Analytical Detection of Immunoglobulin Heavy Chain Gene Rearrangements in Gastric Lymphoid Infiltrates by Peak Area Analysis of the Melting Curve in the LightCycler System

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Because it is difficult to differentiate gastric mucosa-associated lymphoid tissue (MALT) lymphoma from chronic gastritis in gastric lymphoid infiltrates, molecular detection of monoclonality through immunoglobulin heavy chain (IgH) gene rearrangements is commonly performed. However, heterogeneity in the performance and results obtained from IgH gene rearrangements has been reported. To improve the accuracy in the diagnosis of gastric lymphoid infiltrates, we developed an analytical approach based on one-peak area analysis of the melting curve in the LightCycler System. Using a training-testing approach, the likelihood ratio method was selected to find a discriminative function of 4.64 in the training set (10 gastric MALT lymphomas and 10 chronic gastritis cases). This discriminative function was validated in the testing set (five gastric MALT lymphomas, six abnormal lymphocytic infiltrates with subsequently demonstrated gastric MALT lymphomas, and six cases of chronic gastritis). All but one case of gastric MALT lymphoma, as well as abnormal lymphocytic infiltrates, clustered under 4.64, and all chronic gastritis cases clustered above 4.64. These results were validated by conventional electrophoreses confirming one or two sharp bands in cases of gastric MALT lymphomas and a smear of multiple bands in cases of chronic gastritis. Analytical detection of IgH gene rearrangement in gastric lymphoid infiltrates by one-peak area analysis correctly distinguishes gastric MALT lymphomas from chronic gastritis, even in cases with diagnosis of abnormal lymphocytic infiltrates.

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Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) is the most common primary lymphoma at extranodal sites.¹ The most common organ involved is the stomach followed by skin and orbit.¹ In a subset of gastric MALT lymphomas, neoplasia arises as a consequence of chronic infection with *Helicobacter pylori* (ie, chronic gastritis). Because of these consequences, it is sometimes difficult to differentiate between cases of gastric MALT lymphoma and chronic gastritis. In addition, a limited availability of tissue for histological and immunohistochemical examination is another difficulty when differentiating between these two entities.^{2–4} Flow cytometry immunophenotypic studies for light chain restriction has been demonstrated to be useful in the differential diagnosis.⁵ Unfortunately, this technique is applicable only to fresh tissue, a type of sample not always available on clinical grounds. Molecular diagnostic studies such as Southern blot or polymerase chain reaction (PCR), based on the detection of heavy chain (IgH) gene rearrangement at the complementary-determining region III (CDR-III), are also alternative approaches.⁶ In recent years, PCR has gained more acceptance because it can be performed on formalin-fixed, paraffin-embedded (FFPE) tissue from endoscopic biopsies with a 100% specificity and 87% sensitivity.^{7–9} Therefore, the diagnosis of gastric MALT lymphoma based on PCR analysis is made when one or two sharp bands are seen on size fractionation by gel electrophoresis indicating monoclonal IgH gene rearrangement. Conversely, the diagnosis of chronic gastritis is proposed when a smear of multiple bands is seen on the gel, meaning polyclonal IgH gene rearrangement.¹⁰ Unfortunately, this type of analysis relies on large heterogeneity and subjective

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methodologies for measuring the amplicon(s) size(s), such as ethidium bromide agarose gel or polyacrylamide gel electrophoresis (PAGE), or for detecting gel mobility variations on sequence-dependent conformational changes.^{7,11–14}

With the development of the fluorescence-based measurements during the PCR reactions or post-PCR using melting curve analysis, it has become possible to analyze the products of PCR reactions on a real-time bases.¹⁵ Particularly in the melting curve analysis, a PCR product can be identified by virtue of its length, sequence, and GC content, which together determine the melting temperature of the duplex DNA molecule.¹⁵ Recent reports using real-time PCR with consensus primers for IgH gene rearrangement followed by melting curve analysis demonstrated that melting curve analysis was highly specific and sensitive relative to PAGE.^{16–19} However, these novel real-time PCR methods rely on subjective methodologies for measuring the amplicon(s) size(s) of PCR products. In this article, we explore a quantitative method for analyzing the melting curve to the detection of IgH gene rearrangement as a means of identifying monoclonal B-cell populations in small gastric endoscopic biopsy specimens.

