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Analysis of supernumerary clonal gamma T-cell receptor gene rearrangements in
cutaneous T-cell lymphoma

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Whitney Lynn Tolpinrud

2011

Abstract:

Background: Cutaneous T-cell lymphoma (CTCL) is a rare malignancy of the skin and fatal if untreated. The mid 1980s introduction of molecular tests for clonality revolutionized the capacity to diagnose the disease. Polymerase chase reaction (PCR) is now routinely performed on blood and involved skin to detect clonal T-cell receptor (TCR) gene rearrangements. It has been widely assumed that CTCL arises from a monoclonal T-cell population. Detection of one or two uniform γ TCR gene rearrangement sequences is expected, depending on whether one or both alleles have undergone rearrangement in the original tumor progenitor cell. However, three or more clonal sequences are sometimes detected, and thus far no clear explanation for this finding has been offered. Clarification of the mechanism responsible for these multiple monoclonal populations is a prerequisite to the understanding of the pathogenesis of CTCL and for appropriate application of PCR determinations in the diagnosis and management of CTCL.

Objective: To elucidate the origin of γ TCR gene rearrangements in CTCL tissues of single patients in which more than two clonal γ TCR gene rearrangements have been detected by PCR and to determine if CTCL consists of a polyclonal T-cell population.

Methods: PCR of the γ TCR genes was performed using DNA from a skin specimen showing high grade CTCL with three apparent clonal γ TCR rearrangements. The amplified products were separated by gel electrophoresis, molecularly cloned in bacteria, and subjected to sequence analysis.

Results: Sequence analysis of the molecularly cloned PCR products revealed one clonal V γ 9,10 TCR gene rearrangement (184 base pair fragment) and two clonal

V γ 1-8 TCR gene rearrangements (234 and 237 base pair fragments). The sequences of the two V γ 1-8 rearrangements were 86% similar. The V γ 9,10 sequence was 17% similar to the 234 base pair V γ 1-8 sequence and 19% similar to the 237 base pair V γ 1-8 sequence.

Conclusion: The dissimilarity in DNA sequences among the three rearranged clonal γ TCR rearrangements suggests that either more than one T-cell clone is present in this patient's CTCL or that CTCL in this patient arose from a progenitor cell in which both γ TCR alleles had not yet undergone rearrangement. This case is illustrative of the importance of understanding the complexities of tests for T-cell clonality, rather than expecting a simple positive or negative "malignancy" answer.

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Introduction:*Clinical Presentation of Cutaneous T-cell Lymphoma*

Cutaneous T-cell Lymphoma (CTCL) is a malignancy of the skin characterized by proliferation of skin-homing T-cells. CTCL is distinct from other malignancies in that it is often difficult to diagnose and to classify. The term *CTCL* was initially coined by Edelson in the 1980's (1) and has since been used as an umbrella term to describe a group of cutaneous lymphomas with varying presentations and clinical courses. Some examples include: mycosis fungoides (2), Sézary syndrome, peripheral T-cell lymphoma, lymphomatoid granulomatosis (3), as well as a number of other neoplastic processes. Further adding to the complexity of classification, the distinction between CTCL and chronic benign T-cell skin proliferative diseases such as lymphomatoid papulosis (LyP), is unclear. The skin lesions of LyP appear histologically neoplastic but only 15-19% of LyP patients will eventually develop CTCL (4). With all this in mind, it is clear that CTCL is an extremely complex disease entity with a spectrum of clinical and histological presentations creating ample opportunities for scientific inquiry.

CTCL is a rare disease of unknown etiology. The overall incidence was estimated to be 2.8 per million in 1973-1977 and 9.6 per million in 1998-2002 (5). African-Americans and males are two times more likely to develop the disease than whites and females. Although the average age at presentation is 50 years, CTCL can be diagnosed in patients as young as 30 (3).

