Clonality analysis of B- and T-cell extranodal Non-Hodgkin lymphoma

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Publications directly related to the subject of the dissertation

I. **Gurbity PT**, Bagdi E, Groen NA, Bundel LM, Abbou M, Krenács L, Dinjens WNM: Increased sensitivity of B-cell clonality analysis is formalin-fixed and paraffin-embedded B-cell lymphoma samples using an enzyme blend with both 5'→3' DNA polymerase and 3'→5' exonuclease activity. Wirchows Arch 2003; 443:643-648 (IF: 2.445)

II. **Gurbity PT**, Bagdi E, Krenács L, Borbényi Z: A molekuláris biológiai módszerek jelentősége primer gyomor MALT lymphomában. Hematológia Transzfuziológia 2006; 39:99-105

III. Török L, **Gurbity PT**, Kirschner Á, Krenács L: Panniculitis-like T-cell lymphoma clinically manifested as alopecia. British Journal of Dermatology 2002; 147:785-788 (IF:2.227)

IV. Török L., **Gurbity PT**, Kirschner Á, Krenács L: Alopecia képében manifesztálódó subcutan panniculitisszerű T-sejtes lymphoma. Orvosi Hetilap 2002; 143:607-609

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List of abbreviations

Bp base pairs

CD celiac disease

CHR complete haematological remission

CTCL cutaneous T-cell lymphoma

DLBCL diffuse large B-cell lymphoma

ETL enteropathy-type T-cell lymphoma

FFPE formalin-fixed and paraffin-embedded

FL follicular lymphoma

GC germinal center

Hp Helicobacter pylori

IC inconsistent

IEL intraepithelial lymphocytes

IgH immunglobulin haevy chain

IgL immunglobulin light chain

M monoclonal

MALT mucosa-associated lymphoid tissue

MF mycosis fungoides

Nd-PAGE nondenaturating poliacrilamid gelelectrophoresis

OC oligoclonal

P polyclonal

PCR polymerase chain reaction

pGC post-germinal center

RCD refractory celiac disease

RS refractory sprue

SPTCL subcutaneous panniculitis-like T-cell lymphoma

U unsuccesful

1. Introduction

Clonality analysis of lymphoid cell populations plays an increasing role in the primary diagnosis as well as follow up of lymphomas and lymphoid leukemias. There are special entities which are best recognized by the aid of molecular methods, while in others these methods are required only if the immunomorphology is not decisive. In lymphoid lesions, multiple antigen receptor gene sequences can be suitable targets as clonal markers[1-3]. The recombination of antigen receptor genes results in an individual gene sequence unique for each differentiating B- or T-cell[1-3]. This rearranged pattern in the individual cell's life span does not change considerably, whereby its fingerprint-like genotype becomes utilizable. If one of these maturing or matured cells becomes malignant, this cell line becomes dominant in the overall lymphocyte population and, therefore, their individual antigen receptor gene rearrangement combination also becomes dominant in the overall rearrangement pattern at the population level[1-3].

Malignant lymphomas aries from the lymphoreticular tissue, by the clonal proliferation of the lymphoid cells. We distict nodal and extra-nodal lymphomas. The gastrointestinal tract is the commonest site of primary extra-nodal lymphoma, accounting for 30-50% of cases. All categories of lymphoma that tipically arise in the peripheral lymph nodes may arise in the gastrointestinal tract, but two lymphomas, namely mucosa-associated lymphoid tissue (MALT) lymphoma and enteropathy-type T-cell lymphoma (ETL), are unique in that their clinical features and histology reflect the important differences between lymphomas derived from nodal tissue and MALT. This distinctiveness of these two lymphomas has been underlined, by their unique molecular properties[4].

Southern blotting is one of the most reliable methods in detecting lymphoid cell clonality, but it is labor-intensive and requires large amounts of high-molecular-weight DNA, therefore, nowadays it is practically replaced by polymerase chain reaction (PCR) based methods[5-7]. Besides high sensitivity, speed and cost effectiveness, the great advantage of the PCR techniques is that they do not require intact DNA, therefore well suit the routine diagnostic use[7-9]. The highest sensitivity of PCR detection is generally expected on DNA isolated from fresh or frozen material, while using templates from formalin-fixed and paraffin-embedded (FFPE) tissue samples may impair performance [7-12]. Unfortunately, in the majority of the diagnostic cases and in retrospective studies,

only FFPE tissue samples are available for molecular genetic studies. DNA isolated from FFPE archived tissue samples contains mainly strongly fragmented, chemically modified, small – generally less than 200-300 base pairs (bp) – sequences[13-15].

1.1. Gastrointestinal lymphoid tissue

There are four lymphoid compartments in the gastrointestinal tract and they include organized mucosal lymphoid tissue exemplified by the Peyer`s patches in the terminal ileum, the lamina propria, intraepithelial lymphocytes and the mesenteric lymph nodes. Organized lymphoid nodules are concentrated in the terminal ileum, where they collectively form the Peyer`s patches, the generic term applied to this compartment of MALT. Peyer`s patches are encapsulated aggregates of lymphoid cells which closely resemble lymph nodes. Each patch nodule consists of B-and T-cell area. The B-cell area consists of follicle comprised by a germinal center surrounded by a follicular mantle. Surrounding the mantle zone is a board marginal zone which extends towards the mucosal surface and some marginal zone B-cells enters the overlying dome epithelium to form a lymphoepithelium that is defining feature of MALT.

1.2. MALT lymphoma

The concept of MALT has progressively developed during the last 20 years regarding the lymphoid component observed in various organs that do not correspond to peripheral sites of the immune system. It has been first proposed in the early 1980s by Isaacson and Wright. Two types of MALT tissue can be identified; the native type which consists of the lymphoid tissue physiologically present in the gut (i.e. the Peyer`s patches); and the acquired MALT which develops after inflammatory events in response either to infectious agents (e.g. Hp. gastritis in the stomach) or autoimmune processes (e.g. Hasimoto`s tyreoiditis and Sjögren`s syndrome). In the contest of these prolonged lymphoid reactive proliferations a pathological clone can progressively substitute the normal lymphoid population, so originating the MALT lymphoma. The MALT lymphoma has been incorporated in the new Revised European-American Classification of lymphoid neoplasms (REAL Classification) as a distinctive lymphoma type, under the designation “extranodal marginal zone B-cell lymphoma”. It is also recognized entity in the new version (1997) of the World Health Organization (WHO) classification of haematolymphoid tumors. The prototype of low-grade MALT lymphoma is that of

the stomach, which is the most common site for its occurrence, and also has been most intensively studied.

1.2.1. Helicobacter pylori and gastric MALT lymphoma

The fact that the stomach is the commonest site of MALT lymphoma is paradoxical since the normal stomach unlike the intestine is devoid of any organized lymphoid tissue. However, organized lymphoid tissue can be induced following Hp. infection, which causes active chronic inflammation of the gastric mucosa. There are several lines of evidence showing that gastric MALT lymphoma arises from MALT acquired as a consequence of Hp. infection and this plays a critical role in the genesis and development of this tumor. Hp. can be demonstrated in the gastric mucosa of the majority of cases of gastric MALT lymphoma[16 17]. Importantly, the MALT lymphoma B-cell clone can be detected in Hp.-associated chronic gastritis that precedes the lymphoma [18]. In vitro studies have shown that the growth of gastric MALT lymphoma cells can be stimulated by heat-killed Hp. and this effect is via Hp. specific T-cells involving direct interaction between B- and T-cells through co-stimulating molecules CD40 and CD40L[19,20]. Finally, eradication of Hp. in most cases results in regression of gastric MALT lymphoma[21, 22] .

1.2.2. Treatment of gastric MALT lymphoma

Treatment of gastric MALT lymphoma has attracted considerable attention since the first report by Wotherspoon et al in1993. In these study six patients with early stage Hp.-positive gastric MALT lymphoma were treated with antibiotics alone and showed complete regression of the tumor in five cases 9 months after eradication of Hp. as judged by endoscopic, histological, and molecular examination[21]. With further follow-up, the remission in these patients have been stable for more than six years[22]. The following studies from different centers have confirmed that 75% of gastric MALT lymphomas can be successfully treated in this way[23-27]. In most cases, remission is achieved within 12 months after Hp. eradication. In 50% of cases, the tumor clone can be detected by PCR analysis of the rearranged Ig gene on post-remission gastric biopsies that do not show histological evidence of a tumor[23,25]. The clinical significance of this residual tumor cell population is not totally clear. Early observations suggest that it gradually disappears during longer follow-up. In some of these cases the recurrence of the lymphoma can be detected, highlighting the suppressive rather than ablative effect

of antibiotic therapy. It would be extremely useful to be able to identify the 25% of cases of gastric MALT lymphoma that do not respond to eradication of Hp.. The prognostic value of clinical staging has been extensively examined by endoscopic ultrasonography, which allows assessment of the extent of tumor invasion into the gastric wall and regional lymph nodes. In general, lymphomas of stage II_E or above do not respond to Hp. eradication[25,26]. The majority of gastric MALT lymphomas at diagnosis are stage I_E and 20% of these cases will not respond to Hp. eradication. Thus deep invasion of the stomach wall as well as extra gastric dissemination implies that the lymphoma has escaped from T-cell dependency. Genotypic investigations along with careful assessment of the histological grade and by both endosonographic and conventional staging should be performed for gastric lymphoma at the time of diagnosis to help to choose the best treatment strategy for these patients.

