, however, that chromosome translocation alone is not sufficient to produce tumours. Additional genomic alterations have therefore been thought to play important roles in lymphomagenesis. Since the advent of array comparative genomic hybridization (array CGH) technology, copy number alterations have been analysed and it was discovered that each disease subtype has a characteristic alteration pattern [1]. Investigation now focuses on how these genetic alterations participate in lymphoma development and/or manifestation of clinicopathological features. During the course of our analyses, we found that there is intra-tumour clonal heterogeneity derived from the same original clone. The biological significance of clonal heterogeneity caused by clonal evolution during the development of malignant lymphoma will be discussed.

Genome profile is characteristic of each disease entity

We and other researchers have found that genomic alteration patterns are characteristics to each disease entity. Such characteristic genomic alteration regions are thought to contain candidate genes involved in subtype-specific lymphoma development. Mantle cell lymphoma (MCL) shows genomic alterations predominant in loss regions [2–4], which are very different from those observed in diffuse large B-cell lymphoma (DLBCL) [5]. There are also common genomic alterations among different subtypes. For example, the 6q23.3-q24.1 deletion is frequently found in both MCL and activated B-cell like (ABC) type DLBCL but not in germinal center B-cell like (GCB) type DLBCL. Further study revealed that the candidate gene for this region is *TNFAPI3/A20*, which is not involved in GCB type DLBCL [6]. These characteristic genomic alterations are most likely involved in lymphoma development after an initial genetic hit such as chromosome translocation or virus integration, although how and when they collaborate with such initial hits remains unknown.

Genome profiles of adult T-cell leukaemia/lymphoma (ATLL) and peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS)

Adult T-cell leukaemia/lymphoma is a leukaemia/lymphoma of mature $CD4^+$ T-cells and is induced by Human T-cell leukaemia virus type 1 (HTLV-1) infection. HTLV-1-infected individuals (HTLV-1 carriers) are prevalent in the southwestern part of Japan where ATLL is endemic. It is known that only 2–5% of HTLV-1 carriers develop ATLL after a long latency period of 40–60 years, suggesting that HTLV-1 infection alone is not sufficient for ATLL development [7]. The approximate number of independent genetic events occurring before ATLL manifestation was estimated as five according to the Weibull distribution model [8]. In an attempt to explore such genetic events, we analysed genome profiles of ATLL by means of array CGH and found that acute-type and lymphoma-type ATLL show different genomic alteration patterns [9] which are also different from the previously examined B-cell-type lymphomas. We further examined PTCL, NOS and found that this disease entity shows variable genome profiles, with the number of alteration regions ranging from none to the 30s [10]. When the PTCL, NOS with genomic alterations were grouped, the genome profile becomes very similar to that of lymphoma-

type ATLL. Histological observations showed that this group of PTCL, NOS possessed pleomorphic nuclei and nuclear atypia. This histology is indistinguishable from that of lymphoma-type ATLL. The prognosis also overlaps that of lymphoma-type ATLL, which implies that lymphoma-type ATLL and PTCL, NOS with genome alterations are difficult to differentiate if HTLV-1 information is not available. These data indicate that lymphoma-type ATLL and PTCL, NOS with genomic aberrations may belong to the same disease entity which shares common genetic alteration pathways that lead to disease manifestation except for the initial hit [10].

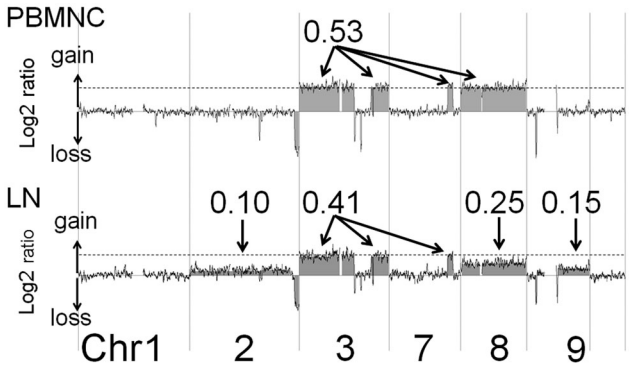

Figure 1. Log2 ratio for acute-type ATLL patient samples of peripheral blood mononuclear cells with CD4+ selection (PBMNC) and lymph node (LN). The PBNMC sample shows less frequent genomic alterations and a constant log2 ratio among different genomic regions, while LN shows more frequent genomic alterations with variable log2 ratios. (Modified from Figure 1 of Umino *et al.*[12])