CTCL is an indolent disease characterized by multiple clinical stages: patch, plaque, tumor, and disseminated disease (6). The systemic form occurs when T-

cells lose affinity for the skin and migrate to lymph nodes, viscera, and peripheral blood. Early detection and treatment is essential as the disease is often fatal when systemic; patients with progression to the tumor or disseminated (erythrodermic) stages have a 5-year survival rate of 40% (7). The distribution is classically confined to areas hidden from sun (“bathing suit” distribution) and includes the flanks, breasts, inner thighs, inner arms, and axillae (8). Generally, patients present with pruritus, chronic nonspecific dermatitis, poikilodermatous skin lesions, or generalized erythroderma (9).

Histology of Cutaneous T-cell Lymphoma

The histology of CTCL is often used as a diagnostic aid because the clinical presentation is so diverse. However, the presence of multiple histologic variants makes agreement on classification of diagnostic criteria difficult (10). Furthermore, the T-cells in benign or non-aggressive diseases such as lymphocytoma and parapsoriasis may present with atypical cytologic features similar to those found in CTCL (11-13), further complicating classification. Although the histology of benign and malignant T-cell processes often overlap, microscopic analysis is still frequently utilized by physicians to assist with accurate diagnosis.

Specifically, examination with light-microscopy often reveals small- and medium-sized lymphocytes with cerebriform nuclei (6) and papillary dermal fibrosis with polymorphic monocyte, plasma cell and eosinophil infiltrate (14). In a minority of cases, pautrier microabscesses may be visualized (14). In a retrospective review of CTCL biopsies, the following characteristics were found to

differentiate between CTCL and other disease processes: haloed lymphocytes, exocytosis, epidermotropism, large epidermal lymphocytes, hyperconvoluted intraepidermal lymphocytes, and lymphocytes aligned within the basal layer (15). Because of the difficulty of diagnosing CTCL, patients with suspected CTCL warrant review by an experienced pathologist with specific emphasis on clinico-pathological correlation (16).

Pautrier microabscesses are pathognomonic of CTCL and contain nests of T-cells attached to dendrites of Langerhans cells (17). They are rarely seen in the initial patch-stage (18) but are sometimes found in biopsies of patients with plaque-stage CTCL (19). Interestingly, the T-cells in pautrier microabscesses are assumed to be malignant, but no study has verified this widely accepted hypothesis.

Immunohistochemical staining techniques have shown that in early stages of CTCL, the malignant T-cells localize to the epidermis, a phenomenon referred to as epidermotropism (20, 21). Interestingly, non-malignant T-cells are often confined to the dermis while the malignant cells can freely cross the epidermal-dermal junction and enter the epidermis. This begs the question of whether malignant cells are attracted to the epidermis by a specific antigen or if normal T-cells are inhibited from entering the epidermis. A review of the literature reveals only a single study examining malignant T-cell migration to various epidermal and dermal micro-compartments of the skin during various stages of CTCL. In this study, Gellrich *et al* demonstrated that all of the T-cells in the patch stage of CTCL are clonal in the epidermis and polyclonal in the dermis, the majority of T-cells in the plaque stage are clonal in both the epidermis and the dermis, and the majority of clonal T-cells in

the tumor stage are found in the dermis (22). The findings of this study are limited by the fact that samples were obtained from just five patients. Clearly, additional studies using a similar experimental design are needed to verify the reliability and generalizability of the conclusions of Gellrich *et al.* Specific determination of the localization of malignant T-cells during various stages of disease may prove to be of great benefit in elucidating the pathophysiology of CTCL and ultimately in improving diagnosis of this elusive diagnostic entity.

Molecular Diagnosis of Cutaneous T-cell Lymphoma

As previously mentioned, the clinical diagnosis of CTCL is quite difficult because of the diverse clinical and microscopic presentations of the disease. Fortunately, the introduction of molecular tests to identify T-cell clonality in the 1980's proved to be of great benefit in the diagnosis of CTCL. Although there are no diagnostic cell surface markers for malignant CTCL T-cells (23), molecular genetic methods in conjunction with histologic findings and clinical presentation are used to enhance the accuracy of diagnosis. In order to understand these tests, it is essential to understand the concept of clonal expansion and the structure of the T-cell receptor (TCR).

