Technical Advance

Demonstration of Light Chain Restricted Clonal B-Lymphoid Infiltrates in Archival Bone Marrow Trephines by Quantitative Real-Time Polymerase Chain Reaction

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Assessment of clonality either by demonstrating light chain restriction or showing monoclonal immunoglobulin gene rearrangement is a valuable and indispensable adjunct to diagnosis in hematopathology. The study of light chain restriction by immunohistochemistry on archival material is hampered by a very low sensitivity especially regarding low grade lymphomas of B cell origin. DNA rearrangement studies of the immunoglobulin locus do improve sensitivity markedly but for lymphomas of follicle center origin they are prone to false negative results due to hypermutations. Therefore we developed a new clonality assay based on the quantification of immunoglobulin light chain transcripts using real-time polymerase chain reaction technology, which is also suitable for the analysis of archival bone marrow trephines. We tested the reproducibility and sensitivity of this approach by comparatively analyzing a series of bone marrow trephines with multiple myeloma ($n = 26$), reactive lymphoid hyperplasia ($n = 37$), and focal infiltration by low grade B cell lymphoma ($n = 29$). We could raise the detection rate of clonality from an average of 17% by immunohistochemistry and 66% as assessed by polymerase chain reaction rearrangement studies to 83% by this new technique. Despite false negative results due to light chain hypermutation in some cases, the detection rate of clonality could be improved even for B cell lymphomas of follicle center origin (follicular lymphoma or marginal zone lymphoma) thus making this novel approach a valuable additional tool for the hematopathologist. (*Am J Pathol* 2001, 159:2023–2029)

The detection of clonality has become a standard tool in the differentiation of reactive from neoplastic lymphoproliferative lesions in hematopathology. This can be achieved by DNA rearrangement studies or by the demonstration of restricted light chain expression using either immunohistochemistry or mRNA *in situ* hybridization.^{1,2} The expression studies are hampered by several technical difficulties concerning primarily the reproducibility, sensitivity, and the quantitative evaluation of staining results.³ This is especially a problem in the analysis of archival specimens. Rearrangement studies using polymerase chain reaction (PCR) are prone to false negative results due to hypermutations or ongoing mutations at the immunoglobulin heavy chain genes and to false positive results caused by amplification of non-functional junctions and pseudogenes or the random preferential amplification of one allele in a small B cell population. These problems are especially prominent when analyzing formalin-fixed paraffin-embedded (FFPE) archival biopsies.^{4,5} Quantitative mRNA analysis for light chain expression would provide an alternative approach to detect clonality

Therefore, in this study we established for the first time a consensus real-time PCR assay^{6,7} for the quantification of immunoglobulin light chain transcripts suitable for use on FFPE and even decalcified bone marrow biopsies. (Since only formalin-fixed biopsies are processed at our institution, we focus our analysis on this type of fixation.) This assay is based on a simplified and robust RNA extraction protocol, which is also suitable for the processing of formalin-fixed and plastic-embedded biopsies.⁸ We validated this new assay in a well defined biological system showing its superior sensitivity and high repro-

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ducibility for neoplastic B cell proliferations in routinely prepared bone marrow biopsies.

Materials and Methods

Patient Samples and Cell Lines

Altogether 92 FFPE bone marrow biopsies comprising 26 cases of multiple myeloma, 37 cases of reactive lymphoid hyperplasia, and 29 cases with infiltration of the bone marrow by chronic lymphocytic leukemia (CLL: CD5⁺, CD10⁻, CD20⁺, CD23⁺, CD43⁺, CD79⁺, *n* = 15), follicular cell lymphoma (FCL: CD5⁻, CD10⁺, CD20⁺, CD23⁻, CD43^{-/+}, CD79⁺, bcl-2⁺, *n* = 12), and marginal zone B-cell lymphoma (MZBCL: CD5⁻, CD10⁻, CD20⁺, CD23⁻, CD43⁻, CD79⁺, *n* = 2)⁹ were retrieved from the archival files of the Bone Marrow Registry at the Institute of Pathology, Hannover Medical School, Hannover, Germany. All bone marrow biopsies were fixed in 0.1 mol/L K-acetate/0.5% glutaraldehyde/1.1% formaldehyde for at least 18 hours, decalcified using EDTA for 48 to 72 hours at neutral pH and paraffin embedded.

