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Topical Update –
The Hong Kong College of Pathologists

Editorial note:

Chronic lymphocytic leukaemia (CLL) is the commonest chronic lymphoproliferative disorder of mature B-cells. Most cases have typical morphological and immunophenotypic profiles; on the contrary, they have extremely heterogeneous clinical courses. Cytogenetics and molecular cytogenetics classify the disease into different prognostic subgroups according to the genetic abnormalities in addition to enable us to understand the pathogenesis of the disease. In this topical update, we would like to introduce the common genetic abnormalities encountered in CLL and their role in pathogenesis, prognosis and treatment. In addition, our local experience in the application of cytogenetics and FISH in CLL will be shared. We welcome any feedback or suggestions. Please direct them to Dr. Wong Wai Shan (e-mail: sws_wong@yahoo.com.hk) of Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists.

Chronic lymphocytic leukaemia – the role of conventional and molecular cytogenetics

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Introduction

Chronic lymphocytic leukaemia (CLL) is the commonest chronic lymphoproliferative disorder of mature B-cells and affects mainly elderly. It is characterized by the presence of $\geq 5 \times 10^9$/L monoclonal and often CD5+ CD23+ B-lymphocytes in peripheral blood. Haematogists usually have no problem in reaching the diagnosis as the majority of the cases have classical morphological and immunophenotypic features; however, it is an extremely heterogeneous disease clinically with highly variable clinical course.

Some patients are asymptomatic and do not require treatment while others progress early and require aggressive treatment. A number of clinical and biological parameters as well as molecular biomarkers have been demonstrated to predict the clinical outcome of the disease [1]. Molecular diagnostics has greatly improved the understanding of pathogenesis of CLL by pointing to candidate genes, for example 17p13 deletion, a common genetic aberration seen in CLL, corresponds to a tumour suppressor gene *TP53*. Moreover, different genetic subgroups have been shown to be associated with different prognosis:

poor survival in 17p or 11q deletions and better survival in trisomy 12, normal karyotype or 13q deletion with the best survival found in isolated 13q deletion [2]. Cytogenetic studies may also help in the diagnosis of problem cases with atypical morphology or immunophenotypic profiles.

Cytogenetic and Fluorescence in-situ hybridization (FISH) – Basic concepts

Cytogenetic study is the analysis of the morphology of chromosomes in metaphase nuclei. Chromosomes are identified by their differences in length and position of the centromeres. A few drops of peripheral blood or bone marrow are added to tissue culture medium and stimulated to divide. After 48 to 72 hours, the dividing cells are arrested in metaphase of cell cycles with chemicals which inhibit mitotic spindle, collected, and treated with hypotonic solution to swell the cells and separate the chromosomes. Chromosomes are then fixed, spread on glass slides and stained. The most common staining method used by clinical laboratory is Giemsa staining which produces a non-uniform staining of the chromosome in a repeatable pattern called banding (G-banding in this particular case). The chromosomes occur in 23 pairs, 22 pair of autosomes and 1 pair or sex chromosomes. The sex chromosomes are termed X and Y; X for the female determining chromosome and Y for the male determining chromosome. Chromosomal aberrations occurring in haematolymphoid malignancies can be numerical (for example, monosomy 7 in myelodysplasia or hyperdiploidy in acute lymphoblastic leukaemia) or structural (for example, translocation between chromosome 9 and 22 in chronic myelogenous leukaemia).

Fluorescence in-situ hybridization (FISH) combines the DNA hybridization technique with fluorescence microscopy. During FISH, a unique DNA fragment or a mixture of DNA fragments are tagged with a fluorochrome. This combination is termed a probe. A slide with metaphase or interphase cells are subjected to conditions which allow the chromosome DNA strands to separate. It is then incubated with labeled DNA fragment (probe) and allowed to hybridize with complementary DNA sequence. The slide is then examined under ultraviolet light of appropriate wavelength and any region where the probe has bound will fluoresce. It allows the evaluation of the presence or absence of a particular DNA sequence or the number of chromosome.

Application of Cytogenetic and FISH in CLL

Conventional cytogenetic study has been performed in chronic lymphocytic leukaemia for years but the detection rate of genetic aberrations is unsatisfactory due to the low mitotic rate of leukaemic lymphocytes. Even with the addition of B-cell mitogens such as 12-*O*-tetradecanoly-phorbol-13-acetate (TPA), the yield improves to 40 to 50% only. Moreover, it is labour intensive and time consuming. FISH allows the detection of chromosomal aberrations not only in dividing cells but also in interphase nuclei of non-dividing cells. In addition, FISH is able to detect cryptic (submicroscopic) changes like deleted 13q (Figure 1a and 1b). Overall interphase FISH is able to detect genetic abnormalities in up to 80% of CLL cases. The major drawback is that it is limited to the specific FISH probes used and also unable to detect complex karyotypes, an adverse prognostic factor in CLL. Recently, CpG-oligodeoxynucleotides (ODNs) have been applied to stimulate the CLL cells' response to cytokines and improve the detection rate of conventional cytogenetic to 80%, comparable to FISH [3].

