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Pyrosequencing as a Fast and Reliable Method in Detecting the *MYD88* p.L265P Mutation in Decalcified Formalin-Fixed and Paraffin-Embedded Tissues

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Dear Editor,

MYD88 p.L265P has recently been identified as a considerably recurrent mutation in lymphoplasmacytic lymphomas with an IgM monoclonal protein (Waldenström's macroglobulinemia [WM]). The mutation was also described as a common event in several other lymphoproliferative disorders, including diffuse large B-cell lymphoma and primary central nervous system (CNS) lymphoma [1].

Detecting *MYD88* p.L265P mutations was subsequently proposed as critical to the differential diagnosis of WM, multiple myeloma, and marginal zone lymphoma [2, 3]. A more recent study indicated that *MYD88* mutation frequency in WM was slightly lower than was previously assumed. Patients with wild-type *MYD88*, however, often show amplifications on chromosome 3 at the 3p22 locus, which includes the *MYD88* gene [4]. Because *MYD88* plays a role in activating nuclear factor (NF)-κB signaling, its mutation will likely be relevant to targeted therapeutics [5].

Previously published methods for the detection of *MYD88* p.L265P include high-resolution melting analysis (HRMA), allele specific polymerase chain reaction (AL-PCR), and direct DNA sequencing [2, 6].

The purpose of this study was to establish a pyrosequencing assay using decalcified formalin-fixed and paraffin-embedded (dFFPE) bone marrow trephine biopsies from 14 patients with WM and 10 patients with multiple myeloma. To extend the application of the technique, we used the assay to evaluate fresh bone marrow mononuclear cell samples (n=5) and peripheral blood samples (n=5) collected from five of the 14 WM patients (Cases 1, 4-7). All samples were collected as part of standard clinical care and diagnosed at the Reference Center for Lymph Node Pathology and Hematopathology, University Hospital of Schleswig-Holstein, Campus Luebeck, Germany. All studies were approved by the Ethics Committee at the University of Luebeck and were in accordance with the Declaration of Helsinki.

Pyrosequencing was performed as described previously [7]. DNA was extracted with the QiaAmp Mini Kit 250 (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A short sequence of DNA encompassing the mutation site was amplified by using a specific pair of primers, one of which was biotinylated (in this case the reverse primer). Next, a single strand of the amplified mutation region was prepared by using streptavidin-coated Sepharose beads to specifically bind the biotin tag on the reverse primer. Sequencing was subsequently performed on a PyroMark Q24 platform (Qiagen) following incubation with a forward sequencing primer. Allele frequency was quantified utilizing the PyroMark Software (Qiagen). Primers

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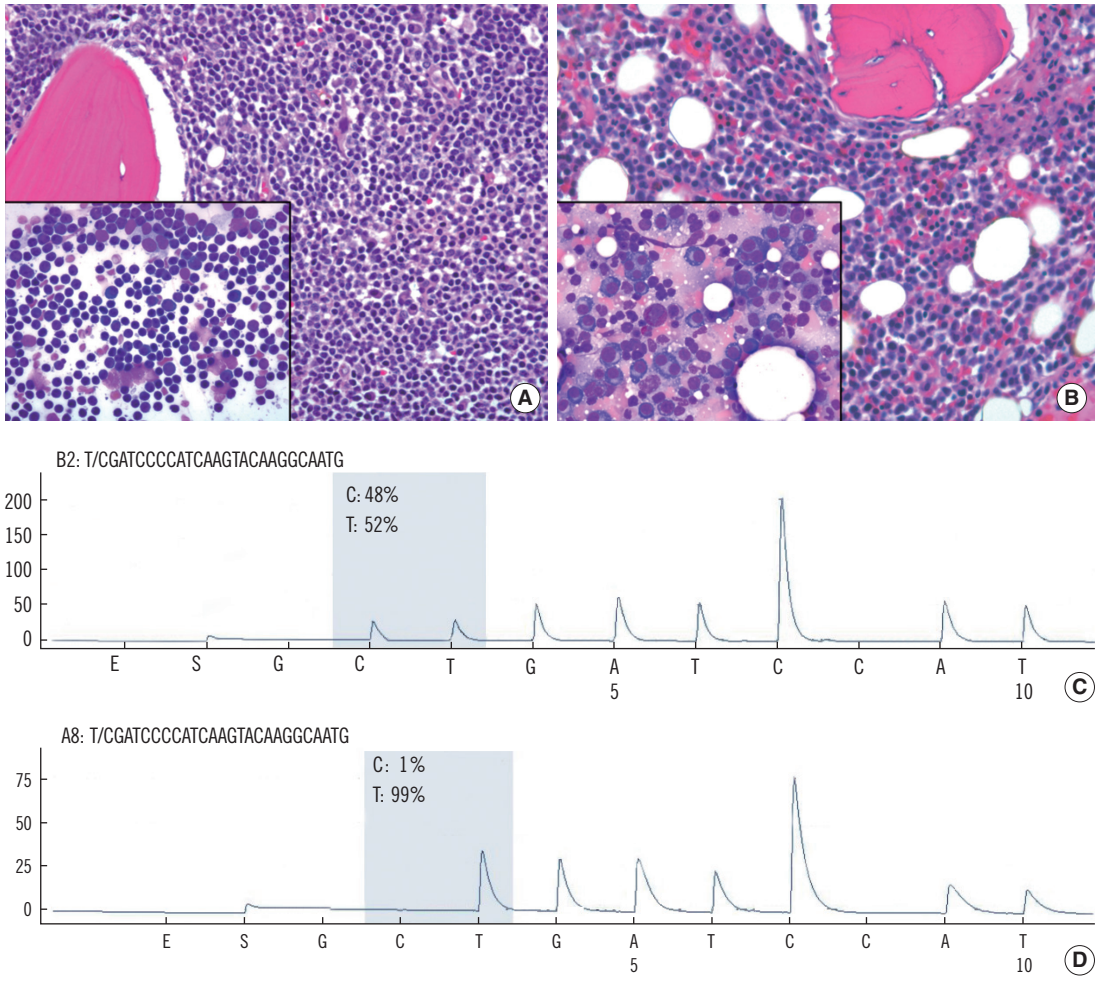