The immune system can be divided into two different parts: the innate immune system, comprised of macrophages, monocytes, and eosinophils, and the adaptive immune system, comprised of B- and T-cell lymphocytes. Lymphocytes are able to generate a wide variety of antigen-binding receptors, B-cell receptors and TCRs, through their unique ability to undergo germline gene rearrangement. T-cells

are further divided into $\alpha\beta$ T-cells or $\gamma\delta$ T-cells based upon the type of antigen-binding TCR they express on their cell surface membrane (24). $\alpha\beta$ T-cells comprise approximately 95% of the total number of T-cells found in the body, and the remaining 5% are $\gamma\delta$ T-cells. The majority of CTCL T-cells are CD4+ $\alpha\beta$ T-cells (25).

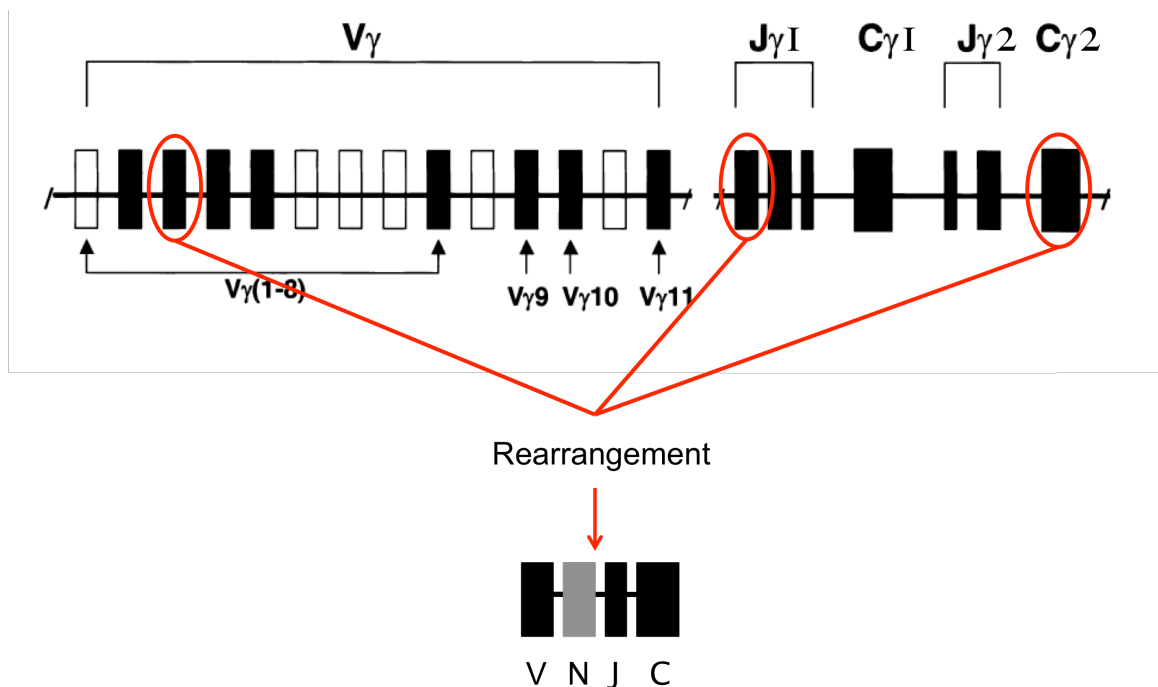
There are four TCR genes that encode the antigen-binding receptors: α , β , δ , and γ . All mature T-cells, even $\alpha\beta$ T-cells, carry the γ TCR gene which has undergone rearrangement early in T-cell development in the thymus (26). The δ TCR gene is the first to rearrange followed by γ TCR and half of the β TCR genes. The $\gamma\delta$ TCR genes can then be transcribed, and the cell may express the $\gamma\delta$ TCR. Alternatively, the δ TCR gene is deleted, and the α TCR and remainder of the β TCR genes are rearranged allowing for expression of the $\alpha\beta$ TCR (27, 28).