Materials and Methods

Patient Samples

In this study, we used a training-testing approach.²⁰ As a training set, we selected 10 surgically resected gastrectomy specimens with diagnoses of gastric MALT lymphoma by morphological and immunophenotypic criteria and 10 gastric biopsies with definitive diagnosis of chronic gastritis. Gastric MALT lymphoma cases were classified according to the World Health Organization system for the classification of hematolymphoid neoplasms.²¹ Chronic gastritis cases were classified according to Sydney's system.³ As a testing set, 11 cases of gastric MALT lymphoma, in which endoscopic biopsies and surgically resected specimens were available, and six endoscopic biopsies of chronic gastritis were included. Among these 11 cases of gastric MALT lymphomas, five had the diagnosis of gastric MALT lymphoma in both endoscopic biopsy and surgically resected specimens. However, in the other six cases, only one surgical specimen had the diagnosis of gastric MALT lymphoma. Endoscopic biopsies of these cases had the diagnosis of abnormal lymphocytic infiltrates (ALI).²² All cases were retrieved from the files of the Laboratory of Pathology, Instituto Chileno Japonés de Enfermedades Digestivas at Santiago-Chile between 1993 and 2001 and were archival material (FFPE tissue). This study was approved by the Institutional Review Board at San Borja-Arriaran Hospital.

DNA Extraction

Five 15- μ m sections from paraffin blocks with representative areas of gastric MALT lymphoma or chronic gas-

tritis were cut and placed into a 0.5-ml tube for DNA extraction. DNA extraction was performed in 100 μ l of extraction solution (1 mol/L Tris, pH 8.0, 50 mmol/L ethylenediamine tetraacetic acid, and 0.5% Tween 20) with 1 mg/ml proteinase K (Sigma) for 12 hours at 55°C. Proteinase K was inactivated by boiling at 100°C for 10 minutes, and DNA was purified by phenol-chloroform extraction and ethanol precipitation according to standard protocols.²³ DNA concentration was determined by the absorbance measured at 260 nm.

LightCycler System Amplification and Melting Curve Analysis

One μ l (1 ng) of extracted DNA in 10 μ l of reaction mixture was used to amplify the IgH CDR-III gene rearrangement region by the LightCycler System (Roche Diagnostic GmbH, Mannheim, Germany) using a semi-nested protocol.^{12,24} The reaction mixtures contained 1 $\times$ FastStart DNA Master SYBR Green I (Roche Diagnostic GmbH), 2.5 mmol/L MgCl₂, and 0.5 μ mol/L of each primer. In the first round of amplification primers, FR3A (5'-ACACGGC(C/T)(G/C)TGTATTACTGT-3') and LJH (5'-TGAGGAGACGGTGACC-3') were used with a thermal profile of 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 0 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 10 seconds. One μ l (1:10 diluted) of this PCR product was used as a template for the second round of amplification with similar conditions using the same FR3A primer but an internal VLJH (5'-GTGACCAGGGTNCCTTGCCCCAG-3') as a reverse primer. After amplification, DNA melting curve analysis was performed. For this purpose, amplified products were denatured at 95°C for 5 minutes, followed by annealing at 60°C for 30 seconds. Then, the temperature was increased slowly from 60 to 98°C at a transition rate of 0.05°C/second during continuous fluorescence monitoring at 521 nm. The melting curve program was selected and instructed to quantitatively calculate one-peak area under the curve according to the manufacturer's specification (LightCycler Data Analysis Software, version 3.5). DNA extracted from LM cell line (derived from B-cell leukemia) was used as monoclonal positive control and distilled H₂O was used as negative reaction mix.

PAGE Analysis

To compare melting curve analysis with PAGE results, a 10- μ l sample of each PCR reaction that had previously been analyzed by melting curve analysis was electrophoresed through an 8% polyacrylamide gel (Tris-borate-ethylenediamine tetraacetic acid buffer, pH 8.0, 19:1 acrylamide/bis-acrylamide) at 200 volts, constant voltage, for 1.75 hours. Following electrophoresis, each gel was stained using ethidium bromide and photographed under UV transillumination. Gel lanes were scored as monoclonal (one or two distinct bands migrating in the range of 75 to 150 bp) or polyclonal (more than two distinct bands or a smear of multiple bands migrating in

Figure 1. Amount of monoclonal IgH gene rearrangement by melting curve and one-peak area analysis in the LightCycler System. A scale from 0.01 to 100 ng of DNA from the lymphoblastoid B-cell line LM was diluted into genomic DNA extracted from human tonsil and amplified by seminested protocol.

the range of 75 to 150 bp). The results obtained with PAGE were considered the gold standard for the purpose of this study and used to validate the results obtained by melting curve analysis.