Clonal heterogeneity

Adult T-cell leukaemia/lymphoma is composed of four clinical subtypes, namely, acute, lymphoma, chronic and smouldering [11]. Subtype diagnosis is sometimes not always clearly defined because diagnosis of acute-type ATLL is made even when the other three subtypes show progression. Therefore, it is puzzling why the significant genomic difference exists between acute-type and lymphoma-type ATLL. One possibility is that the samples used for array CGH were different, with the acute type being from peripheral blood mononuclear cells and the lymphoma type being from lymph node [9]. Thus, we analysed paired samples of peripheral blood and lymph node from the same patients [12]. Interestingly, genome profiles of peripheral blood tumour samples in 70% of patients were different from those of lymph node samples. Genomic alterations of the former samples were less frequent and more stable regarding log2 ratios when compared with those of lymph node samples. In other words, the log2 ratios of lymph node samples are quite variable among genomic regions. One such example is shown in Figure 1. We call these variable log2 ratios, a log2 ratio imbalance.

A log2 ratio imbalance can be artificially made by mixing two different cell lines with variable ratios (Figure 2A). Importantly, the log2 ratios correlate with the percentage of tumour cells (Figure 2B and 2C). Thus, the accurate evaluation of log2 ratios allows speculation concerning the percentage of tumour cells with genomic alterations of interest (Figure 3A). The existence of a log2 ratio imbalance indicates the existence of multiple clones (clonal heterogeneity) with different genomic alterations (Figure 3B). These log2

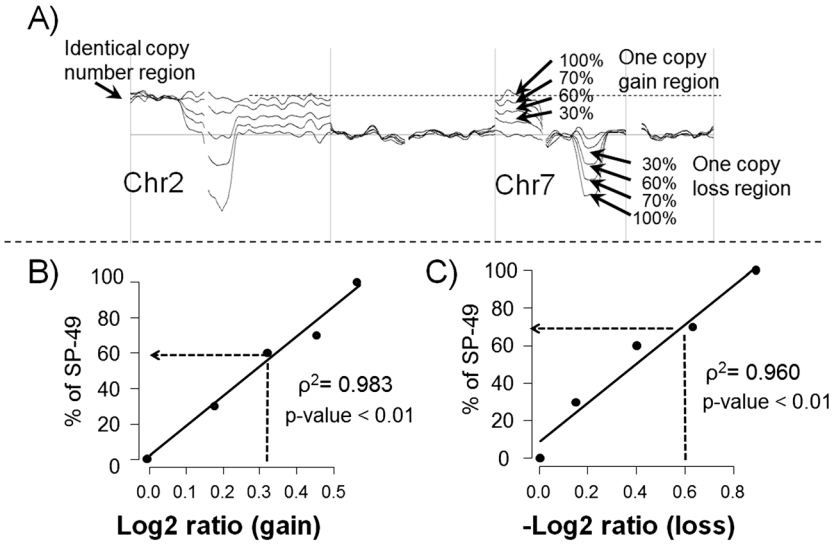

Figure 2. Correlation between log2 ratio and the percentage of tumour cells. When HANK-1 and SP-49 cells are mixed at various ratios, log2 ratio values change accordingly. The log2 ratio of a region with the same copy number gain (identical copy number region) never changes, whereas that of a gain or loss region in SP-49 does change accordingly (A). (Modified from Figure 4 of Umino *et al.*[12]). Linear correlation between the percentage of SP-49 and log2 ratios of gain (B) or loss (C) regions

