IgH PCR of Zinc Formalin-Fixed, Paraffin-Embedded Non-Lymphomatous Gastric Samples Produces Artifactual “Clonal” Bands Not Observed in Paired Tissues Unexposed to Zinc Formalin

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***Helicobacter pylori* (HP) causes dense gastritis that can be difficult to distinguish morphologically from MALT-type lymphoma (ML). Immunoglobulin heavy chain (IgH) gene analysis by polymerase chain reaction (PCR) is often used to resolve diagnosis. However, monoclonal bands have been reported in nonmalignant cases of gastritis. Retrospectively, 16 gastric ML with both formalin-fixed, paraffin-embedded (FF-PE) and ethanol-fixed samples (EF), and 9 cases of FF-PE HP-gastritis were analyzed by IgH PCR to document the presence of non-reproducible bands in HP-gastritis, but not ML samples. In duplicate analyses, 12 of 16 ML yielded identical monoclonal bands in FF-PE and EF samples whereas 3 of 9 FF-PE gastritis cases yielded different-sized (ie, non-reproducible) “clonal” bands. Sequencing of two PCR products from a gastritis case confirmed IgH gene sequences. To investigate whether FF-PE had a direct effect on producing these non-reproducible bands, 7 gastrectomy samples were prospectively divided into EF and FF-PE halves for IgH PCR. All 7 samples demonstrated polyclonal smears in EF portions while 4 of 7 FF-PE portions yielded either multiple distinct bands or non-reproducible bands. In conclusion, IgH PCR of FF-PE tissue can create artifactual “clonal” bands, which are the appropriate product size, contain IgH sequences, and, if not performed in duplicate, may confuse interpretation of B-cell clonality. (J Mol Diagn 2002, 4:159–163)**

Helicobacter pylori (HP) infection occurs widely in the general population and can cause a dense gastritis of the stomach that can be difficult to morphologically separate from malignant lymphomas of MALT type (ML).^{1–3} HP has been implicated as a stimulus for the transformation of a B-cell clone, due to the existence of a prolonged reactive lymphoid population, that may give rise to a ML.^{4–6} The eradication of HP in low-grade MALT lymphomas has been used for treatment, inducing histological remission in 70 to 80% of patients.⁷

Diagnostic material from stomach lesions commonly consists of small biopsies that are processed for histological examination. If the lesion is suspicious for lymphoma, the processed tissue is often tested for lymphoid clonality by semi-nested polymerase chain reaction (PCR) or single step PCR.^{4,6,8,9} However, B-cell monoclonality by immunoglobulin heavy chain (IgH) PCR has been reported in HP-gastritis^{9–11} and after histological remission in many cases.^{12–15} These reports of monoclonal B cells in treated cases of ML in histological remission or HP-gastritis would lead one to view monoclonal PCR bands from gastric samples with caution. The focus of this study is to address in which situation this “monoclonality” may represent an artifact of the fixation paraffin-embedding process, not to define diagnosis or clinical application of monoclonality. In this study, we retrospectively examined the results of PCR amplification of the IgH genes in gastric MALT and benign gastric inflammatory infiltrates from formalin-fixed, paraffin-embedded (FF-PE) and ethanol-fixed (EF) samples, to confirm observations reported in the literature. Prospectively, we obtained random sections from non-lymphomatous, surgically resected stomachs with gross evidence of gastritis, fixed half of the samples in either zinc formalin or 50% ethanol, and evaluated the effect of histological processing by comparing IgH gene amplification results.

Materials and Methods

Case Selection and Histological Review

Retrospectively, 16 biopsies of gastric MALT lymphomas from the University of Florida Shands Hospital files were randomly selected based on the availability of excess FF-PE and EF tissues, which are routinely prepared for diagnostic purposes. HP-gastritis samples with HP organisms identified on biopsy were only available in FF-PE tissue and 9 were selected. These cases were reviewed and

Accepted for publication May 31, 2002.

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Table 1. Results of PCR Amplification of IgH Genes in Either EF or FF-PP Gastrectomy Samples

Case	Primary histologic diagnosis	EF	FF-PE
A	Squamous cell carcinoma	polyclonal	multiple distinct bands
B	Adenocarcinoma	polyclonal	multiple distinct bands
C	Leiomyosarcoma	polyclonal	polyclonal
D	Adenocarcinoma	polyclonal	non-reproducible distinct bands
E	Adenocarcinoma	polyclonal	multiple distinct bands
F	Squamous cell carcinoma	polyclonal	polyclonal
G	Refractory peptic ulcer disease	polyclonal	polyclonal

confirmed histologically by two authors, (R.B. and N.A.). Prospectively, samples from 7 gastrectomies, removed for other than lymphoma diagnosis, were identified. See Table 1 for comparison studies between EF and FF-PE.