1.2.3. The evolution of gastric MALT lymphoma

The evolution of gastric MALT lymphoma is a multistage process, which comprises sequential development of chronic Hp. associated gastritis, low-grade and high-grade lymphoma. Isaacson et al[4] formulated a hypothesis for the pathogenesis of gastric MALT lymphoma which is illustrated in *Figure 1.*

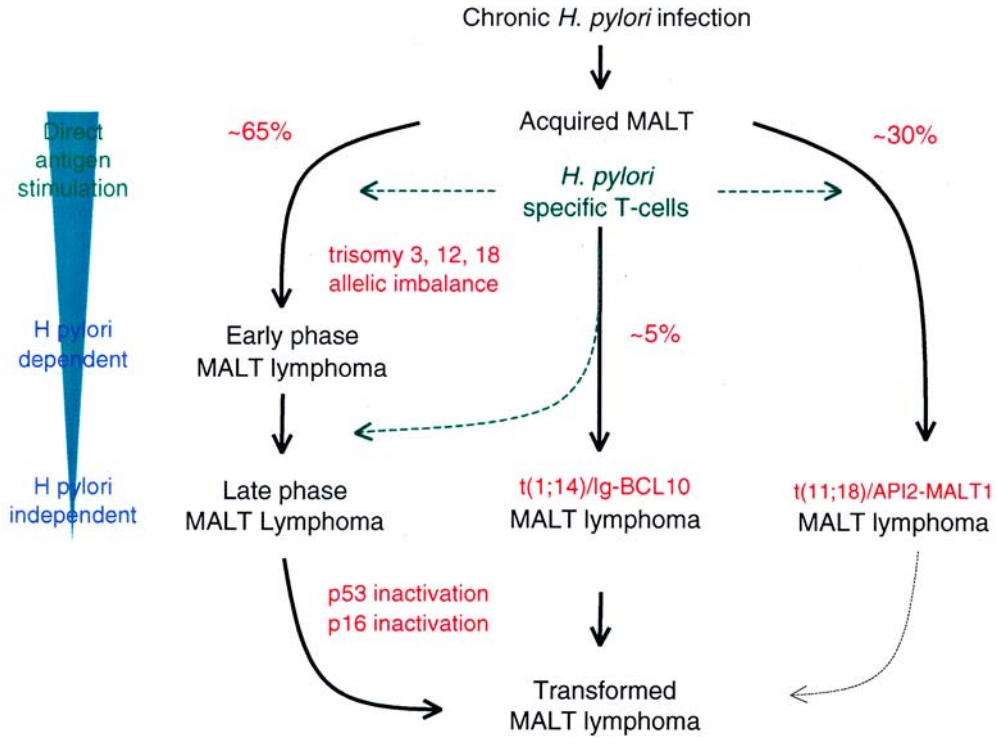

Figure 1.

This scheme suggests that the lymphoma cells gradually accumulate genetic abnormalities and gain the ability for autonomous growth while gradually losing dependence on immunological stimulation during tumor evolution. This multistage process starts with Hp. infection that result in the recruitment of B- and T-cells and other inflammatory cells to the gastric mucosa. The infiltrating B-cells are stimulated both by autoantigens and more importantly, by Hp.-specific T-cells, and may undergo high-grade transformation due to the accumulation of genetic abnormalities. Lymphomas with t(11;18)(q21;q21) gain autonomous growth ability and do not respond to Hp. eradication. They rarely develop into transformed MALT lymphoma. Lymphomas with t(1;14)(p22;q32) or variants are also Hp. independent and may undergo high-grade transformation[28,29]. Lymphomas without these chromosomal translocations, although frequently carrying trisomies 3, 12, and 18, are Hp. dependent at early phases. At this stage the tumor is most frequently confined to the stomach and will regress following eradication of Hp. However, they can progress and become Hp. independent, and transform in high-grade lymphomas following inactivation of the tumor suppressor genes p53 and p16, or acquisition of chromosomal abnormalities involving c-myc and BCL6 locus.

1.2.4. Rearrangement of immunoglobulin (Ig) heavy (IgH) and light chain (IgL)

The characterization of Ig gene rearrangement has become an important and crucial step in the diagnosis of B-cell lymphoid malignancies. During physiological B-cell maturation, IgH gene overcomes VDJ sequence recombination. After antigen contact, the rearranged IgH variable (IgV_H) gene sequences are further modified in germinal center reaction, by means of somatic hypermutation mechanism. The Igκ gene locus undergoes rearrangement after IgH during B-cell differentiation. In the case in which functional κ chain products are not obtained, the Igλ locus subsequently undergoes rearrangement[30,31]. Studies have shown that in all Igκ+ and in a very large majority of Igλ+ B-cell malignancies, either productive or nonproductive Igκ products are formed. Finally, only a small subset of Igλ+ B-cell neoplasms show biallelic deletions of the Igκ variable region (17%) or Igκ in germline configuration (2%)[32,33]. Therefore, the rearranged genomic products of Igκ represent an excellent marker for B-cell clonal analysis.

1.2.5. Detection of the rearranged IgH and IgL

Discrimination between reactive and neoplastic B-cell proliferations is often difficult by morphological methods alone. In samples without immunohistochemically detectable immunoglobulin light chain restriction, application of the molecular techniques can be inevitable. The rearranged IgH and IgL gene segments can be analyzed as clonal markers in distinction of polyclonal and monoclonal B-cell populations. The Ig gene rearrangement can be demonstrated by Southern blotting, with high specificity, but because of the reasons described above for detection of B-cell clonality in FFPE pathological samples, especially in smaller biopsies, the Ig PCR assay is the method of choice.

Several IgH PCR procedures have been published, including different DNA isolation methods, single, seminested, and nested PCR protocols, different degenerative primers and the utilization of various DNA polymerases. The detected monoclonality rate varies between 50-80 %. Most studies have revealed that the highest rate can be achieved on DNA obtained from fresh or frozen tissue with primers directed at the conserved framework 3 area (FR3) and joining gene (JH) segments[34-36].

Efficient IgH PCR analysis can be hampered by primer mismatches, originating from random sequence variability in the template DNA due to somatic hypermutation and ongoing mutational activity[37,38]. A mismatch at the 3' terminus of the primer due to point mutation or single nucleotide deletion of the template DNA significantly influence the outcome of the PCR amplification. High fidelity DNA polymerases or polymerases with "proofreading" (3'→5' exonuclease) activity have the ability to correct any misincorporated nucleotides on the strand of DNA being synthesized, and thus to remove mismatched bases at the 3' terminus of the primers, resulting in sufficient amplification[39-41]. High fidelity PCR can be carried out either with a single high fidelity polymerase or using an optimized polymerase mixture.

1.3. Enteropathy type T-cell lymphoma

Enteropathy type T-cell lymphoma is a distinct clinicopathological entity often associated with longstanding and untreated celiac disease (CD)[42]. There is strong evidence that ETL is indeed a consequence of CD. The histology and distribution of enteropathy in ETL are those of CD and HLA types DQA1 and DQB1 are shared by patients with ETL and CD[43]. Further evidence for this association includes the

demonstration of gluten sensitivity in ETL patients and detection of the ETL clone in cases of CD that no longer respond to gluten withdrawal[44,45].

ETL may involve any part of the small intestine and other parts of the gastrointestinal tract. It is usually multifocal and forms ulcerating nodules, plaques, strictures or large masses. There is sometimes little macroscopic evidence of disease in the intestine. The histological features of ETL show great variation. The most characteristic appearance is uniformly sized large cells with pleomorphic nuclei. The histology of the small intestine apart from the site of the tumor is important in the diagnosis of ETL. In most cases, the changes are identical to those of CD, such as: villus atrophy, with crypt hyperplasia, plasmocytosis of the lamina propria, and increase in intraepithelial lymphocytosis. The villus arhitecture may be normal and the only hint of CD is an increase in intraepithelial lymphocytes (IELs) best seen in immunostained preparations. Despite their innocuous appearance, it seems that these cells are a part of the neoplastic clone. In the most cases of ETL, the tumor cells express CD3, CD7, CD103, and cytotoxic proteins such as granzyme B. They are usually CD5-, CD4-, and CD8- negative and they do not express γ/δ T-cell receptor[4].

ETL mainly occurs in the sixth and seventh decade. It may complicate longstanding CD, or more usually follows a short history of adult CD. In some cases, there is no history of malabsortion only jejunal villus atrophy and crypt hyperplasia are found when the tumor is excised. The clinical course is very unfavorable, in most cases, the lymphoma involves multiple segments of the small intestine, and has already disseminated at the time of the diagnosis, making resection impossible. Most commonly the malabsorption accompanied with abdominal pain reappears in patients with a history of adult CD who previously responded to gluten-free diet. Other patients may present with the sudden onset of severe malabsorption or as an abdominal emergency following hemorrhage or intestinal perforation. There is another group with a long latent interval which is characterized by severe malabsorption unresponsive to gluten-free diet, so-called refractory celiac disease (RCD), or refractory sprue (RS)[46]. In some of these patients, the disease is complicated by intestinal ulceration and stricture formation, known as ulcerative jejunitis (UJ).

1.3.1. Refractory celiac disease

Some cases of CD become unresponsive to gluten-free diet or may be unresponsive de novo. The term RCD has been widely used for these cases[46]. Studies of the rearranged

TCR genes in both ETL and RCD have now elucidate the relationship between these two conditions. Using PCR followed by sequence analysis of the rearranged TCRγ chain genes, Murray et al showed that there was a T-cell population in the `uninvolved` small intestine mucosa adjacent to ETL that shared the same monoclonal TCRγ rearrangement as the lymphoma[47]. Others confirmed this finding and showed TCRγ monoclonality in the non-specific `inflammatory` ulcers that accompanied ETL, as well as in the jejunal ulcers and intervening mucosa of RS. In cases where ETL subsequently developed, the same clone could be detected[45]. Cellier et al showed that in RCD monoclonal T-cell populations were present and it was made up of phenotypically abnormal IELs expressing intracellular CD3ε but no CD4, CD8. More importantly, they clarified the relationship between CD and RCD, by showing the presence of CD-specific anti-endomysial or anti-gliadin antibodies together with other characteristic features of CD such as previous response to gluten-free diet or the typical HLA DQA1 and HLA DQB1 phenotype. Additionally, they showed that in all truly refractory cases, the IELs were either monoclonal, expressed an abnormal immunophenotype, or both [48,49]. Interestingly these clonal and immunophenotipically aberrant IELs were often present in the crypt epithelium, while in uncomplicated CD they are confined to the surface epithelium.