The γ TCR gene has a limited number of possible rearrangements compared to the $\alpha\beta$ TCR. There are 70 possible V region combinations compared to 2500 in the V region of the $\alpha\beta$ TCR gene (29). Approximately 3×10^6 possible $\alpha\beta$ TCRs and 5×10^3 possible γ TCRs can be generated through rearrangement of these regions (30). Although most peripheral T-cells express the $\alpha\beta$ TCR, they also possess the rearranged γ TCR gene (31). Consequently, given its relative simplicity compared to the $\alpha\beta$ TCR gene, the γ TCR gene is often used to detect polyclonal and clonal (malignant) T-cells.

The translated rearranged γ TCR genes produce a wide variety of receptors able to detect diverse antigens. The γ TCR germline gene, located on chromosome 7 (32), contains 14 (8 functional and 6 nonfunctional) variable (V), 5 joining (J), and 2

constant (C) regions (Figure 1). This gene undergoes rearrangement in the thymus early during lymphoid differentiation. During this process V, J, and C gene segments are randomly spliced together, and a various number of nucleotides are deleted and inserted between the V and J segments (33) creating T-cells with unique γ TCR gene sequences.

Figure 1: γ TCR germline gene rearrangement schematic. Variable (V), joining (J), and constant (C) genes are spliced together, and a random number of nucleotides (N) are inserted between V and J regions. The γ TCR germline gene rearrangement process creates a unique genetic sequence in mature T-cells and ensures the γ TCR will recognize a wide variety of antigens.



All lymphomas, including CTCL, are clonal expansions of malignant cells containing the same genetic sequence as the original progenitor cell. Since T-cells,

possess the unique ability to undergo gene rearrangement each individual T-cell population has its own unique genetic fingerprint. Analyzing the DNA sequences of TCRs can help identify malignant T-cell clonal population in lymphomas, including CTCL; detecting multiple copies of the same TCR sequence indicates the presence of a clonal population. β TCR gene analyses first detected malignant T-cell populations in histologically ambiguous CTCL (34). However, since the β TCR gene is more complex, polymerase chain reaction (PCR) of γ TCR gene rearrangements is currently the molecular method of choice used to identify clonality in CTCL (35).

PCR has the capability of synthesizing millions of copies of a specific DNA sequence (36) including the rearranged γ TCR gene. The principle of PCR involves the following steps: denaturation of the double-stranded DNA, annealing of the primers, and primer extension (37). Two oligonucleotide primers hybridize to both sense and antisense DNA strands and flank the sequence of interest. A thermostable DNA polymerase (*Taq* polymerase) extends the primers annealed to the DNA making a new template strand of DNA. The process is then repeated. Ultimately, an exponential expansion of specific DNA fragments is accomplished. Twenty cycles of PCR yields approximately one millionfold amplification (36).

The PCR products are often analyzed using capillary electrophoresis. Capillary electrophoresis is a high-resolution system that separates amplified PCR products by size. The system detects the number of nucleotides in the PCR product and generates fluorescence intensity data for each peak; this roughly correlates with the amplified PCR product concentration. The printout summarizing the results converts the PCR product size and concentration into peaks. The x-axis represents

the number of nucleotides, and the y-axis represents fluorescence intensity or the concentration of DNA. A polyclonal population of T-cells generates multiple short peaks. A monoclonal T-cell population amidst polyclonal reactive T-cells generates multiple short peaks surrounding one or more tall peaks.

It can be difficult to subjectively determine which peaks are *tall* and which are *short*, therefore, objective criteria for peak analysis have been determined. The height ratio between any peak of interest and the average height of the 2 flanking peaks is termed an Rn value and is often calculated to aid in clonality determination. Rn values <1.9 are unlikely to be monoclonal, Rn values between 1.9-3.0 are considered indeterminate, and Rn values >3.0 are thought to be monoclonal (38).