DOI: 10.1002/hon

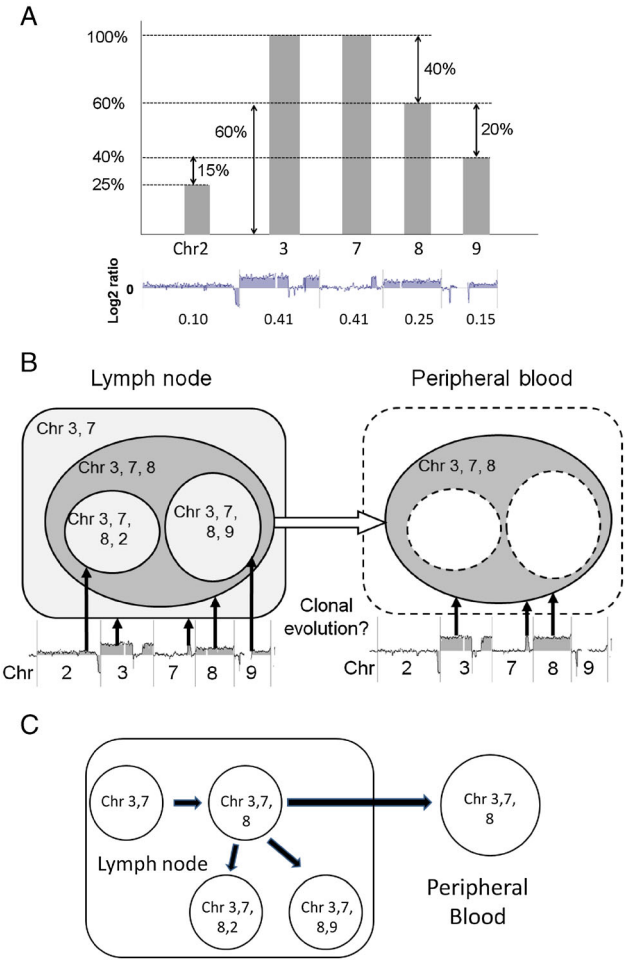

Figure 3. Variable log2 ratios, clonal heterogeneity and a clonal evolution model. Different genomic regions are shown as a percentage in a bar graph calculated using a log2 ratio of 0.41 as 100%. The variable log2 ratios reflect multiple subclones with different genomic alterations. The bar graph indicates that all tumour cells have one copy gain in chromosomes 3 and 7, whereas differences in chromosomes 2, 8 and 9 show variable percentages of tumour cells with respective genomic alterations (A). The ATLL patient of Figure 1 showing a selected clone of peripheral blood derived from lymph node tumour cell subclones is shown schematically (B). A hypothetical clonal evolution model based on the genome profiles of peripheral blood and lymph node tumour cells (C)

ratio imbalances in a patient can be explained by a clonal evolution model (Figure 3C) [12]. The log2 ratio imbalance indicates that multiple subclones derived from the same original clone exist at diagnosis in about 70% of acute-type ATLL patients [12]. Multiple subclones were also found in PTCL, NOS [13] and MCL [14].

Does a hierarchy of genomic alterations exist?

The presence of clonal heterogeneity with variable genomic alteration patterns suggested that genomic alterations may have a hierarchy during the course of lymphoma development. MCL has been shown to have a characteristic genome profile by several investigators, including our group [2–4]. The genomic alterations found in MCL have been thought to play essential roles in establishing MCL in collaboration with initial genomic alteration, *CCND1/IGH@* translocation. However, it has been shown that each MCL patient has a different genomic alteration pattern [14]. Representative genomic regions occurring in MCL patients are summarized in Table 1, where the percentage of tumour cells with respective genomic alteration regions is shown. Table 1 indicates that no common genomic regions are found in 100% of tumours that are shared with all of the listed patients, although 11q loss is more frequent than alteration of any other region, suggesting that the former is involved at an earlier stage of tumour development, and other alterations are involved at a later stage. This indicates that *CCND1/IGH@* translocation collaborates with various combinations of genetic alterations for disease manifestation, and these collaborating genetic events are not strictly restricted in sequence. In other words, the early stage of MCL development may not need any specific genomic copy number alterations. Therefore, *in situ* MCL or *in situ* follicular lymphoma is expected to provide an important clue concerning how the initial phase of malignancy takes place.

Table 1. Summary of clone frequencies for the four frequent chromosomes loss regions in eight MCL cases with multiple subclones

Pt no.	Proportion to main clone				Number of clones
	Chr1p (%)	Chr 9q (%)	Chr11q (%)	Chr13q (%)	
1	-	88	100	-	3
2	-	95	-	100	5
3	100	64	100	100	4
4	100	83	87	-	2
7	100	-	-	31	3
12	89	-	-	100	2
13	40	100	34	51	4
19	38	68	65	100	3

Chr, chromosome; -, no aberration.
Representative cases were selected from Table 2 of the report by Liu *et al.*[14].

Clonal evolution of B-cell lymphocytic leukaemia

About 5% of B-cell lymphocytic lymphoma (B-CLL) patients develop a secondary aggressive lymphoma, usually DLBCL, which is termed Richter's transformation (RT). Mao *et al.* analysed clonality of RT cases with the IGHV sequence as a marker and found that 18 of 23 (78%) classic RT cases had an identical IGHV sequence