Tissue Fixation, DNA Isolation, and Sample Preparation

The cases for the retrospective study included MALT lymphoma samples that had been processed for routine histological examination (fixed in 10% zinc formalin with overnight processing and paraffin embedding) and stored in a solution of 50% ethanol in RPMI medium over 5 years at room temperature. HP-gastritis samples were only processed for histological examination. Prospectively, sections of the non-lymphomatous gastric tissues were excised and each sample divided into two approximately equal portions. One portion was fixed in 10% zinc formalin (Anatech Limited, Battlecreek, MI) and subsequently processed for paraffin embedding, and the other fixed in a solution of 50% ethanol in RPMI medium for at least 24 hours at room temperature. EF of tissue or cells has been shown to preserve high molecular weight DNA and is comparable to using DNA extracted from fresh or frozen samples.¹⁶ Zinc-formalin fixation of tissue exerts damage similar to neutral buffered formalin (NBF) formalin with size degradation of DNA template. To extract DNA, FF-PE tissue sections were processed as described elsewhere.¹⁷ In short, tissue sections were deparaffinized in xylene, hydrated through graded alcohols to 50%, and partially dried on a heating block. The pellet was re-suspended in 100 to 500 μ l of 10 mmol/L Tris-HCl (pH 7.5) with 60 μ g/ml of Proteinase K (Boehringer-Mannheim, Indianapolis, IN) and digested overnight at 55°C. EF tissue was washed twice in a phosphate-buffered saline solution and DNA was isolated by a non-organic salt-extraction procedure according to Miller et al.¹⁸

IgH Semi-Nested PCR

Primers to the immunoglobulin heavy chain gene amplified either a Framework 2A (FR2A) or Framework 3A (FR3A) variable region and a segment of the joining region (LJH and VLJH). Primer sequences were as described previously.^{19,20} The initial amplification included 0.2 μ g DNA from EF or 1 μ g DNA from FF-PE tissues. The amount of DNA used for EF or FF-PE tissue was optimized for maximum sensitivity (3 to 5%) using dilutions of ML in normal tonsil. The reaction mix contained PCR

buffer (10 mmol/L β -mercaptoethanol, 67 mM Tris-HCl (pH 8.8), 16.6 mmol/L NH_4SO_4 , 0.02% filtered gelatin), 100 μ mol/L of each dNTP (Amersham Pharmacia, Piscataway, NJ), 0.3 units of AmpliTaq DNA polymerase (PE Applied Biosystems, Branchburg, NJ), 100 ng of LJH, and 400 ng of FR2A or FR3A primer with 1.5 mmol/L or 2.0 mmol/L MgCl_2 respectively, in a total volume of 50 μ l. Re-amplification of 1 μ l of product from the first reaction was carried out using 200 ng of the internal primer to the joining region VLJH and 200 ng of the same FR2A or FR3A primers, in a total volume of 25 μ l. The MgCl_2 concentration was raised to 2 mmol/L and 3 mmol/L for FR2A and FR3A, respectively. PCR reactions were performed in a PE Biosystems 9600 Thermocycler, (PE Applied Biosystems), following Tamara et al²¹ with minor modifications. In short, the samples were loaded onto the thermocycler at 96°C in a modified "hot start" for 5 minutes. This was followed by five initial highly stringent cycles at 63°C for 30 seconds for primer annealing and then 35 cycles at 57°C. The DNA denaturation (96°C, 15 seconds) and primer extension (72°C, 30 seconds) were kept constant for all cycle steps. The cycling was followed by a 5-minute extension at 72°C. For re-amplification the stringent primer annealing conditions were kept constant at 63°C for 30 seconds and 25 cycles. The number of cycles was decreased by five for both steps with FR3A. FR2A products were electrophoresed through a 2.75% high-resolution Metaphor gel (FMC, Rockland, ME) with an expected size of 240 to 280 bp. FR3A products were electrophoresed through a 4% Metaphor gel with an expected size of 80 to 120 bp. "Clonal" bands were indicated by a distinct band with or without a polyclonal background smear. "True" monoclonal bands were distinct bands of the same size and intensity in duplicate reactions. Each sample was also amplified for β -globin, a common housekeeping gene, to ensure adequate DNA for PCR.

Sequencing of PCR Products

PCR products were purified using a QIAquick Column (Qiagen, Valencia, CA), labeled with Big Dye Terminators and cycle-sequenced using FR2A and VLJH internal primers on an ABI Prism 310 (PE Applied Biosystems, Foster City, CA).