Fig. 1. Bone marrow trephine biopsy and smear from a case of Waldenström's macroglobulinemia harboring the *MYD88* p.L265P mutation with an allele frequency of 46% as determined by pyrosequencing assay (A), and a patient suffering from multiple myeloma without the *MYD88* p.L265P mutation (B) (hematoxylin-eosin, ×200, Pappenheim stain, ×400). Corresponding quantification of allele burden using the PyroMark software in the above cases of Waldenström's macroglobulinemia (C) and multiple myeloma (D).

were designed and synthesized (Tib Molbiol, Berlin, Germany) as follows: *MYD88*-265-forward: 5′-GAATGTGTGCCAGGGGTACTT; *MYD88*-265-reverse: 5′-Bioteg-TCAGGATGCTGGGGAACTA; *MYD88*-265-sequencing-forward: 5′-CCCATCAGAAGCGAC.

In agreement with the literature, 11 of 14 (78.6%) WM samples contained the *MYD88* p.L265P mutation [2]. Wild-type sequence was found in 3 samples (21.4%). Poulain et al. [4] recently suggested that an alternative genomic aberration affecting the *MYD88* gene, e.g., 3p22 amplification, might be relevant in such cases promoting a functionally equivalent activating effect on NF-κB signaling. All cases of multiple myeloma tested negative. Morphological and molecular aspects of two selected cases are displayed in Fig. 1. *MYD88* p.L265P mutations with an allele frequency of 5% or higher were reproducibly detected with the pyrosequencing assay. For comparison, all samples were sequenced by the Sanger method, which generated a sensitivity cut-off at an allele burden of approximately 20%. Results from the analysis of fresh bone marrow and peripheral blood samples showed that the sensitivity was comparable to that seen in the dFFPE samples, detecting *MYD88* mutations in all five cases (allele burden 8-48%). Clinical, hematological and molecular features of the study group are briefly summarized in Table 1.

Compared to previously published methods, pyrosequencing provides a fast, reliable, highly sensitive, and economic method to detect *MYD88* p.L265P [2, 6]. Because it quantifies the allele burden, pyrosequencing is useful for follow-up diagnostics and monitoring disease activity. Its robustness and applicability to dFFPE samples render it useful for routine hematopathological diagnostics.

Table 1. Clinical, hematological and molecular features of the study group

Case No.	Sex	Age (yr)	Diagnosis	Hb (g/dL)	WBC ($\times 10^9$/L)	PLT ($\times 10^9$/L)	*MYD88* p.L265P allele burden in dFFPE samples, BM and PB-Pyro*	*MYD88* p.L265P-Sanger†
1	M	60	WM	11.9	3.6	234	Positive 6% (BM: 8%, PB: 8%)§	No
2	F	61	WM	10.7	4.6	134	Negative	No
3	F	74	WM	12.3	6.9	221	Negative	No
4	M	69	WM	9.2	7.2	254	Positive 42% (BM: 44%, PB: 37%)§	Yes
5	F	54	WM	11.3	4.5	178	Positive 48% (BM: 48%, PB: 39%)§	Yes
6	M	72	WM	10.4	5.3	146	Positive 36% (BM: 39%, PB: 28%)§	Yes
7	M	49	WM	12.8	3.8	157	Positive 27% (BM: 28%, PB: 24%)§	Yes
8	F	71	WM	10.6	7.4	187	Negative	No
9	M	53	WM	11.9	5.6	261	Positive 61%	Yes
10	F	73	WM	8.1	3.8	197	Positive 52%	Yes
11	F	54	WM	8.2	6.1	520	Positive 23%	Yes‡
12	M	58	WM	9.4	7.9	384	Positive 24%	Yes‡
13	M	74	WM	8.7	6.7	267	Positive 5%	No
14	F	75	WM	10.2	5.8	310	Positive 24%	Yes
15	M	49	MM	11.6	3.8	178	Negative	No
16	F	49	MM	13.1	5.3	242	Negative	No
17	M	68	MM	12.3	5.2	244	Negative	No
18	M	35	MM	11.3	6.4	216	Negative	No
19	F	71	MM	10.8	7.4	188	Negative	No
20	M	73	MM	12.2	8.1	321	Negative	No
21	F	78	MM	8.8	6.9	178	Negative	No
22	M	62	MM	11.4	5.4	228	Negative	No
23	F	70	MM	10.9	4.7	199	Negative	No
24	F	66	MM	9.8	4.8	167	Negative	No

*As detected by pyrosequencing; †Detection of *MYD88* p.L265P by means of Sanger sequencing; ‡Reliable detectability by means of Sanger sequencing yet in close proximity to the sensitivity cut-off; §Results from pyrosequencing on dFFPE samples confirmed by comparative investigation of fresh bone marrow mononuclear cells (BM) and peripheral blood samples (PB).
Abbreviations: M, male; F, female; WBC, white blood cell; PLT, platelets; dFFPE, decalcified formalin-fixed and paraffin-embedded; WB, Waldenström's macroglobulinemia; MM, multiple myeloma.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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