DOI: 10.1002/hon

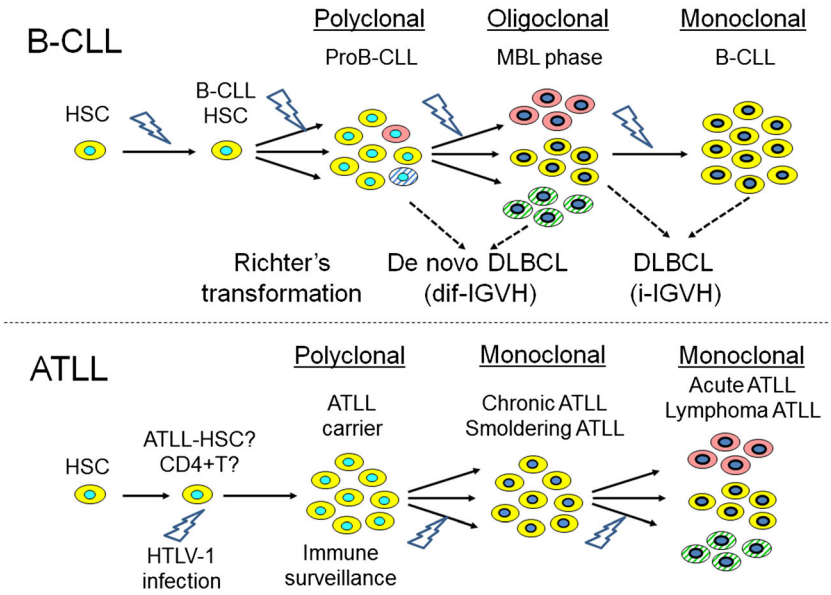

Figure 4. A clonal evolution model for B-cell lymphocytic lymphoma (B-CLL) and adult T-cell leukaemia/lymphoma (ATLL). Kikushige *et al.* used a mouse xenograft model and demonstrated that B-CLL stem cells (HSC) exist in the CD34+ fraction [16]. Their model revealed that at least two fractions of B-CLL HSC before and after *IG* rearrangement can proliferate in NOG-SCID mice. This model fits well the findings of Mao *et al.* who analysed 40 cases of RT (upper panel). ATLL is initiated by virus integration. About 95% of virus carriers do not develop ATLL, but 2–5% show clinical manifestation with the accumulation of independent genetic events. Paired sample analysis revealed multiple subclones in lymph node tumour cells (lower panel), dif-IGVH, different IGVH sequence; i-IGVH, identical IGVH sequence

as the original CLL clones, indicating that clonal evolution took place [15]. The remaining five cases (22%) had different IGHV sequences, suggesting that these are *de novo* DLBCL, although there is some skew in VH gene usage [15]. Recently, Kikushige *et al*. analysed B-CLL stem cells in a xenograft mouse model and found that the stem cell fraction exists in CD34+ bone marrow cells but not in peripheral circulating tumour cells [16]. We find it interesting that the clones obtained from the same patients frequently showed different IGHV sequences although VH1, VH2 and VH4 usages were predominant. These results indicate that B-CLL stem cells (cells predisposed to B-CLL) exist before *IG* rearrangement takes place. This xenograft mouse model fits well with the finding of Mao *et al*. [15] that 22% of DLBCL had a different IGHV. The 78% of RT cases with an IGHV sequence identical to the original clone in Mao's report can be explained by the supposition that there are cell fractions with additional genetic alterations, one step advanced from Kikushige's stem cells predisposed to B-CLL, which were not able to expand under xenograft conditions. A possible explanation of the findings by Mao *et al*. in relation to Kikushige's model is shown in Figure 4 (upper panel). It seems that there are at least two pathways for B-CLL RT. One involves the B-CLL stem cell fraction before the *IG* gene rearrangement stage, which has a growth advantage. The second fraction comprises B-CLL stem cells after *IG* gene rearrangement, which probably contains enough genetic alterations to expand in the human body. It is likely that the majority of classic RT with an identical IGHV sequence is derived from this fraction. RT cases with a different IGHV sequence are likely to be derived from the B-CLL stem cell fraction before *IG* gene rearrangement. This process is somewhat similar to HTLV-1 carriers. The majority of carriers do not develop ATLL, whereas 2–5% develop ATLL after a long latency period of 40–60 years (Figure 4, lower panel). A portion of these individuals develop chronic-type ATLL, and most of the patients develop acute-type ATLL. When genomic profiles of these two types of ATLL are compared, common regions are detected, which are probably collaborating with HTLV-1 (Yoshida *et al*., manuscript in preparation). We find it interesting that the genomically altered regions are more frequent and there exist regions specific to the acute type, suggesting that chronic-type ATLL may be one or two steps less advanced in comparison with the acute type (Yoshida *et al*., manuscript in preparation).

Conclusion

Malignant lymphoma is a monoclonal neoplasm with clonal heterogeneity resulting from clonal evolution. Genome profile analysis demonstrated that genomic alterations may have a hierarchy regarding occurrence but do not strictly adhere to one specific sequence. This clonal

DOI: 10.1002/hon

heterogeneity suggested that early genetic events and late events may exist. Reports of B-CLL clonality suggested that cells predisposed to B-CLL before *IG* gene rearrangement are likely to exist. In this context, genomic analyses, whole-genome sequencing of B-CLL stem cell clones, and investigation of *in situ* lymphomas of MCL and FL will provide important information concerning how and when the initiation mechanisms towards a definite malignant stage work. It is essential that these early events are explored in future studies. Investigation of the molecular mechanisms of genomic instability causing clonal heterogeneity is also an important issue because patients with clonal heterogeneity have a poorer prognosis.

Conflict of interest

The author declares no competing financial interests.

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DOI: 10.1002/hon