Cell lines were obtained from the ATCC, Rockville, MD (Daudi κ , Raji κ , Ramos κ , BL 60–2 λ) and the DSMZ, Braunschweig, Germany (RPMI 8226 λ , IM-9 κ , Namalwa λ , JVM-13 λ). All cell lines were cultured in RPMI 1640 medium with 10% heat inactivated fetal calf serum (Biobrom KG, Germany) with 100 U/ml penicillin and 100 μ g/ml streptomycin.

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected from healthy volunteers after informed consent using the Ficoll gradient method. The anonymous lymph nodes without any morphological detectable alteration were retrieved from the tissue bank of the Department of Pathology.

RNA Extraction

Total RNA from cell lines, PBMCs, and fresh frozen lymph nodes was isolated using the TRIzol reagent (Gibco BRL, Germany) according to the manufacturer's instructions. The RNA was isolated from the bone marrow samples essentially as described.⁸ Briefly, three to six 10- μ m sections were cut from each paraffin block. The sections were incubated overnight at 55°C in a vigorously agitating thermoshaker in a solution containing 4 mol/L guanidinium isothiocyanate; 0.25 mol/L sodium citrate; 0.5% sarcosyl, 0.1 mol/L β -mercaptoethanol; and 5 mg of proteinase K. After extraction with water-saturated phenol and chloroform, the RNA was precipitated from the aqueous phase with isopropanol and glycogen as a carrier.

cDNA Synthesis, Real-Time PCR, and IgH-PCR

1 μ g of total RNA was transcribed using 200 units of SuperScript II RNase⁻ reverse transcriptase (Gibco BRL, Germany) following the manufacturer's protocol. The PCR amplification was performed using a 96-well tray and optical caps (Applied Biosystems, Weiterstadt, Germany) with a 30- μ l final reaction mixture containing 250

A) Sequences of primers and hybridization probes

Name	Sequence
C κ	5'-AGCAGGCACACAACAGAGGC
V κ 1a	5'-AGSCTGAAGATKTTGSAGTTTATTACTG
V κ 1b	5'-AGSCTGAAGATKTTGSAACTTATTACTG
V κ 2a	5'-CAGATTTACWCTSAAAATCAGC
V κ 2b	5'-AGATTTACWCTSAACATCAGC
κ probe	5'-FAM-CCAGATTTCAACTGCTCATCAGATGGCG-TAMRA
C λ	5'-CTATCTGCCTTCCAGGCC
V λ 1	5'-TGAGGATGAGGCTGATTATTACTG
V λ 2	5'-CTGACCGATTCTCTGGCTCC
λ probe	5'-FAM-CACACCAGTGTGGCCTTGTGGCTTG-TAMRA

B) Location of primers and probes

Figure 1. A: Sequences of all primers and hybridization probes used in this study. **B:** Schema of primer and probe binding sites in the immunoglobulin light chain transcripts.

nmol/L each primer, 150 nmol/L probe, 0.75 units of platinum *Taq* (Gibco BRL, Germany), 200 μ mol/L each of dATP, dCTP, dTTP, and dGTP in 1X Platinum *Taq* reaction buffer and 4 μ l of cDNA. The reaction mixture was preheated at 95°C for 5 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. For each light chain all primers and the hybridization probe were combined in a single reaction. The primer and probe sequences and their location are given in Figure 1. The IgH rearrangement was detected as described.¹⁰

Data Evaluation

The average difference of $C_T(\lambda)$ and $C_T(\kappa)$ in a series of 37 FFPE biopsies displaying reactive hyperplasia $\pm$ the double SD ($\Delta C_T \pm 2\sigma$) was defined as the normal range [ΔC_T : solid line in Figure 3A], $\pm 2\sigma = 1.2$ to 4.8, dotted lines in Figure 3A]. A sample with a $\Delta C_T(\lambda - \kappa) < 1.2$ or > 4.8 was classified as monoclonal concerning the light chain expression. This corresponds to a $\kappa:\lambda$ ratio of greater than 8:1 or smaller than 0.75:1.

Results

Development of a New Consensus Real-Time PCR for Immunoglobulin Light Chain Transcripts

The aim of this study was to establish the quantification of immunoglobulin light chain mRNA as a novel assay for the detection of monoclonal B-cell populations. For the design of new consensus primers and hybridization probes suitable for the analysis of FFPE samples, all available sequences of functional V region and C region exons retrieved from VBASE and NCBI GenBank were

aligned to cover the sequence variability as completely as possible.

Two regions of nearly identical sequences were identified in each set of V region exons and appropriate primers were designed (two $V\lambda$ upstream primers and four $V\kappa$ upstream primers, see Figure 1). In each set of the C region exons, a sequence displaying 100% conservation and long enough to design a consensus primer and a consensus hybridization probe for the real-time PCR assay was identified.