A limited number of PCR primers can be used to amplify most possible γ TCR gene rearrangements. The primers used in the PCR reaction are designed to anneal to the V and J regions of the γ TCR gene since these regions share a great deal of homology and the number of possible V and J rearrangements is restricted (39). Furthermore, PCR can only amplify DNA segments less than 500 base pairs (33). After rearrangement, the distance between the V and J regions is small enough to allow for amplification of the whole gene product.

PCR of the γ TCR gene is capable of detecting T-cell rearranged genes only; the nonrearranged germline γ TCR genes are too large to be amplified. Furthermore, polyclonal and monoclonal T-cells can be distinguished from one another because all T-cell populations contain different sequences. PCR analysis using automated high-resolution capillary electrophoresis can detect and report slight differences in

product size (38). If malignancy is present, PCR will detect multiple copies of DNA with the exact same size and electrophoresis will report a dominant PCR product.

Although most patients with CTCL produce monoclonal γ TCR PCR products, two clonal γ TCR PCR products are often reported (22, 40-42). Each chromosome in diploid T-cells has the ability to undergo gene rearrangement therefore creating a maximum of two different γ TCR sequences per cell, a process called biallelic gene rearrangement (22). CTCL is currently thought of as a monoclonal T-cell malignancy. However, in our experience, a minority of patients (approximately 10-15%) display oligoclonality.

The supernumerary clonal γ TCR gene rearrangements seen in CTCL have not been addressed in the literature. In fact, most dermatologists assume multiple clones are due to inflammation because the high sensitivity of the PCR method can cause certain lymphoproliferative diseases to produce false-positive clonality results. Some examples include: lymphomatoid papulosis (30), pityriasis lichenoides et varioliformis acuta (PLEVA) (43), chronic dermatitis, cutaneous lymphoid hyperplasia (33), drug-induced dermatoses (44), parapsoriasis (45), lichen planus (46), and lichen sclerosis (47). We contend these multiple clones represent multiple malignant T-cell populations.

Many questions regarding the pathophysiology of CTCL still remain unanswered. Most importantly, is CTCL a monoclonal or polyclonal malignancy? Moreover, why are malignant T-cells isolated to the epidermis during early stages of the disease? How does the migration pattern of T-cells change during different stages of disease? Are all the T-cells in pautrier microabscesses malignant and if so,

did they arise from the same progenitor cell? These are the unanswered questions which form the basis for the process of inquiry described here.

Statement of Aim and Hypothesis:

We hypothesize CTCL is a polyclonal T-cell lymphoma. We propose that the multiple clonal γ TCR sequences identified in the minority of CTCL patients represent multiple malignant clones. The first aim of this project is to determine the origin of the supernumerary (more than two) γ TCR clonal sequences routinely detected by conventional TCR PCR and capillary electrophoresis. The fundamental question is whether CTCL arises out of multiple sets of T-cells or whether a single malignant progenitor T-cell creates subsequent subclones. We hypothesize that the multiple clonal populations have arisen from a single progenitor T-cell.

We also hypothesize that clonal T-cells preferentially migrate to the epidermis in early CTCL and later migrate to the dermis. The second aim of this project is to determine what part of the epidermis and dermis the clonal CTCL T-cells migrate to during different stages of disease. We ultimately hope to clarify the pathophysiology of CTCL.