Statistical Analysis

The likelihood ratio (LR) method was selected to find a discriminative function from the ratio peak area/ $(-dF1/dT)$ equal to $LR = 1$ from the training set cases.²⁵ This number was tested in the validation set cases to distinguish between monoclonal versus polyclonal IgH gene rearrangement. The LR analysis was performed using the program LOGDIS²⁶ in a Visual Basic for Applications version (VBA) to estimate the peak area with $LR = 1$ with a 95% confidence interval.

Results

We first determined the amount of monoclonal DNA to obtain a clear melting curve in the LightCycler System. A scale from 0.01 to 100 ng of DNA from the lymphoblastoid B-cell line LM was amplified using a seminested protocol. A clear sharp melting curve was obtained in a range from 0.01 to 100 ng of DNA (Figure 1). Next, 1 ng of genomic DNA from each of the training set cases (10 gastric MALT lymphoma and 10 chronic gastritis) was chosen as a standard amount for LightCycler amplification. After seminested amplification, melting curve analysis yielded temperature melting point (T_m) values (mean $\pm$ SD) of 84.10 ± 0.87 and $86.71 \pm 0.41^\circ\text{C}$ and one-peak area yielded values of 3.00 ± 0.82 and 7.69 ± 2.40 for gastric MALT lymphoma and chronic gastritis cases, respectively (Table 1 and Figure 2). To confirm these results, a 10- μl sample of each LightCycler amplification was electrophoresed as described in Materials and Methods, confirming one or two sharp bands in all cases of gastric MALT lymphomas and a smear of multiple bands in all cases of chronic gastritis (Figure 3). To

Table 1. One-Peak Area Analysis in the Training Set Cases

Case ID	Histology	LightCycler T_m ($^\circ\text{C}$)	One-peak area analysis	LR value	PAGE
1	Gastric MALT lymphoma	84.56	2.77	0.55	M
2	Gastric MALT lymphoma	85.19	3.75	0.85	M
3	Gastric MALT lymphoma	85.02	2.67	0.50	M
4	Gastric MALT lymphoma	85.14	4.64	1.0	M
5	Gastric MALT lymphoma	83.57	2.43	0.3	M
6	Gastric MALT lymphoma	83.76	2.6	0.40	M
7	Gastric MALT lymphoma	82.88	3.19	0.70	M
8	Gastric MALT lymphoma	83.01	3.78	0.90	M
9	Gastric MALT lymphoma	83.47	2.12	0.20	M
10	Gastric MALT lymphoma	84.42	2.09	0.15	M
11	Chronic gastritis	86.04	6.24	1.25	P
12	Chronic gastritis	86.29	5.65	1.20	P
13	Chronic gastritis	87.33	10.88	1.50	P
14	Chronic gastritis	87.02	6.5	1.35	P
15	Chronic gastritis	86.99	9.56	1.45	P
16	Chronic gastritis	86.91	5.28	1.10	P
17	Chronic gastritis	86.49	9.15	1.40	P
18	Chronic gastritis	86.62	6.49	1.30	P
19	Chronic gastritis	87.08	5.43	1.15	P
20	Chronic gastritis	86.33	11.74	1.52	P

LR value, likelihood ratio values for each case using a discriminative function equal to $LR = 1$; M, monoclonal IgH gene rearrangement; P, polyclonal IgH gene rearrangement.

Figure 2. One-peak area analysis of monoclonal IgH gene rearrangement after melting curve analysis in the LightCycler System. **A:** Example of gastric MALT lymphoma case (case 5 in Table 1). **B:** Example of chronic gastritis case (case 20 in Table 1). The peak areas command with one-peak area was chosen to calculate the area under the curve restricted to the largest peak present. *x* axis indicates temperature (°C); *y* axis indicates plotted of the negative first derivative of the fluorescence versus temperature ($-dF/dT$). Blue line is the melting curve. Red line is one-peak area analysis.

find a specific one-peak area that distinguished gastric MALT lymphoma from chronic gastritis, we applied the LR method. As shown in Table 1, $LR = 1$ was found with a one-peak area value of 4.64. Thus, all cases of gastric MALT lymphoma were clustered under 4.64, but cases of chronic gastritis were clustered above 4.64.

To determine the minimum detectable percentage of monoclonal IgH gene rearrangement of gastric MALT lymphoma necessary to demonstrate one-peak area below 4.64, 1 ng of DNA from three independent gastric MALT lymphoma cases were serially diluted (50, 12.5, 6.25, 3.12, and 1.56%) in a chronic gastritis case. After seminested amplification in the LightCycler System, one-peak area below 4.64 was clearly detected at the 1.56% level (Figure 4).