Validation of the Multiplex Consensus Real-Time PCR

The specificity and sensitivity of the newly designed primers were evaluated using RNA isolated from lymphoid cell lines expressing only λ or κ transcripts (see Materials and Methods). Mixing experiments showed that the combination of the four $V\kappa$ upstream primers in a single reaction did not reduce the reaction efficiency. This enabled a simple multiplex approach completely covering the whole spectrum of known framework region sequences in one reaction tube.

The λ -expressing cell line RPMI8226 showed no cross-reactivity using κ primers and the κ -expressing cell line Daudi was also completely negative using the λ primers, thus demonstrating a very high specificity of the chosen primers and probes (see Figure 2 A)). The transcripts from 10 λ light chain-expressing cells could be detected in a background of 10^6 κ light chain-expressing cells (Figure 2B, left). A similar sensitivity could be shown for the detection of κ transcripts (Figure 2B, right).

To apply the direct calculation of the ratio of λ and κ transcript levels in a given sample from the comparison of the C_T values (ΔC_T -method,¹¹) the reaction efficiencies have to be identical. Since the length of the PCR products is different for both primer probe systems (Figure 1B), we used two complementary approaches to determine this important index (Figure 2C). In the first approach, we measured the C_T values for a dilution series of a given cDNA sample. The reaction efficiency E can be calculated from the slope of the regression line in a $C_T/\log(\text{conc.})$ -diagram ($s = -1/\log[1 + E]$, Figure 2C, left). In the second approach, we measured the slope of the amplification plot in the linear range for a group of different samples ($s = \log[1 + E]$,¹² Figure 2C, right). A reaction efficiency of $91\% \pm 2\%$ was measured.

In a series of PBMC samples from healthy volunteers ($n = 8$) and fresh frozen lymph nodes ($n = 4$) without any morphological detectable alteration, we found the expected physiological ratio of κ versus λ transcripts of approximately 2:1 (data not shown).

Determination of the Normal Range for $\Delta C_T(\lambda - \kappa)$ in FFPE Biopsies

We chose 37 bone marrow biopsies showing reactive lymphoid hyperplasia to test the reproducibility of the RNA extraction protocol and this novel real-time PCR

assay. All samples showed small T-cell-dominated nodular lymphoid aggregates in the central bone marrow spaces and light plasmacytosis (below 10% of nucleated bone marrow cells) with slight predominance of κ light chain expression by immunohistochemistry. It would be expected that the ratio of κ and λ transcripts is constant in all samples, reflected by a constant difference between $C_T(\lambda)$ and $C_T(\kappa)$. The results shown in Figure 3 A demonstrate a constant ratio of the immunoglobulin light chain transcripts for all samples (open circles). This constant ratio defining the normal range was used in all following calculations as the reference value for the quantification of the ratio of immunoglobulin light chain transcripts (see Materials and Methods).

Analysis of Multiple Myeloma

We analyzed 26 unselected FFPE bone marrow biopsies with an unequivocal diagnosis of multiple myeloma, a neoplastic plasma cell proliferation with high levels of monoclonal light chain expression. By real-time PCR (Figure 3) a significantly altered ratio of λ and κ transcripts could be demonstrated in all cases. Evaluation of the preferential light chain expression gave 100% concordance between the results of immunohistochemistry and real-time PCR (data not shown).

Analysis of Lymphoproliferative Lesions

To further test the reliability and sensitivity of this new approach and compare it to established clonality assays we analyzed a series of bone marrow biopsies with infiltrates by low grade B cell lymphomas. The samples chosen were typical examples of chronic lymphocytic leukemia (CLL, $n = 15$), follicular cell lymphoma (FCL, $n = 12$), and marginal zone B cell lymphoma (MZBCL, $n = 2$) with an extended bone marrow infiltrate exceeding 30%. By immunohistochemistry¹³ monoclonal light chain restriction could be demonstrated for 20% and 8% of the cases of CLL and FCL, respectively.

In IgH rearrangement studies¹⁰ 93% of the CLL and 42% of the FCL cases were monoclonal, but both MZBCL cases appeared polyclonal. By contrast, the real-time PCR analysis of the immunoglobulin light gene transcripts demonstrated clonality for 100% of the CLL, 58% of the FCL, and both cases of MZBCL. The combined detection rate of monoclonality for FCL reaches nearly 70% (see Table 1). As expected, the detection rate is lower for FCL than for CLL due to hypermutations occurring in the precursor cells in the germinal center. These hypermutations interfere with primer binding. The detection rate for FCL using IgH-PCR is in accordance with reports in the literature.^{2,14,15} In cases of FCL and MZBCL, the novel real-time PCR clearly improves existing methodology for the analysis of clonal B cell lesions.