1.4. Cutaneous T-cell Lymphoma

Cutaneous T-cell Lymphoma (CTCL) is generally classified as a type of non-Hodgkin's lymphoma. CTCL consists of a heterogeneous group of lymphoproliferative disorders that primarily present in the skin and are composed of malignant clonal T lymphocytes. Indolent subtypes include mycosis fungoides (MF) and its variants, primary cutaneous anaplastic large cell lymphoma, lymphomatoid papulosis (LP), subcutaneous panniculitis-like T-cell lymphoma (SPTCL), and primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma. Aggressive subtypes include Sézary syndrome, primary cutaneous natural killer (NK)/T-cell lymphoma nasal type, primary cutaneous aggressive CD8+ T-cell lymphoma, and primary cutaneous γ/δ T-cell lymphoma[50]. More than 65% of cutaneous lymphomas are T-cell disorders. B-cell lymphomas account for approximately 25% of cases, and up to 10% of cases are unspecified[50]. Accurate diagnosis of early CTCL is difficult because of the varied clinical and histological expressions of the disease and also because of a lack of

uniformity in diagnosis and treatment. Further in the study we focused on MF, since it is the most common subtype of CTCL, accounting for almost 50% of all primary cutaneous lymphomas. In addition we examined 2 cases of SPTCL.

1.4.1. Mycosis fungoides

The course of MF is usually indolent, with slow progression, but it can be unpredictable. Most patients experience a prolonged survival with little morbidity, while some patients develop a fulminate course with rapid dissemination and death. The malignant cells in MF are CD4+ T cells, which have encountered antigen and thus express CD45R0[51]. There are many clinical variants of MF. The three classical cutaneous phases are patches, infiltrated plaques, and tumors that are distributed in sun-protected areas. The disease may progress through each of these phases, which frequently overlap or occur simultaneously. Variants with distinctive clinicopathological features include folliculotropic MF, granulomatous MF and pagetoid reticulosis[52,53]. The diagnosis of MF is established with a skin biopsy. A diagnosis of MF is often delayed for many years as it can masquerade clinically other entities such as dermatitis or fungal infection. The staging of a patient with MF is performed at initial diagnosis and depends on the type and extent of skin lesions and the presence or absence of nodal, visceral, and peripheral blood involvement. Prognosis is related to the stage of disease at the initial diagnosis and to the presence of extracutaneous disease. According to one study, the 10 year survival of patients with MF in stage I disease was equivalent to the normal age-matched population, however, decreased for all other stages[54]. Successful treatment of MF often depends on the stage of the disease at diagnosis. For treating early-stage disease topical glucocorticoids, topical nitrogen mustard, PUVA photochemotherapy and both broad-bend and narrow-bend UVB phototherapy are used. For more extensive disease systemic therapy is necessary[55,56].

1.4.2. Subcutaneus panniculitis-like T-cell lymphoma

SPTCL is an uncommon cutaneous T-cell lymphoma with clinical and histological features that can mimic benign panniculitis[57-62]. SPTCL is regarded to be a tumor of activated cytotoxic T-cells[60,61]. Since 1984, about 50 cases of SPTCL have been documented in the literature, although some cases were originally described as cytophagic histiocytic panniculitis (CHP)[60, 63]. In 1980, Winkelmann and Bowie[64] were

the first to stress the novel pathogenetic aspects of panniculitis. Although the clinical picture of their case of CHP was similar to that of the well-known forms of the disease, histological findings showed activated histiocyte infiltration of the fat tissue. Cytologically the benign-looking histiocytes containing cell debris ("bean-bag" cells) are very typical. Although the exact relationship between CHP and SPTCL is not yet fully understood, it is assumed that most cases mentioned above are ab novo T-cell lymphomas[60-62]. The average age of SPTCL patients is 46 years and women are more frequently affected than men. The disease is characterized by the development of subcutaneous inflammatory nodules most frequently on the trunk and extremities; other general symptoms, including fever, loss of body weight, night sweating, and fatigue, contribute to the clinical features[60-63,66,67]. Lymphomatous dissemination to lymph nodes and other organs is uncommon and usually occurs late in the clinical course[61]. Haemophagocytic syndrome is a frequent complication in SPTCL. It usually indicates a downhill clinical course and is the cause of death in the majority of patients[61-63]. Histomorphological investigation shows atypical T-cell infiltration in the subcutis with coagulation necrosis and karyorrhectic nuclear derbis. The presence of the "rimming" phenomenon, which occurs in the fat cells by the atypical lymphoid cells, helps in the differentiation between malignant and benign panniculitis[60,61]. Immunohistochemically, the atypical lymphocytes reveal an activated cytotoxic T-cell phenotype with CD8 positivity in most cases, but CD4 and CD8 double negative or CD4+ cases can also be found[60-63]. Cytotoxic granular proteins, such as TIA-1/GMP-17, granzyme B and perforin, are consistently expressed, indicating cytotoxic T-cell characteristics [60,61,63]. In typical cases, the cytomorphological atypia and the characteristic immunophenotype are sufficient for the diagnosis. Nevertheless, detection of monoclonal TCR gene rearrangement can confirm the diagnosis[60,61,63].

2. Aims of the study

2.1. Our study was addressed to increase the detection rate of clonality analysis in FFPE samples of mature B-cell lymphomas of germinal center (GC) or post-germinal center (pGC) origin[69], and in particular in small endoscopic biopsies. Follicular lymphomas (FL), gastric extranodal marginal zone B-cell lymphomas of MALT lymphomas, and gastric Diffuse Large B-cell Lymphomas (DLBCL) were included[69-71] GC/pGC lymphomas demonstrate random sequence alterations in the IgV_H regions related to somatic hypermutation and ongoing mutations[69], which result in a lower chance to amplify these gene segments by conventional PCR methods[40, 72-74]. In order to at least partly overcome this problem, first we have utilized a DNA polymerase combination, providing both 5'→3' polymerase and 3'→5' exonuclease activities. Secondly, we performed the Igκ PCR in addition to IgH PCR on a subset of gastric low- and high-grade MALT lymphoma samples. We applied universal FR3κ and Igκ light chain joint region (Jκ) primers (designed by Jerry Z. Gong et al) that expand the hypervariable complementary region (CDR3) of Igκ chain[36]. This single pair of degenerate oligoprimers should be able to recognize the large majority of all Igκ light chain variable region members within the six different families of the κ gene.

2.2. In consideration of the unfavorable course and the late diagnosis of ETL our aim was to facilitate early diagnosis, in order to start early specific treatment and to improve the survival by the utilization of PCR based TCRγ gene rearrangement detection on tissue samples from different stages of ETL patients. Furthermore we wished to show that monoclonal IELs in RCD and ETL patients are involving most of the gastrointestinal tract, by detecting the same clonal amplicon of the lymphoma and non-lymphomatous specimens taken from different sites of the gastrointestinal tract. We also addressed to prove that the IELs detected by immunohistochemical methods in RCD are already the counterpart of the malignant cell population.

2.3. TCR gene rearrangement analysis, using Southern blot or PCR methods, helps to confirm early or atypical CTCL when the histology is suggestive but not diagnostic. We utilized the TCRγ PCR analysis in a small number of CTCL cases in order to establish the sensitivity of this method in the conformation of the diagnosis and in distinguishing clinically indolent and aggressive forms of tumors.

3. Patients and Methods

3.1. Tissue specimens and controls

FFPE samples of 6 FL cases; 15 gastric MALT lymphoma cases, and 22 gastric DLBCL cases were retrieved from the files of the Department of Pathology, Josephine Nefkens Institute, Erasmus Medical Center (EMC), Rotterdam, The Netherlands. Samples from 14 gastric MALT lymphoma and 17 gastric DLBCL cases were small endoscopic biopsies (*Table 1*). All cases included were histologically and immunophenotypically fully characterized at diagnosis. DNA samples from reactive tonsils and 2 nodal B-cell lymphoma cases with well-established clonal IgH rearrangement were employed as polyclonal and monoclonal controls, respectively. Two cloned and sequenced FR3 fragments from B-cell lymphomas (gift of Dr. van Belzen, Department of Hematology, EMC) were also used as controls in studying effect of the 3' end mismatch. DNA from a human epithelial cell line (LNCap) was used as negative control. Water controls were included in all rounds to monitor contamination.