Results

Retrospectively, 16 gastric ML with both FF-PE and EF samples and 9 cases of FF-PE HP-gastritis were ana-

Figure 1. PCR amplifications of IgH genes using FR3A region primers in 3 representative formalin-fixed, paraffin-embedded (FF-PE) MALT lymphoma (ML) cases performed in duplicate. **Lane 1:** 20-bp ladder. Case in **lanes 2-3** show polyclonal smears. Cases in **lanes 4-5** and **6-7** show reproducible monoclonal bands. Twelve of 16 cases of gastric ML yielded reproducible monoclonal bands of the same size from both FF-PE and EF samples.

lyzed by IgH PCR in duplicate reactions to document the presence of non-reproducible bands in HP-gastritis, but not ML samples. Twelve of 16 cases of gastric ML yielded reproducible monoclonal bands of the same size from matched EF and FF-PE samples. There were no false negatives in FF-PE as compared to EF- paired samples. Results from three representative ML cases are shown in Figure 1. In contrast to ML, 3 of 9 HP-gastritis cases demonstrated distinct bands of variable intensity in both lanes but of different sizes (ie, non-reproducible). Representative HP-gastritis case results are shown in Figure 2.

One HP-gastritis case with non-reproducible bands underwent 10 PCR reactions to the FR2A region (Figure 3). In 3 separate reactions, clear bands of different sizes were observed. Two of these bands from lanes labeled 11 and 12 were re-amplified from the first PCR reaction before nesting, and the results confirmed the initial amplification (data not shown). The products from these amplifications were sequenced in both directions using VLJH and FR2A primers (Figure 4). The sequences were compared to files in the BLAST program from the National Center for Biotechnology Information (NCBI) and confirmed to be IgH rearranged sequences. As noted in Figure 4 by the single underlined nucleotides, there was approximately 75% sequence homology between both bands. However, unique sequences were also observed for each product. The boxed regions contained the VLJH and FR2A sequences used in the initial PCR. Homology to the FR3A primers, indicated by the double underlined nucleotides, confirmed that these products were not the result of non-specific primer binding.

Prospectively, DNA extracted from 7 gastrectomy specimens that were divided into EF and FF-PE portions,

Figure 2. PCR amplifications of IgH genes using FR2A region primers in 4 representative cases of FF-PE HP-gastritis (each indicated by a **bracket**). **Lane 1:** 20-bp ladder. Three of 9 FF-PE HP-gastritis cases demonstrated distinct bands of variable intensity and size in duplicate reactions (**lanes 2-3** and **6-7** show two examples).

were PCR-amplified for the heavy chain genes and demonstrated polyclonal smears in duplicate reactions in all of the EF samples. In contrast, 3 of 7 FF-PE samples demonstrated a pattern of multiple distinct bands. One of the 7 samples had non-reproducible bands, which appeared clonal but were of different bp sizes (Figure 5).

Discussion

As reported by other investigators in the literature we have detected the presence of "clonal" products in HP-gastritis by IgH PCR in FF-PE tissues. Torlakovi et al⁹ found that HP-gastritis FF-PE cases had an increase in the number of oligoclonal or monoclonal bands as compared to reactive lymph node specimens. By single-step IgH PCR using FR11 primers, these authors found that 17 of 20 cases of HP-gastritis with lymphoid hyperplasia yielded single or dominant, apparently monoclonal

Figure 3. **Lanes 2-8** and **10-12** show 10 replicas of PCR amplifications of IgH genes using FR2A region primers from a single case of FF-PE HP-gastritis (**lanes 6-7** in Figure 2). "Monoclonal" bands of different sizes are seen in lanes 2, 3, 7, 11, and 12. **Lanes 1** and **9:** 20-bp ladder.

PRODUCT "11": GT-- AC CNA AGG TNC CTT GGC CCC A AG TAG T-----CAA ACC CT
PRODUCT "12": GT GAC CCA AGG TNC CTT GGC CCC A AGG GGT CGA AAT CAA ACC CT

ATC TTG AGC CAG CCA CTG CCC CCC CTA TCT TTC GCA CAA GTA ATA TAC GGG CCG
TCA TAG CTG TAC CAC ----- TC GCA CAA GTA ATA CAC AAG CCG

TGT CCT CGG GCT CTC AGG CTG TTC ATT TGC AGA TAC ACC GTG TTC TTG GAA TTG
TGT CCT CGG C-T CTC AGG CTG TTC ATT TGC AGA TAC AGT GAG TTC TTG GCG TTG

TCT CTG GAG ATG GTG AAC CGG CCC TTC ACG GAG TCT GCG TAG TAT GTG CTA CCA
TCT CTG AAG ATG GTG AAT CGG CCC TTC ACT GAG TCT GCG TAG TAT ATG TAA CTA