Discussion

In the present study we have developed a novel multiplex consensus real-time PCR assay for the reliable quantifi-

A) Specificity

B) Sensitivity

C) Efficiency

D) Efficiency

Figure 2. A: Demonstration of specificity of the consensus PCR approach. No cross-reactivity was observed. **B:** For determination of the technical sensitivity 10^6 cells of one cell line were mixed with a decreasing amount of cells of the other cell line. Subsequently, RNA was isolated and analyzed. **C:** The reaction efficiency E was calculated from the slope in a C_T value/log(conc.) diagram generated by analyzing a dilution series of a given cDNA ($s = -1/\log(1 + E)$) (**left**). In addition we calculated the efficiency from the slope of the amplification plot in the exponential reaction phase ($s = \log(1 + E)$) for several different cDNAs (**right**). For both measurements RNA isolated from FFPE biopsies was used to mimic the analysis of patient samples as accurately as possible. **D:** Demonstration that the multiplex PCR does not reduce amplification efficiency. In all samples tested, the primer mix (yellow curve) is as efficient as the best individual primer (blue curves). The relative fluorescence intensity ΔR_n is higher for the primer mixture because the primer concentration is higher and more than one reaction contributes to the emission of fluorescence.

Figure 3. Quantification of immunoglobulin light chain transcripts by real-time PCR in archival bone marrow trephines in a series of multiple myeloma ($n = 26$) and reactive lymphoid hyperplasia ($n = 37$). **A:** Ratio of κ and λ light chain mRNA in reactive lymphoid hyperplasia ($\circ$) and multiple myeloma ($\blacklozenge$) measured as the difference of the C_T value for κ and λ transcripts. **Solid line,** $\Delta C_T(\lambda - \kappa)$; **dotted lines,** $\pm 2\sigma$ range (see Materials and Methods). **B:** Amplification plot of a sample displaying κ light chain restriction. **C:** Amplification plot of a reactive lymphoid hyperplasia. **D:** Amplification plot of a sample displaying λ light chain restriction. **B–D:** All reactions were performed in duplicates. ΔRn is the normalized relative fluorescence intensity measured on-line during the PCR process. The **thick horizontal line** is the threshold of detection.

cation of immunoglobulin κ and λ transcripts. This real-time PCR method is a new tool for the detection of monoclonal B-cell populations.

Most published primer pairs for the amplification of λ and κ transcripts^{16–19} are not suitable for the analysis of FFPE biopsies because the distance between the primer binding sites and hence the PCR product is much greater than the average fragment size of RNA extracted from FFPE samples.²⁰ Also in these studies several separate primer mixes have been used. Therefore, new consensus primers had to be developed. To cover the sequence variability as completely as possible, all available sequences of functional V region and C region exons were aligned. The number of degenerate positions in the PCR primers was kept at a minimum to ensure optimal amplification efficiency. The equal reaction efficiency for both κ and λ primer/probe systems (see Figure 2C) enabled

the direct calculation of the ratio of λ and κ transcript levels in a given sample from the comparison of the C_T values.¹¹ This relative quantification has several advantages. First, the difficult and laborious construction and storage of standard solutions is not necessary. Second, only signals generated from the same cDNA preparation (C_T value for κ and λ , respectively) are compared, excluding the variability of RNA preparation and the real-time reaction. This is especially important when analyzing fragmented RNA extracted from fixed tissue samples. Third, since only the ratio of κ and λ mRNA is measured, the amplification of standard solutions for a determination of absolute template concentrations is not necessary. This greatly increases the throughput of the system and reduces the costs per sample.