Furthermore 42 FFPE small endoscopic biopsy samples of 26 primary gastric MALT-type lymphoma patients (20 low-grade and 6 high-grade), 10 chronic gastritis cases, 40 (25 resection- and 15 endoscopic-) FFPE samples of 6 ETL patients and 13 FFPE punch biopsy samples of 12 CTCL patients were retrieved from the files of the Laboratory of Tumor Pathology and Molecular Diagnostics, Institute for Biotechnology, Bay Zoltan Foundation for Applied Research, Szeged, Hungary (*Tables 3, 4*). The cases included were fully characterised histologically and immunophenotypically at diagnosis. MALT lymphoma was diagnosed in 34/42 biopsy samples and 8/42 samples revealed complete haematological remission (CHR; morphological and histological) due to different lymphoma treatment (Hp. eradication, chemotherapy). TCRγ gene rearrangement PCR analysis was carried out in all ETL and CTCL specimens. CD3 and CD8 staining of the IEL samples was carried out only in patients No.1 and 6 (*Table 3*) in order to determine the aberrant phenotype as no overt ETL was detected at the time of the diagnosis. In the remaining cases where the lymphoma was detected first, only the TCRγ PCR was carried out on the samples revealing IELs. Diagnosis was ETL in 15/40, RCD in 4/40 samples and in 21/40 samples morphologically detectable IELs were seen without signs of lymphoma. Samples were taken at different time (T) and from different gastrointestinal sites during the course of the disease. The 13 skin biopsy

samples were diagnosed as MF (8/13), LP (2/13) and SPTCL (3/13). Reactive tonsil DNA was used as polyclonal control. For monitoring DNA contamination water controls were included. Each PCR reaction was carried out twice to avoid detection of "pseudo-monoclonal" bands. In addition one case of SPTCL (patient No. 12, *Table 4*) is described is detail, which presented on the scalp, leading to the clinical impression of alopecia areata.

3.2. DNA extraction

Five paraffin sections of 5 μm (in EMC) and 15 μm (in Hungary) from the selected samples were placed into Eppendorf vials, devaxed, and digested at 58˚C in a lysis buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA; 50 mM NaCl; 0,5% sodium dodecyl sulfate), containing 200 μg/ml Proteinase K. Following an overnight digestion, an additional 10 μl Proteinase K solution (20 mg/ml) was added to the same buffer, for a further 2 hours at 58˚C. Finally, the lysate was boiled for 10 minutes to inactivate the Proteinase K, centrifuged at 14,000 rpm, and the supernatant was used for the study.

3.3. PCR amplification

3.3.1. PCR amplification of IgH with rTth polymerase, XL

For PCR amplification, 15 μl reaction mixture was used, containing 1 μl of target DNA, 0.2 mM dCTP, dGTP, dTTP of each, 0.02 mM dATP and 0.8 μCi α-^{32}P-dATP (Amersham, Buckinghamshire, UK) as well as 20 mmol of each primer. FR3a (5'-ACA CGG CC/T G/C TGT ATT ACT GT-3') [21] and JHC1 (5'-ACC TGA GGA GAC GGT GAC-3') consensus primers were utilized. For conventional method, 0.4 U Taq polymerase (Promega, Madison, WI, USA) was added with 1.5 mM concentration of $MgCl_2$. For the new method, 0.3 U rTth polymerase, XL (GeneAmp® XL Kit, Applied Biosystems, Foster City, CA, USA) enzyme was added with buffer XL and 1.5 mM $MgCl_2$. The primers and the cycling conditions were the same for both enzymes: 45 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 45 seconds and extension at 72˚C for 1 minute. Reactions concluded with a final extension at 72˚C for 10 minutes. The reaction was performed in a Biometra thermal cycler (Biometra, Göttingen, Germany). The expected product size was ~120bp. In order to avoid

"pseudo-monoclonal" band due to minute amount of B-cell DNA, we employed 4 serial dilutions of target DNA (neat; 1:10; 1:100; 1:1000) in each case.

3.3.2. PCR amplification of IgH with conventional Taq

For single PCR amplification, a 50 µl reaction mixture was used, containing 1-3 µl target DNA, buffer (75 mmol/L Tris-HCL (pH 8.8), 20 mmol/L $(NH_4)_2SO_4$, 0,01% Tween 20), 0.2 mmol/L of each dNTP, 1.75 mmol/L $MgCl_2$, 2.0 U Recombinant-Taq DNA Polymerase (Fermentas), and 20 pmol of each primer. FR3a (5'-ACA CGG CC/T G/C TGT ATT ACT GT-3') and JHa (5'-TGA GGA GAC GGT GAC C-3') consensus primers were utilized. The mixture was overlaid with mineral oil and subjected to the following thermal cycling conditions of 40 cycles: denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1 minute, preceded by an initial denaturation at 94°C for 2 minutes and followed by a terminal extension at 72°C for 10 minutes (Bio-Rad Minicycler).

3.3.3. PCR amplification of Igκ

For PCR amplification of Igκ gene rearrangement oligonucleotides designed by Jerry Z. Gong et al[36], recognizing the Igκ FR3 and Igκ J regions were utilized (FR3κ-5' TTC AGC/T GGC AGC GGA/G TCT GGG -3' and Jκ-5' CAG/C CTT G/TGT CCC C/TTG GCC GAA-3'). Two units of Recombinant Taq DNA Polymerase (Fermentas) was subjected to PCR mixture, containing buffer (75 mmol/L Tris-HCL (pH8.8), 20 mmol/L $(NH_4)_2SO_4$, 0.01% Tween 20), 0.2 mmol/L of each dNTPs, 1.5 mmol/L $MgCl_2$, 20 pmol of each primer and 1-3 µl extracted DNA in 50 µl reaction volume. The cycling condition were: 10 minutes of initial denaturation at 95°C, followed by a touchdown PCR (4 cycles of 15 seconds at 94°C, 1 minute at 64°C, 1 minute at 72°C, 4 cycles of 15 seconds at 94°C, 1 minute at 62°C, 1 minute at 72°C, followed by 30 cycles of 15 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C with a final extension at 72°C for 10 minutes).

3.3.4. PCR amplification of TCRγ

For single PCR amplification, a 50 µl reaction mixture was used, containing 1-3 µl target DNA, buffer (75 mmol/L Tris-HCL (pH 8.8), 20 mmol/L $(NH_4)_2SO_4$, 0,01% Tween 20), 0.2 mmol/L of each dNTP, 1.75 mmol/L $MgCl_2$, 1.5U Recombinant-Taq

DNA Polymerase (Fermentas), and 20 pmol of each primer. VG-I (5'-TCT GGG/A GTC TAT TAC TGT GC-3'), VG-II (5'-GAG AAA CAG GAC ATA GCT AC-3'), VG-III/IV (5'-CTC ACA CTC C/TCA CTT C-3') and JG12 (5'-CAA GTG TTG TTC CAC TGC C-3') consensus primers designed by Diss[75] et al were utilized. The mixture was overlaid with mineral oil and subjected to the following thermal cycling conditions of 40 cycles: denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minutes, and extension at 72°C for 1 minute, preceded by hot start when the Taq DNA polymerase is added to the reaction mixture at 54°C after an initial denaturation at 95°C for 5 minutes. The PCR reaction is followed by a terminal extension at 72°C for 10 minutes and the PCR product was kept on 4^{o}C until loading (Bio-Rad Minicycler)

3.4. Post-amplification steps and gel electrophoresis

In EMC for gel electrophoresis, 5 μl of the PCR amplification products were diluted immediately prior analysis with 5 μl of loading buffer (95% formamide, 10mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, pH 8.0). Then the PCR products were denatured at 95˚C for 5 minutes and electrophoresed at 65W for 1.5 hours in a 6% denaturing polyacrylamide gel, containing 42% urea. Gels were dried and exposed to X-ray films at -80˚C with intensifying screens. Because of large differences in signal intensities, multiple different exposures were made. Monoclonality was established if 1 or 2 consistent (identical sized) dominant bands were found in at least 3 different dilutions. Oligoclonal pattern was acknowledged if more that 2 consistent bands and polyclonal product if a ladder were seen. Inconsistent result was accomplished if the dominant band(s) shifted by dilution.

In the study part carried out in Hungary, prior electrophoresis the Igκ PCR products were submitted to heteroduplex analysis, in which equal amounts of PCR products and running buffer (98% formamid, 10 mmol/L EDTA, 0.025% xylene cyanol FF and 0.25% bromophenol blue) are mixed, denatured at 95°C for 5 minutes, renaturated at 37°C for 30 minutes and kept on 4°C until loading. Ten μL of the PCR amplification products were subjected to electorphoresis on 10% nondenaturing-PAGE (nd-PAGE) for 45 minutes (60 minutes for TCRγ PCR) at a power of 150 V (Bio-Rad Mini-Protean-II system) at room temperature. The gels were then stained with ethidium bromide for 10 minutes and destined in distilled water twice for ten minutes and photographed under ultraviolet light. Photographs were taken under different exposure

times. The expected size of the IgH, Igκ and TCRγ PCR products was 70-120bp, 126-144bp and 64-100bp respectively. Monoclonality was established if 1 or 2 consistent (identical sized) dominant bands were found in both PCR reactions. Polyclonal pattern was acknowledged if ladder was seen. The reaction was unsuccessful when no bands were seen at all.

3.5. Control experiments

To test the effect of the 3'→5' exonuclease activities on the PCR result, two cloned and sequenced fragments were amplified with both polymerases. One fragment has a complete primer match while the other fragment has a 3' end primer mismatch. In addition, from all 8 cases with a monoclonal pattern with the rTth enzyme and a polyclonal pattern with the Taq enzyme, the FR2 fragments were amplified with the rTth enzyme in a semi-nested procedure. For the FR2 amplification, we used primers FR2 (5'-TGG A/GTC CGC/A CAG G/CCT/C T/CCN GG-3') and JHC1 in the first round and FR2 and VLJH (5'-GTG ACC AGG GTN CCT TGG CCC CAG-3') in the second round. PCR products were purified and bidirectionally sequenced with FR2 and VLJH primers using Applied Biosystems Taq DyeDeoxy terminator cycle sequencing. The sensitivity of detection of a monoclonal population was determined by diluting DNA from a clear monoclonal sample with polyclonal tonsil DNA.