To validate the clinical utility of one-peak area analysis, we used a testing set of 11 endoscopic biopsies of gastric MALT lymphomas (five with diagnosis of gastric MALT lymphoma and six with diagnosis of ALI) and six endoscopic biopsies with the diagnosis of chronic gas-

Figure 3. Polyacrylamide gel electrophoresis analysis and ethidium bromide staining of monoclonal IgH gene rearrangement after seminested amplification in the LightCycler System. **A:** Examples of gastric MALT lymphoma case showing one band consistent with monoclonal IgH gene rearrangement (lanes 1 to 6). **B:** Examples of chronic gastritis cases showing a smear of multiple bands corresponding to polyclonal IgH gene rearrangement (lanes 1 to 6). MW, molecular weight marker (100 bp; Invitrogen).

tritis. After seminested amplification, melting curve analysis yielded T_m point values (mean $\pm$ SD) of 81.19 ± 4.08 , 83.24 ± 1.86 , and $84.47 \pm 2.05^\circ\text{C}$ and one-peak area values of 3.95 ± 1.07 , 4.11 ± 0.58 , and 7.24 ± 2.16 for gastric MALT lymphoma, ALI, and chronic gastritis cases, respectively (Table 2). Interestingly, all but one of

Figure 4. Detection of the minimum percentage of monoclonal IgH gene rearrangement by melting curve and one-peak area analysis. DNA from a gastric MALT lymphoma case (case 4) was diluted 50, 12.5, 6.25, 3.12, and 1.56% in a chronic gastritis case (case 14). The one-peak area analysis was 4.30 for undiluted, 4.64 for 50%, 4.25 for 25%, 4.64 for 12.5%, 3.98 for 6.25%, 4.58 for 3.21%, and 4.44 for 1.56% dilution of gastric MALT lymphoma in chronic gastritis case. The one-peak area analysis was 6.60 for the chronic gastritis case.

Table 2. One-Peak Area Analysis in the Testing Set Cases

Case ID	Histology	LightCycler T _m (°C)	One-peak area analysis	Likelihood ratio value	PAGE
21	ALI	81.93	2.95	0.60	M
22	ALI	84.93	4.44	0.95	M
23	ALI	84.50	4.27	0.95	M
24	ALI	80.21	4.50	0.95	M
25	ALI	83.23	4.34	0.95	M
26	Gastric MALT lymphoma	84.58	4.03	0.95	M
27	Gastric MALT lymphoma	82.66	3.88	0.90	M
28	Gastric MALT lymphoma	82.23	5.01	1.10	M
29	Gastric MALT lymphoma	82.37	4.62	1.0	M
30	ALI	84.61	4.14	0.95	M
31	Gastric MALT lymphoma	74.09	2.23	0.25	M
32	Chronic gastritis	83.05	5.38	1.15	P
33	Chronic gastritis	85.68	7.32	1.35	P
34	Chronic gastritis	85.24	5.28	1.10	P
35	Chronic gastritis	86.67	11.04	1.52	P
36	Chronic gastritis	81.06	6.32	1.25	P
37	Chronic gastritis	85.12	8.09	1.35	P

M, monoclonal IgH gene rearrangement; P, polyclonal IgH gene rearrangement.

the cases of gastric MALT lymphoma or ALI displayed a one-peak area analysis below 4.64. To confirm these results, a 10- μ l sample of each LightCycler amplification was electrophoresed as described confirming one or two sharp bands in gastric MALT lymphoma or ALI and a smear of multiple bands in all cases of chronic gastritis (data not shown). Thus, testing set data suggest that the one-peak area analysis with a discriminative function of 4.64 might be clinically useful to distinguish gastric MALT lymphomas from chronic gastritis.

Discussion

In this study, we explore a quantitative method for the evaluation of the melting curve of the IgH gene rearrangement in the LightCycler System as a means of identifying monoclonal B-cell populations in small gastric endoscopic biopsy specimens. We used a training set of cases (10 gastric MALT lymphoma surgical specimens and 10 chronic gastritis endoscopic biopsies) to define a discriminate function of the one-peak area analysis. Next, we used a testing set of cases (11 gastric MALT lymphoma endoscopic biopsies and six chronic gastritis endoscopic biopsies) to confirm our prediction model system. Using the LR approach, our training set defines a one-peak area of 4.64 as a discriminative function to distinguish gastric MALT lymphoma from chronic gastritis. This definition was further evaluated in the testing set, in which six cases have the diagnosis of ALI with involvement by gastric MALT lymphoma subsequently demonstrated. The one-peak area analysis correctly identified monoclonal IgH gene rearrangement in all six ALI cases. These findings suggest that one-peak area analysis thus helps to support the diagnosis of MALT lymphoma when histological findings are suspicious for possible lymphoma.