Common genetic abnormalities seen in CLL and their clinical implications

Chromosomal abnormalities in CLL are mostly deletions or amplifications of the involved chromosome regions while translocations are rare. The most frequent genetic aberrations are deletions in 13q, 11q, 17p or 6q and trisomy 12, with deleted 13q being the commonest (55%) [4]. The latter is often an interstitial deletion at 13q14 and is cryptic in nature, i.e. it cannot be detected by conventional cytogenetic study. No genes in this region have been identified to show a pathogenetic role in CLL although deletion and thus down-regulation of the micro-RNA genes at 13q14 have been described [5].

Structural aberrations of chromosome 11 have been reported in 12% to 25% of CLL, frequently involving q22 and q23 where the ataxia telangiectasia mutated (*ATM*) gene, a tumour suppressor gene, is located. CLL with 11q deletion has a poor prognosis and is associated with advanced disease, extensive lymphadenopathy and rapid lymphocyte doubling times [6]. A recent study has shown that 3 new candidate genes, *NPAT, CUL5 and PPP2R1B*, with roles in cell cycle regulation and apoptosis, were significantly down-regulated in CLL with 11q deletion [7].

17p deletion (Figure 2) is another poor prognostic marker that occurs in 10 to 15% of CLL. It results in the loss of *p53* located at 17p13. The *p53* gene is a tumour suppressor gene and plays an integral role in inducing cell cycle arrest and apoptosis after DNA damage. Patients with 17p deletion usually present at an advanced stage and have a high incidence of transformation. Loss of p53 is also associated with resistance to fludarabine and alkylating agent-based therapies [8].

Trisomy 12 (Figure 3) is found in 20% to 40% of CLL by FISH. It is more common in those cases with atypical morphological and immunophenotypic features, including lymphoplasmacytoid appearance, cleaved nuclei, bright CD20 expression and FMC7 positivity. A number of genes including CDK2, CDK4, STAT6, APAF-1 and MDM-2 which play important roles in cell cycle regulation, apoptosis and oncogenesis are located in chromosome 12 but none of them are well characterized in the pathogenesis of CLL. It is associated with an intermediate prognosis [9].

Deletion 6q are detected in up to 9% of CLL by FISH. It is characterized by a high incidence of atypical morphology, classical immunophenotype and intermediate incidence of IGVH somatic hypermutation [10].

Abnormalities involving chromosome 14q32, at which Ig heavy chain (IgH) is located, are seen in 6% to 14% of CLL. The *IgH* locus is fused with other partner genes. One such translocation involves the *BCL3* gene at 19q13. Cases with translocation (14;19)(q32;q13) usually have atypical morphology, increased prolymphocytes and cleaved/indented nuclei. Such patients present at a younger age and have an aggressive clinical course [11].

Local Experience

All Chinese patients diagnosed with CLL in Queen Elizabeth Hospital (QEH) from May 2007 to August 2010 and those from all public hospitals in Hong Kong during the 10-month period of September 2010 to June 2011 were referred to the haematology laboratory of QEH for both conventional cytogenetic and FISH analysis. 77 patients were recruited with a median age of 65 years (range: 37-94 years, mean 64.2 years) and a male to female ratio of 2.5:1. Cytogenetic study was performed on bone marrow or peripheral blood lymphocytes by 3-day 12-O-tetradecanolyphorbol-13-acetate-stimulated (TPA) and overnight fluorodeoxyuridine synchronized culture. Metaphase chromosomes were banded by trypsin/Giemsa and karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN) 2009. The cell pellets kept in Carnoy's fixative were used for the FISH study. A panel of locus-specific and centromeric probes were used, namely *ATM* (11q22.3), D12Z3 (CEP12), D13S319 (13q34) and p53 (17p13.1) in 2 cocktails of probe mixes.

Clonal cytogenetic abnormalities were detected in 33.8% of patients (26 out of 77) by conventional cytogenetic study, with trisomy 12 being the most frequent change. Majority had a single chromosomal abnormality (61.5%) and the rest had more complex karyotypes, with evidence of clonal evolution in nine patients.