4. Results

4.1. Effect of rTth polymerase on monoclonal and FR3 controls

The effect of the 3'→5' exonuclease activity on the PCR result is shown in *Figure 2.* The FR3/JH amplification of the cloned and sequenced fragment with complete primer match at the 3' end resulted in identical strong product intensities using either Taq or rTth polymerases (Fig. 2A). Whereas, the fragment with a 3' end primer mismatch was amplified far more efficiently with the rTth than with Taq, the latter showed only a faint product (Fig. 2B).

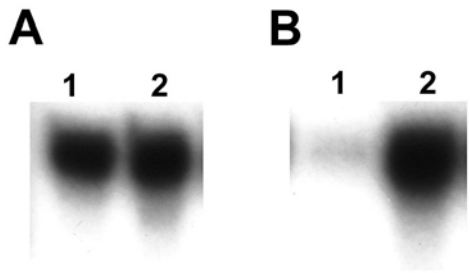

Figure 2. Framework three-area (FR3)/joining genes (JH) amplification of two (A, B) cloned and sequenced FR3 fragments with Taq (lanes 1) and rTth (lanes 2). Sequences FR3a primer: 5'primer-ACTGT-3'. Fragment A: TGACA. FR3a primer: 5'primer-ACTGT-3'. Fragment B: TGACC. Note the primer 3' end mismatch with fragment B, resulting in very minor amplification with Taq (B1) and strong amplification with rTth (B2).

In one of the nodal B-cell lymphomas used as control, the Taq revealed a polyclonal FR3/JH pattern, whereas a clear dominant monoclonal band with a polyclonal background was observed using the rTth DNA polymerase *(Figure 3).*

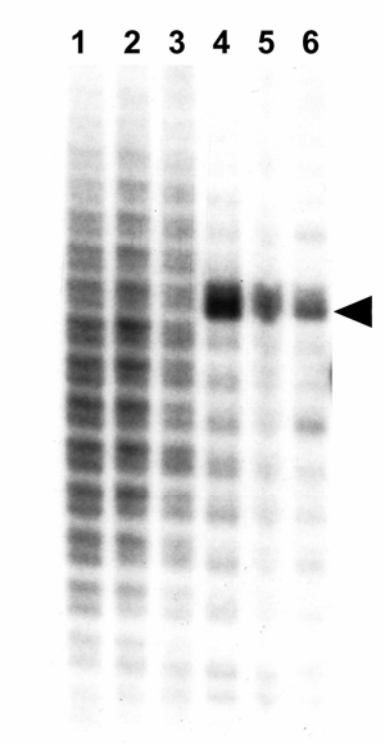

Figure 3. The effect of the rTth enzyme is shown on one of our nodal B-cell lymphoma cases used as control. One genomic DNA FR3/JH sample amplified with Taq (lanes 1-3; undiluted, 1:10, 1:100) and rTth polymerase (lanes 4-6; undiluted, 1:10, 1:100). In this case, the Taq revealed a polyclonal FR3/JH pattern, whereas a clear monoclonal pattern (arrow) in a polyclonal background was observed using the rTth DNA polymerase.

In eight cases, a polyclonal pattern was obtained with Taq and a monoclonal pattern with rTth (*Table 1*). From these cases, FR2/JH fragments were amplified in a semi-nested procedure. PCR products were obtained from 3 cases (*Table 1*, gastric large B-cell lymphoma cases 20 and 21; gastric MALT lymphoma case 11). Lack of FR2 amplification in 5 cases is probably caused by the size of the FR2 fragment. After bidirectional direct sequencing, an unambiguous sequence was obtained from one case (*Table 1*, gastric large B-cell lymphoma case 20). This sequence revealed a FR3a primer 3' end mismatch (AGG ACA CGG CTG TGT ATT ACT G/C/ GCG). The 2 other cases had too many admixtures of polyclonal PCR products to get a clear sequence from the monoclonal population. No FR3A/JH IgH PCR products were amplified from DNA samples from the epithelial tumor cell line LNCap (not shown).

FR3 monoclonal control DNA was diluted in polyclonal tonsil DNA and from these mixtures, FR3 was amplified both with Taq and rTth enzymes. From 500-1000x

dilutions of monoclonal genomic DNA in polyclonal DNA, the FR3 monoclonality can still be detected with both enzymes (results not shown).

4.2. Clonality analysis of lymphoma cases using conventional Taq and rTth

The results are summarized in *Table 1*. Using conventional Taq polymerase, consistent monoclonal product was found in 53% (23/43) of the cases (FL: 67%; MALT lymphoma: 47%; DLBCL: 55%). The rTth polymerase revealed reproducible monoclonal band in 73% (31/43) of the cases (FL: 67%; MALT lymphoma: 73%; DLBCL: 73%) *(Fig.4A).* Oligoclonal pattern was ascertained in 1 gastric DLBCL case with both Taq and rTth. An inconsistent result was seen in 5 cases with Taq polymerase and 4 (identical) cases with rTth polymerase, as the size of the PCR products differed between different dilutions *(Fig 4B).* Two of these inconsistent cases represented endoscopic biopsies (*Table 1*).

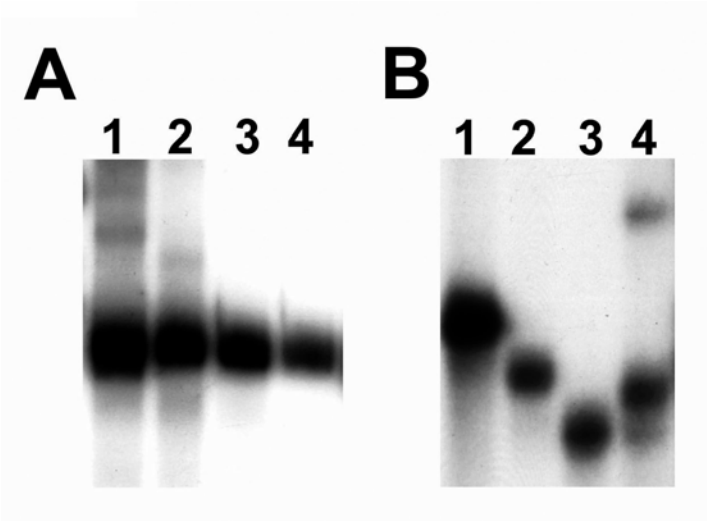

Figure 4. FR3a/JH amplification with rTth of MALT cases in four different serial dilutions. A: consistent clonal pattern is observed. B: An inconsistent clonal pattern is observed.

4.3. Detection rate of IgH and Igκ PCR analysis

Reproducible dominant band was detected in 21/34 (61.76%) lymphoma samples when IgH PCR was performed and in 28/34 (82.35%) when Igκ. All together dominant band was detected in 32/34 (94.12%) histologically confirmed MALT lymphoma samples. Igκ monoclonality was still detected in 4/8 samples revealing CHR, while no IgH

monoclonality was detected (*Table 2*). Polyclonal pattern was seen in the controll group of 10 gastritis.

We had the opportunity to follow up a patient (Patient No. 6 Table 2) being diagnosed with low-grade, I. stage gastric MALT lymphoma who only received Hp. eradication treatment *(Figure 5).* At diagnosis both IgH and Igκ PCR detected monoclonality. The lymphoma was still detectable histologically and genetically on the gastric biopsy repeated immediately after the patient received Hp eradication therapy. Igκ PCR still revealed monoclonality on the biopsy repeated 4 month after Hp. eradication, while IgH PCR showed polyclonal pattern.

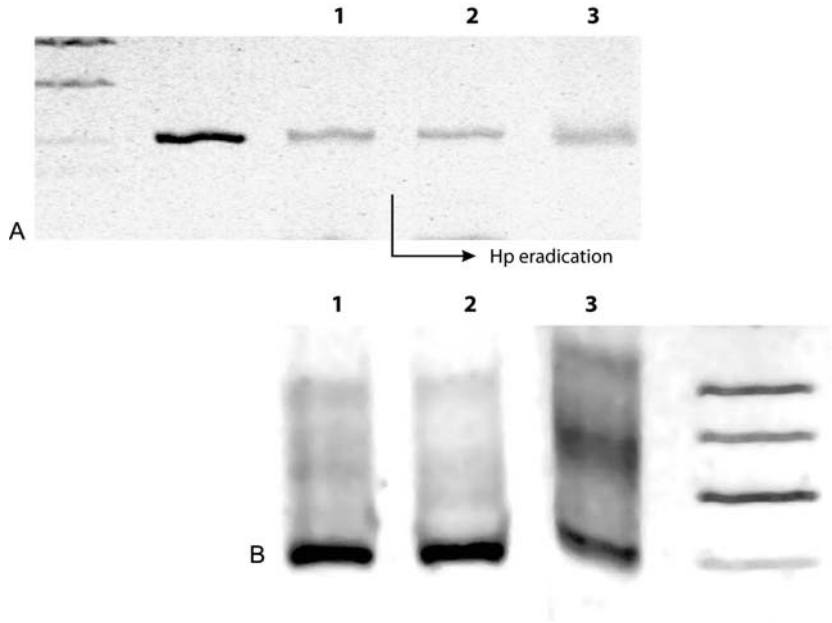

Figure 5. A: single IgH PCR (lane 1: gastric MALT lymphoma, lane 2: histologically detectable lymphoma after Hp. eradication, lane 3: molecular and histological remission). B: single Igκ PCR (lane 1: gastric MALT lymphoma, lane 2: histologically detectable lymphoma after Hp. eradication, lane 3: histological remission but still detectable Igκ monoclonality).