The real-time PCR technology is superior to conventional PCR approaches especially in the setting of a

Table 1. Results of Clonality Analysis of Low-Grade B-Lymphoma Infiltrates in the Bone Marrow

	Immunohistochemistry	IgH rearrangement	Light chain mRNA real-time PCR	IgH + κ/λ mRNA
CLL	3/15 (20%)	14/15 (93%)	15/15 (100%)	15/15 (100%)
FCL	1/12 (8%)	5/12 (42%)	7/12 (58%)	8/12 (67%)
MZBCL	1/2	0/2	2/2	2/2

diagnostic laboratory, because no post-amplification manipulation of samples is necessary thereby greatly reducing the risk of contamination and the workload. In contrast to all *in situ* methods (ie, immunohistochemistry and *in situ* hybridization) the reaction conditions and the data evaluation can be easily standardized in an objective manner for the real-time PCR system. In the future this will greatly facilitate the comparison of results between different laboratories. Flow cytometry also provides objective data but relies exclusively on freshly collected samples²¹ and is especially difficult for the exact quantification of κ and λ light chains.²²

The results concerning the κ/λ transcript ratio in freshly prepared PBMC samples and fresh frozen lymph node biopsies demonstrate the reliability of this new consensus real-time PCR assay in well defined samples. With this non-fragmented mRNA extracted from fresh biological material as a template, the C_T value difference between the κ transcript and the λ transcript is approximately one cycle corresponding to a factor of two (reflecting the physiological κ/λ ratio of 2:1). Due to the structure of the λ transcripts the distance between the λ primers is greater than the distance between the κ primers (Figure 1B) thereby generating longer amplification products. As a consequence the amplification of λ transcripts isolated from fixed tissue specimens will always result in C_T values higher than expected (and therefore also to a ΔC_T values greater than approximately 1 for the reactive lymphoid hyperplasia). This shift of the amplification plot to higher cycle numbers is not caused by a reduction in efficiency for the amplification of the longer λ transcript (see Figure 2C), but due to the well known fragmentation of RNA extracted from formalin-fixed biopsies (see²⁰ and references therein). This fragmentation is responsible for the fact that the concentration of RNA fragments long enough for successful amplification is always lower for λ than for κ . For these reasons the mean $\Delta C_T(\lambda - \kappa)$ -value measured for formalin-fixed and decalcified biopsies showing reactive lymphoid hyperplasia is not approximately 1 (as measured for freshly collected blood) but three ($\pm$ SD). These considerations also argue strongly for a relative quantification strategy as described above in favor to an absolute quantification.

To the best of our knowledge this is the first report of successful and reliable quantification of mRNA extracted from formalin-fixed, decalcified and paraffin-embedded archival bone marrow trephines. The use of unselected archival material in this study, fixed, decalcified, embedded, and sectioned under routine conditions is representative of a realistic clinical practice. The protocol for RNA extraction from FFPE and decalcified bone marrow biopsies described in this study has now been used for the isolation of RNA from several hundred specimens with an overall efficiency of more than 98%.

The constant λ/κ ratios in a large series of bone marrow biopsies displaying benign reactive lymphoid hyperplasia (Figure 3) clearly demonstrate the reproducibility of the RNA extraction from FFPE decalcified biopsies and the following quantification of transcript levels using real-time PCR technology. This reliability was further confirmed by the analysis of a series of multiple myeloma

which all showed a significantly altered light chain transcript ratio.

In this respect our study confirms and extends the results reported by Godfrey et al²⁰ and Specht et al²³ concerning the quantitative analysis of archival biopsies. Preliminary data also show that this new quantitative assay can be used for the analysis of immunostained sections (unpublished observations). In an ongoing study we have extended our methodology to the analysis of archival lymph node biopsies. To date, we have found with this type of biopsy an excellent correlation with the immunophenotype and an overall detection rate for monoclonality of nearly 90% using the PCR system and evaluation algorithm presented in this manuscript.

The analysis of a series of neoplastic lesions clearly demonstrates that in cases of follicular lymphoma the new assay described in this study increases the detection rate of monoclonal B cell populations. The detection of restricted light chain expression in a sample depends on the level of light chain expression, cellular composition of the suspicious infiltrate, and the extent of the inflammatory, polyclonal background. Therefore, it is not possible to define a general threshold of detection. To examine the sensitivity of our method we microdissected small lymphoid infiltrates (containing approximately 1000 lymphoid cells) from bone marrow trephine sections (using the laser-microdissection device from P.A.L.M., Bernried, Germany). In 5 of 6 cases of multiple myeloma monoclonal light chain expression could be clearly demonstrated (Bock et al, unpublished). In certain circumstances the ratio of κ and λ mRNA is altered without clear manifestation of malignancy.²⁴ Therefore the molecular data obtained with this new assay always have to be evaluated in the clinical and morphological context.

The protocols and data presented in this study complement and improve existing methods for detection of monoclonal B cell populations and form the basis for further quantitative studies concerning the gene expression level in morphologically defined archival biopsies. This will now enable large retrospective studies of well documented biopsies for which extensive clinical data are available.

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