CCA CCA CTA CTA ATA GCT TGA GAC -CA CTC CAG CCC CTT CCC CGA AGC CTG TCN
CTA CTA CTA CTA ATG GA- -GA GAC CCA CTC CAG CCC CTT CCC CGA AGC CTG -CG

GGA CCC A
GGA CCC A

Figure 4. Comparison of PCR product band sequences from lanes 11 and 12 in Figure 3. Boxed nucleotides represent VLJH and FR2A primers at the end of respective sequences. Single underlined nucleotides represent homology between products "11" and "12". Unmarked nucleotides represent unique sequences. Double underlined nucleotides represent homology to internal FR3A primers.

bands. However, these bands were only reproducible in 1 of 17 cases. Saxena et al¹⁰ using semi-nested PCR, found that 10 of 20 FF-PE gastritis cases with associated lymphocyte infiltrates had irreproducible bands, while El Zimaity et al¹¹ found monoclonal bands in 15 of 39 cases of FF-PE chronic active gastritis. Clonal products have been described in the literature in follicular, reactive lymphoid, and chronic gastritis, ranging from 4% to 38% of cases reviewed.^{4,11}

In our retrospective study of ML, 75% of the cases yielded monoclonal bands confirming similar reports in the literature, where approximately 25% of MALT lymphomas cannot be amplified with primers to the heavy chain gene due to somatic mutation or lack of annealing of consensus primers.^{7,14,22} The resultant bands were iden-

tical in duplicate samples of EF and FF-PE tissue. In contrast, of 9 FF-PE gastritis cases, 3 showed monoclonal bands of different sizes in duplicate reactions. These non-reproducible bands, if not performed in duplicate, could be considered clonal by most standards: correct size, distinct, and with IgH sequence homology.

To address the question of the effect of histological processing on PCR amplification of IgH genes in non-lymphoma gastric specimens, we prospectively compared results of matching stomach samples processed separately, and found distinct non-reproducible bands in FF-PE but not in EF gastric tissue, a finding that indicates these bands were most likely induced by histological processing. Our sequencing studies indicate these distinct bands are not the result of non-specific primer binding.

Figure 5. Representative PCR amplifications of IgH genes using FR3A region primers in either EF (lanes 2-7) or FF-PE (lanes 9-14) non-lymphomatous gastric tissue samples (see Table 1) performed in duplicate (brackets). Lanes 2-3: case "C". Lanes 4-5: case "D". Lanes 6-7: case "E". Lanes 9-10: case "D". Lanes 11-12: case "E". Lanes 13-14: case "F". The polyclonal smear observed in EF tissue from case "D" (lanes 4-5) yielded distinct non-reproducible "monoclonal" bands in the corresponding FF-PE tissue (lanes 9-10). Three of 7 FF-PE samples demonstrated oligoclonal patterns with multiple distinct bands. All EF tissue produced polyclonal smears. Lanes 1 and 8: 20-bp ladder.

Several authors have postulated that "monoclonal" bands observed in cases of HP-gastritis are due to the paucity of B cells or preferential proliferation of B-cell clones within a limited biopsy area.^{9,23} Elenitoba-Johnson et al²³ elegantly demonstrated the emergence of monoclonal bands from IgH PCR amplifications with simple serial dilutions of DNA from reactive lymphoid tissues into placental DNA. These authors theorized that the limited amount of IgH template allowed DNA amplification from only a few B cells, yielding the appearance of B cell monoclonality. We compared the PCR results from surgical gastrectomy specimens, divided into approximately equal sizes, for either EF or FF-PE that should theoretically contain roughly equivalent numbers of B cells. In our cases, the amplification of a polyclonal smear with the EF samples would indicate that there were an adequate number of B cells. Since non-reproducible bands were only detected in the FF-PE portions, we conclude that these bands were due to the histological processing (perhaps caused by formalin fixation-induced DNA damage), not just a paucity of B cells. Although our study used zinc-formalin fixation, the results should be applicable to other laboratories since its properties for PCR do not seem to differ from NBF.²⁴

In the absence of available fresh tissue that could be subjected to immunophenotypic²⁵ and/or appropriate PCR-based analysis for lymphoid clonality, we recommend that PCR reactions for IgH clonality using FF-PE tissues should be performed in duplicate using high-resolution gels for optimal product size separation to determine reproducibility. Also, in follow-up biopsies, PCR amplification of DNA from an initial diagnostic specimen, together with the current sample, in conjunction with histological and immunohistochemical review may be required for comparison and adequate interpretation of PCR results.

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