4.4. Rearrangement of the TCRγ gene

In our sequential ETL clonality analysis TCRγ monoclonality was found in 15/15 (100%) of ETL-, 4/4 (100%) of RCD- and 13/21 (61, 9%) of IEL samples. Polyclonal pattern was detected in 6/21 (28, 5%) of IEL samples and only 2/21 reactions were unsuccessful (*Table 3*). TCRμ rearrangements of the lymphoma specimens were clonal

with the same length as the nonlymphomatosus mucosa samples of the gastrointestinal tract (not shown). In patient No. 1 (*Table 3*) IELs were detected histologically in the tissue specimens taken from the duodenum and the gastric antrum. The TCRγ PCR reaction revealed an identical size monoclonal population *(Figure 6).*

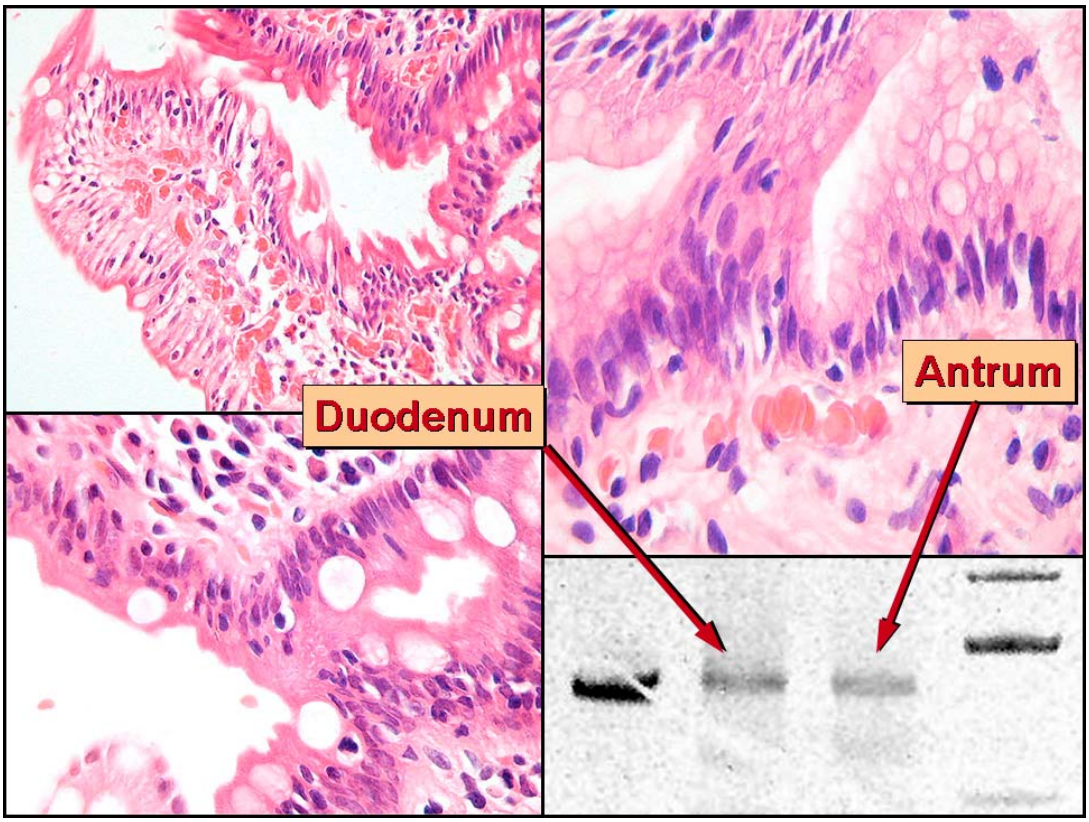

Figure 6. Histological examination confirmed IELs in the tissue specimens of the duodenum and antrum of Patient No. 1 (*Tables 3*). The PCR reaction of TCRγ rearrangement revealed identical size monoclonal bands.

The sequential study of Patient No. 6 showed that specimens taken in different times during the course of the disease has the same clonal amplificate *(Figure 7).*

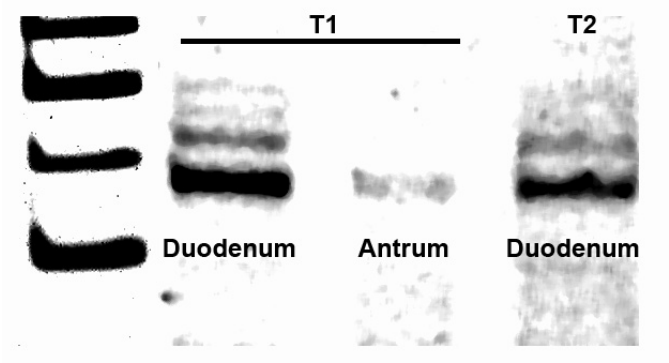

Figure 7. The sequential study of Patient No. 6 (*Table 3*) showed that specimens taken in different times during the course of the disease has identical size monoclonal amplificate. Lane 1: RCD in duodenum, Lane 2: nonlymphomatosus gastric antrum, Lane 3: RCD in duodenum. Specimens shown in lane 1 and 2 were taken at the same time while in lane 3 was taken later during the course of the disease.

TCRγ PCR monoclonality was detected in 85% (11/13) of CTCL samples (7/8 MF, 2/2 LP, 2/3 SPTCL samples). Polyclonal pattern was seen in only two samples (1/8 MF, 0/2 LP, 1/3 SPTCL). No unsuccessful reaction was detected in CTCL samples (*Table 4*).

4.4.1. Case report of patient No. 12

A 45-year-old, otherwise asymptomatic woman presented at the Department of Dermatology, County Hospital, Kecskemét, Hungary in November 1999 with alopecia lesions on her scalp that had persisted for 6 months. On admission, confluent, polycyclic, bright red, hairless areas with uneven surfaces were observed on the parietal and temporal skin. Enlarged lymph nodes were not found. Routine laboratory findings showed no changes. Excision biopsy was performed from typical-looking lesion. The histological sample showed intact epithelium, mild non-specific chronic dermal inflammation and a confluent lobular subcutaneous necrosis with excessive acellular karyorrhectic debris. The latter led to atrophy and a lesion resembling fibrinoid necrosis of the incorporated hair follicles, which explained the alopecia. In the border zone of the necrosis, accumulation of “tingible-body” macrophages and of some small to medium-sized lymphocytes was found. Among these lymphocytes, only scattered medium-sized cells showed irregular hyperchromatic nuclei as well as CD3, CD8, and TIA-1 positivity, while the majority revealed small lymphocyte cytomorphology with a CD3 and CD4+ immunophenotype without expressing the cytotoxic granule protein TIA-

1/GMP-17. No CD56+ cells were detected, excluding the possibility of both gamma/delta T-cell and nasal-type natural killer cell lymphomas[61,63]. Rimming of fat vacuoles due to invagination of lymphocytes in cells was found in only a few fat cells. The morphological and immunohistochemical findings that revealed prevalent necrosis and only a few atypical lymphocytes were insufficient to verify malignancy, and therefore the case was interpreted as "consistent with CHP", although the possibility of a "burned-out" SPTCL was not excluded.

Continuous external and three times repeated intrafocal steroid therapy proved unsuccessful, and long-term doxycyclin therapy was also ineffective; therefore, a further biopsy was performed for histomorphological re-evaluation in February 2000. The second biopsy showed granulomatous panniculitis with areas of confluent necrosis. Atrophy and necrosis of the hair follicles were still observed. Infiltrates contained a heterogeneous population of lymphocytes with the prevalence of small CD3 and CD4+ T lymphocytes. No atypical CD8+ cells were detected in the second biopsy, thus CHP was repeatedly diagnosed. PCR-based analysis of TCRγ gene rearrangement for determination of T-cell clonality became available to us during the summer of 2001. It was performed retrospectively in both biopsies and revealed a monoclonal T-cell population in the first biopsy sample (*Figure 8*) and a polyclonal pattern ("smear") in the second one.

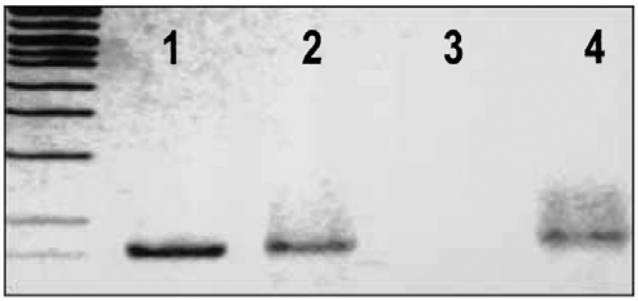

Figure 8. Products of TCRγ PCR of patient No. 12 (*Table 4*) are shown. Lanes 1 and 2: positive controls, lane 3: negative control, lane 4: dominant band in a polyclonal background (smear), revealing presence of a monoclonal T-cell population in the first biopsy.

Thus a final diagnosis of SPTCL was established. After an overall 2-year follow-up period, the patient is physically and subjectively asymptomatic (as of November 2001),

apart from the persisting inflammatory scalp lesions, which showed slight enlargement. Physical examination, chest X-ray and abdominal ultrasonography demonstrated no dissemination to lymph nodes or to internal organs. There was no further follow-up as the patient was lost for unknown reason.

