

Detection and quantification of MBR/JH2 t(14;18) BCL-2 gene rearrangement in follicular lymphoma using a combined real-time polymerase chain reaction assay

We report our experience with a new real-time polymerase chain reaction (PCR) assay applicable for simultaneous quantification and characterization of MBR/JH translocation in follicular lymphomas. This technique, which combines amplification with the FRET probe with SYBR Green I melting curve analysis, allows efficient detection of tumor cells in bone marrow or peripheral blood and their comparison with the original neoplastic clone.

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Follicular lymphoma (FL) accounts for about 30% of newly diagnosed non-Hodgkin's lymphomas. In FL the neoplastic clone is characterized by the chromosomal t(14;18) translocation in up to 90% of patients resulting in a rearrangement of *BCL2* and the immunoglobulin heavy chain (*IgH*) genes.¹ The majority of the patients will relapse, usually those with advanced stage disease, due to the persistence of residual disease. The results of several clinical trials indicate that the absence of neoplastic cells bearing the *BCL2*/*IgH* rearrangement in bone marrow and peripheral blood during the follow-up is strongly associated with a reduced risk of recurrence.²⁻⁵ During the molecular follow-up, polymerase chain reaction (PCR)-detected *BCL2*/*IgH* rearrangements are usually related to the original FL clone but occasionally are unrelated, since the MBR/JH fusion sequence can also be detected in peripheral blood of individuals without tumors.⁶⁻⁹ Consequently, it is crucial to determine whether the cells showing the *BCL2* gene rearrangement in the peripheral blood are iden-

tical to the original tumor clone. Sequencing of the amplified products is a widely accepted technique for analyzing the clonality of rearranged sequences but it is expensive and time-consuming.⁷ Fluorescent melting curve analysis is an alternative real-time PCR approach that is used to characterize a DNA fragment, because the melting temperature of DNA segments depends on their GC content, length and sequence.¹⁰ Unlike the FAM-Tamra labeled TaqMan probe, which may also provide low detection limits,⁶ FRET probes used by the current real time LightCycler instruments provide two detection wavelengths (640 and 705nm), both of which are higher than that of SYBR Green I and thus make melting point analysis possible.

We examined 29 lymph node, 107 peripheral blood and 6 bone marrow samples from 29 patients with different clinical stages of FL. Five milliliters of blood and 2 mL of bone marrow were used for tumor cell detection. The lymphocytes were separated by Biocoll solution (BIOCHROM AG, Germany) and a 300 µL cell suspension, containing mostly lymphocytes, was used for DNA extraction performed with a commercially available DNA isolation kit (High Pure, Roche, Germany). DNA from lymph nodes was isolated using the same kit. PCR was performed using 2 µL FastStart mastermix (Roche, Germany), 5 mM MgCl₂, 0.5 µM MRP 3'LC-705 labeled hybridisation probe (5'-TgTgTTgAAACAggCCACgTAA-LC705-3'), 0.5 µM IgJH2 primer (5'-ACCTgAggAgACggTgAC-3'), 0.5 µM MBR2 internal fluorescently labeled primer (5'-TTTgACCTTTAgAgAgT-f-TgC-3'), 2 µL template DNA (max 1 µg) and PCR grade water to a final volume of 20 µL. TibMolbiol (Germany) manufactured all the primers and probes. Amplification was performed in a Roche LightCycler instrument under the following conditions: incubation: 95°C for 10 min; denaturation, 95°C for 5 sec; signal detection, 50°C for 0 sec (channel F3); annealing, 61°C for 10 sec; extension, 72°C for 12 sec. The melting curve analysis was done after the above-described real time PCR adding 2 µL master SYBR Green I (Roche, Germany) to each capillary. After denaturation for 1 sec at 95°C the samples were cooled to 65°C for 30 sec and heat-