Xu et al^{17,18} originally developed the detection of IgH gene rearrangement by melting curve analysis using the LightCycler System. In their hands, melting curve analy-

sis, compared with PAGE, revealed no false-negative and no false-positive results, yielding both sensitivity and specificity equal to 100%. Later, Dobbs et al¹⁹ set a similar approach using an iCycler instrument, yielding sensitivity of 88.9% and specificity of 100% and indicating that a positive result on melting curve analysis is specific for a monoclonal IgH gene rearrangement but that negative results would require further evaluation by conventional methods. However, in both approaches the criteria to distinguish monoclonal versus polyclonal IgH gene rearrangement were subjective. Conversely, one-peak area analysis is an analytical approach and gives a quantitative parameter to distinguish reliably between monoclonal versus polyclonal IgH gene rearrangement in gastric lymphoid infiltrates.

The design of this analytical approach was based on the use of seminested IgH gene rearrangement amplification and not only external PCR as used previously.^{18,19} Although seminested amplification is cumbersome and there is a risk of contamination, it is more sensitive than external PCR in demonstrating monoclonal IgH gene rearrangement in gastric lymphoid infiltrates.^{22,24} In our hands, we obtained 95.2% sensitivity and 100% specificity, which seem to be similar to other authors.^{22,27} In addition, the use of a seminested protocol might explain our ability to detect as little as 1.56% of monoclonal IgH gene rearrangement from gastric MALT lymphoma in a chronic gastritis background. This range of detection is different from that reported by Xu et al,¹⁸ who achieved 12.5% also using the same LightCycler instrument. However, the use of seminested IgH gene rearrangement gene amplification is a major drawback of one-peak area analysis because it requires a manual transfer with risk of PCR contamination by product carryover.

Currently, the PCR technique is the main standard technique for diagnosis of monoclonal IgH gene rearrangement,^{9,28} particularly because this approach is suitable for lymphoid infiltrates in FFPE. However, the interpretation of PCR products depends on the type of gel

used (eg, ethidium bromide agarose gel or 8% PAGE)^{7,11-13} and seems more subjective than Southern blot.²⁹ In this scenario, one-peak area analysis gives an analytical parameter to evaluate IgH gene rearrangements in gastric lymphoid infiltrates. Because this method is independent from the type of electrophoresis used, one-peak area analysis might be useful to unify different current methods of IgH gene rearrangement among several laboratories. In fact, a multicenter survey of IgH gene rearrangement demonstrated a large heterogeneity in the performance of PCR assays, making it necessary to re-evaluate their methodologies.³⁰

In the statistics used in this study, the LR was chosen because it enables us to convert continuous data to binary data after identifying the one-peak area with LR = 1. Thus, one-peak area was assigned as the discriminative function for the distinction of monoclonal versus polyclonal IgH gene rearrangements in the training set and then validated in the testing set. Although the receiver operating characteristic (ROC) curve is the most popular statistical method to convert continuous data to binary data in diagnostic tests, ROC has several drawbacks.²⁶ ROC curve plots the true-positive rate against the false-positive rate for different choices of discriminative functions. However, half of the area shown on an ROC plot cannot contain any meaningful curves. Furthermore, ROC requires substantial familiarity with the subject to understand how the true-positive or false-positive rate alters the probability of disease.²⁶ Conversely, LR provide clearer descriptors for altering disease probability and can be more easily represented when viewed using positive and negative likelihood ratio axes. In addition, when scaled by base ten logarithms, visual interpretation is made easier for selecting a discriminative function to convert continuous data to binary data.²⁶

In summary, we have presented an analytical method to analyze IgH gene rearrangement in cases of gastric lymphoid infiltrates based on the one-peak area analysis of the melting curve using the LightCycler System amplification. This method gives an analytical interpretation of IgH gene rearrangement and is reproducible in FFPE gastric endoscopic biopsies, even in cases without histological diagnosis of gastric MALT lymphoma. Finally, it might be interesting to evaluate this method in other organs besides the stomach such as lymph nodes, skin, or orbit, where it is sometimes difficult to identify monoclonal B-cell populations.

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