5. Discussion

The sensitivity of a PCR-based assay for IgH gene rearrangements largely depends on lymphoma type and tissue fixation[35] . The GC/pGC lymphomas reveal signs of somatic hypermutation[69-71], resulting in mutations and deletion in the IgV_H gene segments[69], which may cause false negativity due to primer mismatch during annealing[40,72,74] . This fact can be at least partly overcome by application of degenerated primers as well as by using a DNA polymerase with proofreading activity which has the ability to remove mismatched bases at the 3'terminus of the primers and thus to increase the chance of amplification[39-41]. According to this, a significantly increased sensitivity in FR3/JH PCR analysis has been reported by the use of UITma DNA polymerase, a DNA polymerase with 3'→5' exonuclease activity[40,41]. We have tested the UITma DNA polymerase in the Erasmus MC (Rotterdam, The Nederlands) with encouraging results, but the UITma DNA polymerase is not more available. A survey for a similar enzyme has led us to the r*Tth* DNA Polymerase, XL (Applied Biosystems), an enzyme blend of *Thermus thermophilus (Tth)* and *Thermococcus litoralis (Tli)* DNA polymerases, combining both 5'→3' polymerase and 3'→5' exonuclease activities (information provided by the manufacturer). Thus, with the rTth DNA Polymerase, we were able to achieve the expected improvement provided by the 3'→5' exonuclease activity in our PAGE pattern analysis, using 2 cloned and sequenced FR3 fragments (*Figure 2*). In the targeted 43 FFPE mature B-cell lymphomas of GC/pGC origin, the rTth DNA polymerase has markedly improved the sensitivity of the FR3/JH PCR method (from 53% to 73%), as compared with the conventional Taq polymerase. The most significant improvement was noticed in gastric MALT-type lymphoma cases (from 47 to 73%), meanwhile all except 1 cases represented small endoscopic biopsies. These figures concur with the monoclonality rate reported by others using the conventional FR3/JH PCR and DNA extracted from fresh/frozen tissues[35].

Another corner stone of the accurate IgH PCR assays is the use of appropriate amounts of target DNA to ensure the correct assessment of clonality. Minute DNA amount may result in inconsistent size bands that can be misinterpreted as clonal rearrangement[76,77], which is the case of endoscopic biopsies. In order to avoid these arteficial bands, we employed a gradual target DNA dilution. We experienced inconsistent size bands in 5 cases with Taq polymerase and in 4 cases with rTth

polymerase. Nevertheless, only 2 of these cases were endoscopic gastric biopsies, suggesting that this phenomenon is not dependent purely on the size of the tissue and the utilization of the rTth polymerase has no significant influence on it. We believe that this can occur also due to the degraded template DNA, resulting in relative paucity of amplifiable B-cell DNA.

In this part of the study we optimized the IgH PCR assay for FR3/JH segment in FFPE mature B-cell lymphoma samples of GC/pGC origin, using rTth DNA polymerase, an enzyme blend providing both 5'→3' polymerase and 3'→5' exonuclease activities. The application of polymerase significantly increased the detection rate of monoclonal IgH rearrangements. B-cell clonality was determined by FR3/JH single PCR protocol, which amplifies small fragments, therefore it can be easily employed on fragmented DNA retrieved from FFPE pathologhical specimens. The amplification of FR3/JH segment is relatively simple and use only one primer pair in a single PCR reaction[72,73,78]. We demonstrated that the sensitivity of FR3/JH PCR method can be significantly improved by the use of the rTth DNA polymerase, as compared with the conventional Taq polymerase.

We conclude that the utilization of rTth DNA polymerase greatly improved sensitivity of PCR clonality analysis in FR3/JH IgH gene segment, as applied in FFPE FL, gastric MALT lymphoma, and gastric DLBCL samples. Our method is benefited from the 3'→5' exonuclease activity, which appears to help in achieving superior fit of the primers to the rearranged IgH gene segments. The sensitivity of our assay seems to be similar to that PCR methods employ DNA from fresh/frozen tissues. In addition, the serial dilution of target DNA proved to be useful to avoid misinterpretation of inconsistent size bands those may be revealed due to the minute amount or poor quality of the B-cell DNA.

The CDR3 region of Igκ contributes to the greatest variability within the VJκ segments. This variability is largely due to the juxtaposition of VJ regions and the addition of P and N nucleotides. The high degree of variability of the CDR3 region of Igκ is an ideal target for separation of polyclonal and monoclonal products[79,80]. As compared to the IgH locus, Igκ does not contain a diversity (D) region resulting in less diversity in κ VJ than the heavy chain VDJ segments. To facilitate the resolution of the banding in detecting Ig clonality, we used nd-PAGE to analyse the PCR products. Nd-PAGE is optimal in facilitating the resolution of homoduplex/heteroduplex bandings in deteting Igκ clonality. Nd-PAGE when compared with other high resolution gels has the

advantage of small size, simplicity of assemblage, short running time, and economy[81]. This technique was originally developed to evaluate the phylogeny in evolution and was subsequently found to be an excellent tool for the study of Ig gene clonality[81,82] .Under a cycle of denaturation and renaturation of the PCR products, the DNA of monoclonal products will perfectly match each other and form a uniform double-stranded population. This population is composed of a single homoduplex population and appears as a distinct single band on nd-PAGE. On the other hand, each double-stranded product of polyclonal population will form heteroduplex with a varying degree of mismatch. The mismatch alters the conformation of every double-stranded DNA, and this heteroduplex population appears as a smear, which also has slower mobility compared with the homoduplex population.

In the second part of the study we combined the single IgH and Igκ PCR on endoscopic gastric MALT lymphoma samples. Igκ PCR products were submitted to heteroduplex analysis. ND-PAGE was utilised for separating monoclonal and polyclonal products. We were able to increase the monoclonality rate from 61.7% achived alone with IgH PCR to 94.1% when combined with Igκ on our histologically confirmed FFPE gastric MALT lymphoma samples. The combination of IgH- and Igκ PCR/conventional Taq seems to be superior when compared to the results achieved by the utilization of rTth DNA polymerase in single IgH PCR in gastric MALT lymphoma samples (94.1% versus 73%). No monoclonality was detected by IgH PCR in the 8 gastric samples revealing CHR, while in 4 Igκ monoclonality was still detected. This finding suggests that Igκ PCR is more sensitive than IgH PCR in detecting B-cell monoclonality even in samples showing CHR. The clinical significance of this detected monoclonality is unknown; more prospective studies should be carried out.

Finally, our study demonstrated that the unique approach of combined IgH and Igκ gene rearrangement analysis, along with a highly sensitive nd-PAGE method, is a useful tool in the routine clinical laboratory assessment of B-cell clonality in endoscopic gastric MALT lymphoma samples and in the follow up after treatment of MALT lymphoma. Using a simple pair of primers to detect κ light chain rearrangement on nd-PAGE is a sensitive, simple, and cost-effective method as an adjunct to IgH analysis. Owing to the short amplification products in FR3Jκ fragments it is an ideal tool for analyzing small poorly preserved formalin-fixed and paraffin-embedded material, which is most frequently available in routine clinical practice.

The presence of a monoclonal T-cell population in non-lymphomatous enteropathic mucosa has been described in ETL, ulcerative jejunitis and RCD. Cellier et al stated that intestinal IELs from patients with RCD are clonal and display a markedly abnormal phenotype ($sCD3\varepsilon^-$, $cCD3\varepsilon^+$, $CD4^-$, $CD8^-$, $TCR\alpha\beta^-$ and $TCR\mu\delta^-$) in comparison with the major $sCD3^+$, $CD8^+$, $TCR\alpha\beta^+$ populations of T cells that comprise the increased IEL in CD[48, 49]. Initially, the precise nature (reactive, neoplastic) of these T cell populations was uncertain. Evidence has now accumulated suggesting that these T cells are an early manifestation of ETL. In most cases of ETL the neoplastic cells express $CD3\varepsilon^+$ but are $CD4^-$ and $CD8^-$. Sequence analysis has shown that these tumor cells are clonally identical with a T-cell population in the enteropathic mucosa identified as IEL[45, 47]. This points to the direct link between the monoclonal T-cell population in RCD, UJ, enteropathic nonlymphomatous mucosa in ETL and in ETL itself. Some of RCD patients developed overt ETL containing the same clonal rearrangements in their overt lymphoma as in duodenal biopsies. We addressed to show that TCRγ gene rearrangement using a single PCR method in FFPE samples is a simple and sensitive diagnostic method in the routine diagnosis of RCD and ETL as some of the above mentioned studies were carried out on fresh and frozen mucosal specimens and used Southern-blot, nested, semi-nested PCR methods. The findings reported in this study show that the PCR based TCRγ gene rearrangement analysis is sensitive and simple enough to detect monoclonal IELs in RCD, in ETL and in the enteropathic non-lymphomatous mucosa specimens from patients with ETL. Detection of the same clonal amplicon of the lymphoma and non-lymphomatous specimens is suggesting that the monoclonal IELs in RCD and ETL patients are involving most of the gastrointestinal tract. On the basis of previous molecular studies[45-49,75] and the clonality findings of our study , it seems to be safe to state that the monoclonal IEL in patients with RCD and overt ETL are neoplastic although they are not cytologically abnormal and they do not form tumor masses. The accumulation of phenotypically aberrant, monoclonal IEL appears to be the first step in the genesis of ETL. With the recognition that patients with RCD are suffering from a neoplastic T-cell disorder (also called as cryptic lymphoma), involving most of the gastrointestinal tract, systemic therapy is necessary.

TCR gene rearrangement analysis, using Southern blot or PCR methods, helps to confirm early or atypical CTCL when the histology is suggestive but not diagnostic[83]. Most reported cases of CTCL have a clonal rearrangement detected by TCR gene rearrangement. The diagnostic value of TCR clonality analysis by Southern blot is

limited by a low sensitivity since a level of abnormal T-cell clone infiltration below 5% may be too low for detection. PCR has a sensitivity that is at level of magnitude greater than Southern blotting, and the increase in the limit of detection may allow a diagnosis of CTCL in very early disease stages[83, 84]. The TCRγ PCR analysis for detecting monoclonality in CTCL is a promising diagnostic technique. Our results of 85% monoclonality detected in this small series of CTCL punch biopsy FFPE samples suggest that the applied TCRγ PCR method for detecting monoclonality in CTCL is sensitive enough to confirm the diagnosis. Further advances in our knowledge of clonality in CTCL are necessary before PCR can be used as a sole diagnostic test for CTCL, as it is acknowledged that some non-neoplastic T-cell disorders such as pityriasis lichenoides et varioliformis acuta may display some level of clonality[83].