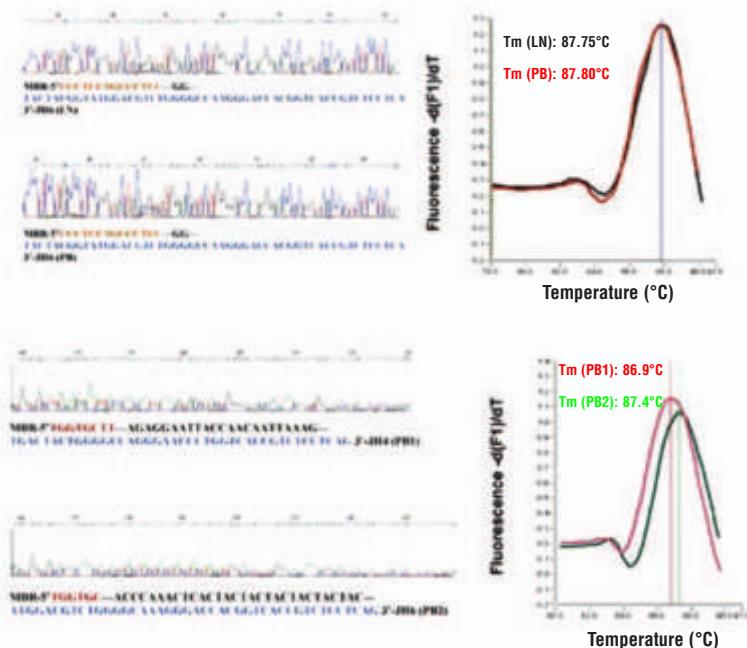


Figure 1. Melting curve and sequence analysis of two cases. Fluorescence melting peaks of the PCR products from the lymph node and peripheral blood of the first patient (case 7) show identical melting temperatures and sequences (the vertical lines indicate the melting points). In the second patients (case 11) two different MBR/JH translocation products are present; the different melting temperatures refer to unrelated clones.

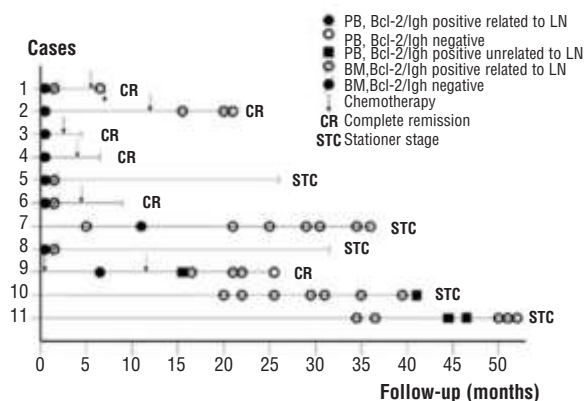


Figure 2. Clinical and molecular analysis of cases: samples taken 0 - 56 months after diagnosis of follicular lymphoma. Dots and squares represent time when blood or bone marrow samples were collected. The length of the line corresponds to the follow up in months.

ed to 95°C (slope 0.1°C/sec). The signal was detected on channel F1. We found that the previous quantification step had no effect on melting curve analysis. The quantitative analysis was assessed with serial dilutions of DNA samples, representing 2, 20, 200 and 2000 tumor cells from the MBR/JH t(14;18)-positive DOHH2 cell line (DSMZ, Germany), starting with an initial template concentration of 160 ng/20µL (~20000 tumor cells; 7.1pg DNA is equivalent to 1 cell).^{6,9} We could routinely detect two tumor cells. Among 18 patients the MBR/JH translocation was not detected in either the peripheral blood or bone marrow throughout the entire molecular follow-up. The clinical and molecular analysis of the remaining 11 patients is shown in Figure 2. Altogether 16% of peripheral blood and bone marrow samples contained cells showing the *BCL2/IGH* rearrangement. These *BCL2/IGH*-positive cells were compared to the corresponding lymph node cells in these 11 patients. In eight cases, evidence of clonal identity was provided by detection of *BCL2* MBR/JH fusion products with identical melting points. Unrelated rearrangements with different melting points were found in three cases. The clonality was also confirmed by direct sequencing (Figure 1). No disease progression was seen in the three patients with unrelated rearrangements by 16-44 months after the initial diagnosis (Figure 2). Ladetto *et al.*⁷ also reported evidence of *BCL2/IGH* rearrangements - occurring more than 3 years after treatment - which were unrelated to the original tumor clone in two autografted FL patients.

Our findings underline the importance of determining the clonal identity between the tumor and residual cells, which is crucial to avoid unnecessary treatment of patients. On the basis of our results we suggest the use of the FRET-labeled probe and primer combined with SYBR Green I fluorescent melting curve analysis. This is a fast, easy and cost-effective method for quantification and characterization of the *BCL2* gene rearrangement and can improve the management of patients with FL.

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