FISH raised the detection rate of cytogenetic abnormality to 81.8% and over half of the cases had a single abnormality (59.7%, 46/77). Loss of 13q14.3 was the commonest abnormality detected (46/77) with the majority being heterozygous deletion (37/46). Translocation involving 13q were detected in 4 of them (12). Trisomy 12 was the second most frequent abnormality (20.8%, 16/77) and loss of *ATM* gene and *p53* are found in 9.1% (7/77) and 6.5% (5/77) respectively.

Our preliminary data show that the abnormality rate and frequency of deleted 13q and deleted 17p

in Chinese patients with CLL in Hong Kong are similar to those of the West. It is also found that deleted 11q is associated with a younger age of onset and deleted 17p with complex karyotypes demonstrated by conventional cytogenetics.

Conclusion

Conventional cytogenetics and FISH have been proven pivotal in separating CLL into distinct clinical subgroups for both prognostication and treatment purposes. They identify genetic aberrations that play a role in the pathogenesis of CLL. Conventional cytogenetics and FISH are complementary in the identification of chromosomal aberrations in CLL, with the former providing a global view while the later identifying cryptic changes. FISH is also useful in providing supplementary information in those cases with a failed culture.

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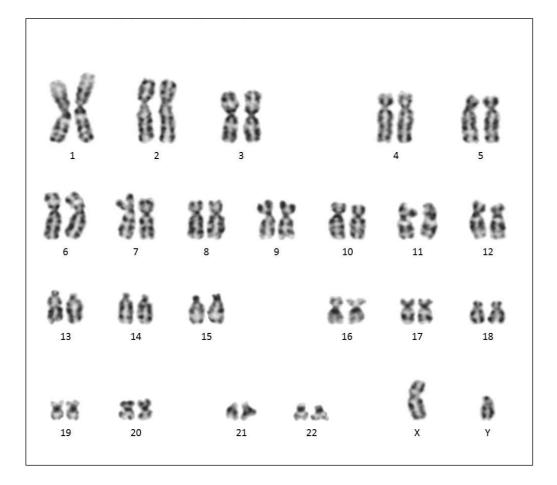
Figure 1a. A patient newly diagnosed CLL and normal karyotype 46,XY by conventional cytogenetics, both chromosomes 13 appear normal

Figure 1b. Metaphase ish with a panel of probes for chromosome 12 (SpectrumGreen, Vysis), 13q14.3 (SpectrumOrange, Vysis) and 13q34 (SpectrumAqua, Vysis) performed on the same patient of Figure 1a. Arrow indicates the abnormal chromosome 13 with loss of 13q14.3 signal (red).
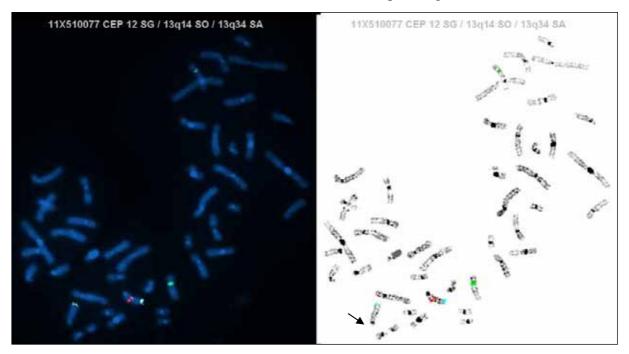
Figure 2. Interphase ish with probes for *ATM* (11q22.3) (SpectrumGreen, Vysis) and *p53* (17p13.1) (SpectrumOrange, Vysis), showing loss of one signal for 17p13.1 (red).
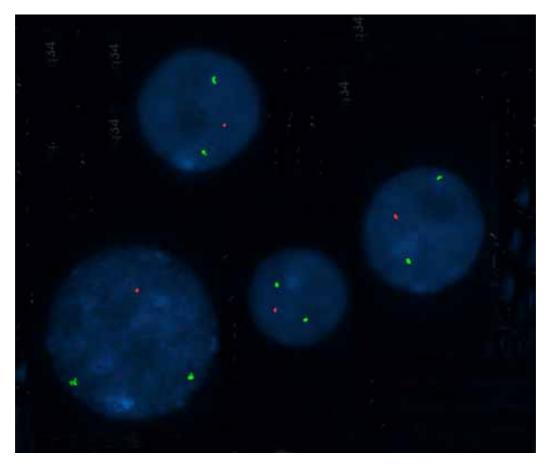

Figure 3. Trisomy 12, shown by G-banding in conventional cytogenetics