Although SPTCL is thought to have an aggressive natural history and poor prognosis, many patients often respond effectively to chemotherapy and show quite a long survival[60, 62, 63, 65, 67]. According to the literature[60-63, 65-67] and our experience, two clinical subtypes of subcutaneous lymphomas can be identified. The first type is characterized by an indolent, CHP-like course. These cases are indicative for primary SPTCL. Most cases with alpha/beta cytotoxic T-cell phenotype are suggestive of an indolent course. The second type has a rapid onset, followed by a progressive clinical course. These lymphomas, however characterized by CD56 positivity, the presence of Epstein-Barr virus, and lack of TCR gene rearrangement[61,68]. In respect of these considerations, the complex evaluation of the clinical features, immunophenotype and molecular data provides an inevitable tool in distinguishing potentially indolent and aggressive cases, thus allowing the employment of the correct therapeutic regimen.

Our case described in detail is noteworthy for many reasons. The patient had panniculitic symptoms in an unusual localization (hairy skin) without signs of lymph node or internal organ involvement. The histopathological evaluation revealed a benign CHP-like appearance and the clinical course proved to be indolent. Furthermore, our case has demonstrated that, apart from the wellknown dermatoses, alopecia can develop also because of SPTCL. This report reinforces the importance of the implementation of immunhistological and molecular methods in the diagnosis of the lympoproliferative skin disorders.

6. Summary

The utilization of rTth DNA polymerase greatly improved sensitivity of PCR clonality analysis in FR3/JH IgH gene segment, as applied in FFPE FL, gastric MALT lymphoma, and gastric DLBCL samples. Our method is benefited from the 3'→5' exonuclease activity, which appears to help in achieving superior fit of the primers to the rearranged IgH gene segments. The sensitivity of our assay seems to be similar to that PCR methods employ DNA from fresh/frozen tissues. In addition, the serial dilution of target DNA proved to be useful to avoid misinterpretation of inconsistent size bands those may be revealed due to the minute amount or poor quality of the B-cell DNA.

Our study demonstrated that the unique approach of combined IgH and Igκ gene rearrangement analysis, along with a highly sensitive nd-PAGE method, is a useful tool in the routine clinical laboratory assessment of B-cell clonality in endoscopic gastric MALT lymphoma samples and in the follow up after treatment of MALT lymphoma. Using a simple pair of primers to detect κ light chain rearrangement on nd-PAGE is a sensitive, simple, and cost-effective method as an adjunct to IgH analysis. Owing to the short amplification products in FR3Jκ fragments it is an ideal tool for analyzing small poorly preserved formalin-fixed and paraffin-embedded material, which is most frequently available in routine clinical practice. The combination of IgH- and Igκ PCR/conventional Taq seems to be superior when compared to the results achieved by the utilization of rTth DNA polymerase in single IgH PCR in gastric MALT lymphoma samples.

On the basis of previous molecular studies and the clonality findings of our study, it seems to be safe to state that the monoclonal IEL in patients with RCD and overt ETL are neoplastic although they are not cytologically abnormal and they do not form tumor masses. The accumulation of phenotypically aberrant, monoclonal IEL appears to be the first step in the genesis of ETL. With the recognition that patients with RCD are suffering from a neoplastic T-cell disorder (also called as cryptic lymphoma), involving most of the gastrointestinal tract, systemic therapy is necessary.

Our results from this small series of CTCL punch biopsy FFPE samples suggest that the applied TCRγ PCR method for detecting monoclonality in CTCL is sensitive enough to confirm the diagnosis. The complex evaluation of the clinical features,

immunophenotype and molecular data provides an inevitable tool in distinguishing potentially indolent and aggressive cases, thus allowing the employment of the correct therapeutic regiment. The detailed report of patient diagnosed with SPTCL reinforces the importance of the implementation of immunhistological and molecular methods in the diagnosis of the lympoproliferative skin disorders.

APPENDIX

Table 1 Results of FR3/JH PCR with Taq and rTth DNA polymerases.

	Specimen type	Taq FR3/JH	rTth FR3/JH
Nodal follicular lymphoma			
1	Lymph node	M	M
2	Lymph node	M	M
3	Lymph node	M	M
4	Lymph node	M	M
5	Lymph node	IC	P
6	Lymph node	IC	IC
Gastric diffuse large B-cell lymphoma			
1	Endoscopic	M	M
2	Endoscopic	M	M
3	Endoscopic	M	M
4	Endoscopic	M	M
5	Endoscopic	M	M
6	Endoscopic	M	M
7	Endoscopic	M	M
8	Endoscopic	M	M
9	Endoscopic	M	M
10	Endoscopic	M	M
11	Endoscopic	P	M
12	Endoscopic	P	M
13	Endoscopic	P	P
14	Endoscopic	P	P
15	Endoscopic	P	P
16	Endoscopic	OC	OC
17	Endoscopic	IC	IC
18	Resection	M	M
19	Resection	M	M
20	Resection	P	M
21	Resection	P	M
22	Resection	IC	IC
Gastric MALT lymphoma			
1	Endoscopic	M	M
2	Endoscopic	M	M
3	Endoscopic	M	M
4	Endoscopic	M	M
5	Endoscopic	M	M
6	Endoscopic	M	M
7	Endoscopic	M	M
8	Endoscopic	P	M
9	Endoscopic	P	M
10	Endoscopic	P	M
11	Endoscopic	P	M
12	Endoscopic	P	P
13	Endoscopic	P	P
14	Endoscopic	IC	IC
15	Resection	P	P

Table 2 Results of the IgH and Ig kappa PCR of gastric MALT lymphoma patients.

Patient No.	Sample No.	Type	IgH	Ig kappa
Low-grade MALT lymphoma				
1	1		P	M
	2	CHR	P	M
2	3		M	M
3	4		M	M
	5		U	M
4	6		P	M
	7		P	M
5	8		P	P
	9	CHR	P	P
	10	CHR	P	P
6	11		M	M
	12		M	M
	13	CHR	P	M
7	14		P	M
	15		U	M
	16	CHR	P	P
8	17		M	M
	18		M	M
9	19		P	M
	20		P	P
	21	CHR	P	P
10	22		P	M
11	23		M	P
12	24		M	M
13	25		M	P
14	26		M	M
	27		M	M
15	28		M	M
16	29		M	M
17	30		M	M
18	31		M	M
	32		M	M
19	33		P	M
20	34		M	M
21	35		P	M
22	36		M	P
High-grade MALT lymphoma				
23	37		P	M
	38	CHR	P	M
24	39		M	M
25	40		M	P
26	41		M	M
	42	CHR	P	M

Table 3 Results of TCRγ PCR in ETL patients.

Patient No.	Sample No.			Site	Specimen type	Histology	TCRγ PCR
	T1	T2	T3				
1	1			Gastric corpus	Endoscopic	IEL	M
	2			Gastric antrum	Endoscopic	IEL	M
	3			Duodenum	Endoscopic	IEL	M
2	4			Duodenum	Endoscopic	RCD	M
	5			Duodenum	Endoscopic	RCD	M
3	6			Jejunum	Resection	ETL	M
	7			Duodenum	Resection	IEL	M
		8		Gastric antrum	Endoscopic	IEL	U
			9	Gastric corpus	Endoscopic	IEL	U
4	10			Ileum	Resection	ETL	M
	11			Ileum	Resection	IEL	M
	12			Ileum	Resection	IEL	M
	13			Small bowel	Resection	ETL	M
	14			Abdominal wall	Resection	ETL	M
	15			Colon	Resection	IEL	M
		16		Gastric	Endoscopic	IEL	M
		17		Small bowel	Resection	IEL	M
		18		Small bowel	Resection	ETL	M
		19		Small bowel	Resection	ETL	M
		20		Small bowel	Resection	ETL	M
		21		Small bowel	Resection	IEL	P
		22		Small bowel	Resection	ETL	M
		23		Small bowel	Resection	ETL	M
			24	Duodenum	Endoscopic	IEL	P
5	25			Small bowel	Resection	ETL	M
	26			Small bowel	Resection	ETL	M
	27			Small bowel	Resection	ETL	M
	28			Small bowel	Resection	IEL	M
		29		Duodenum	Resection	IEL	OC
		30		Duodenum	Resection	IEL	OC
		31		Duodenum	Resection	IEL	OC
		32		Jejunum	Resection	ETL	M
		33		Jejunum	Resection	ETL	M
		34		Jejunum	Resection	IEL	M
			35	Duodenum	Endoscopic	IEL	M
6	36			Duodenum	Endoscopic	RCD	M
	37			Gastric antrum	Endoscopic	IEL	M
	38			Gastric corpus	Endoscopic	IEL	P
		39		Duodenum	Endoscopic	RCD	M
			40	Ileum	Resection	ETL	M

Table 4 Results of TCRγ PCR of CTCL patients.

Patient No.	Sample No.	Histology	TCRγ PCR
1.	1.	MF patch phase	M
2.	2.	SPTCL	P
3.	3.	MF early stage	P
4.	4.	MF early stage	M
5.	5.	LP	M
6.	6.	MF early stage	M
7.	7.	LP	M
8.	8.	MF follicular variant	M
9.	9.	MF patch phase	M
10.	10.	MF patch phase	M
11.	11.	MF patch phase	M
12.	12.	SPTCL	M
	13.	SPTCL	P

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