Methods:

Laser Capture Microdissection

IRB approval was obtained prior to the start of this project. Dr. Antonio Subtil in the Yale Department of Pathology selected CTCL biopsies for analysis determined by the Molecular Diagnostics Laboratory at Yale to have at least one

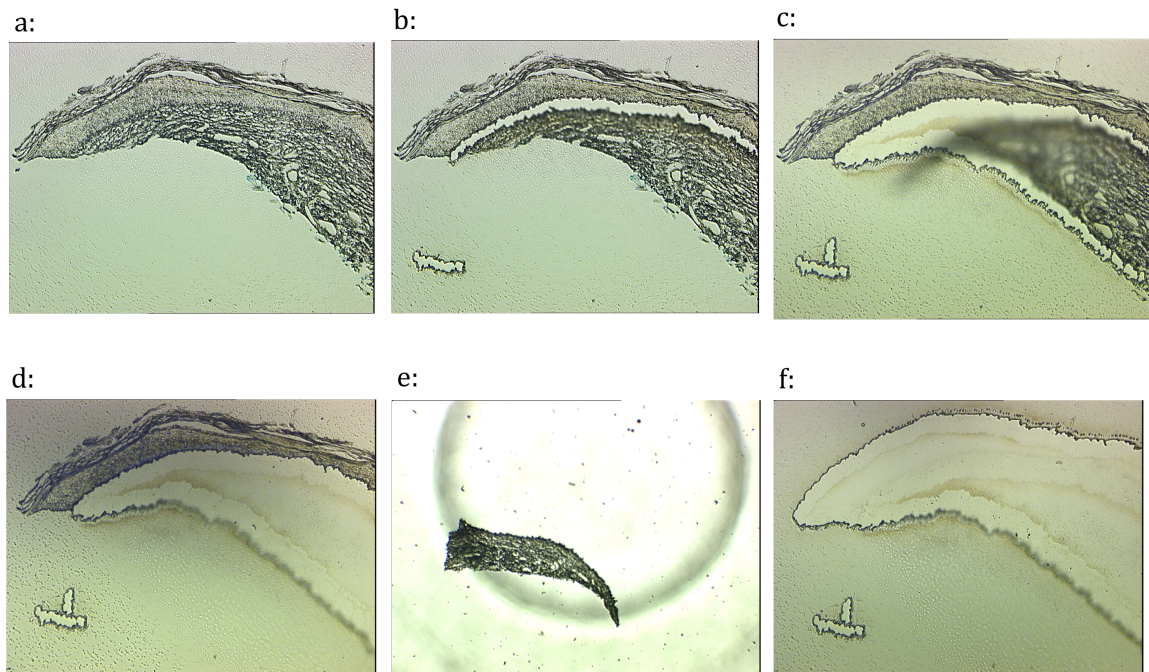
γ TCR clonal rearrangement. The Dermatopathology Laboratory had previously formalin-fixed and paraffin-embedded each sample.

Laser capture microdissection was used to remove the epidermis and dermis from selected biopsies. The biopsy samples were sliced into 6-micron thick sections and mounted on glass slides with a polyethylene naphthalate membrane (Figure 2a). Mr. Vincent Klump in the Dermatopathology Laboratory deparaffinized the samples by using the following protocol: 4 washes in 250 ml of xylene for 2.5 minutes, 2 washes in 250 ml of 100% ethanol for 1 minute, 2 washes in 250 ml of 95% ethanol for 1 minute, and 2 rinses in water. The slides were then air-dried overnight

Mr. Vincent Klump and Dr. Eleanor Knopp assisted with operating the 2006 Leica Laser Microdissection System. The laser was used to separate and to collect the entire epidermis and dermis of each biopsy specimen. First, the laser was used to dissect the epidermal-dermal junction (Figure 2b). The dermis (Figure 2c-e) followed by the epidermis (Figure 2f) was then removed. The laser-dissected cells were collected in 0.5 ml microcentrifuge tube filled with either 40 μ l of AE buffer or buffer ATL (Qiagen) and subjected to DNA extraction.

Figure 2: Dissection of the epidermis and dermis of CTCL biopsies using laser capture microdissection. **a.** The pre-cut skin biopsy. **b.** The biopsy is cut along the epidermal-dermal junction. **c.** The dermis is partially removed. **d.** The entire dermis is removed, and only the epidermis is visualized. **e.** The dermis is inside the

collection tube. **f.** The epidermis and dermis are removed from the slide.

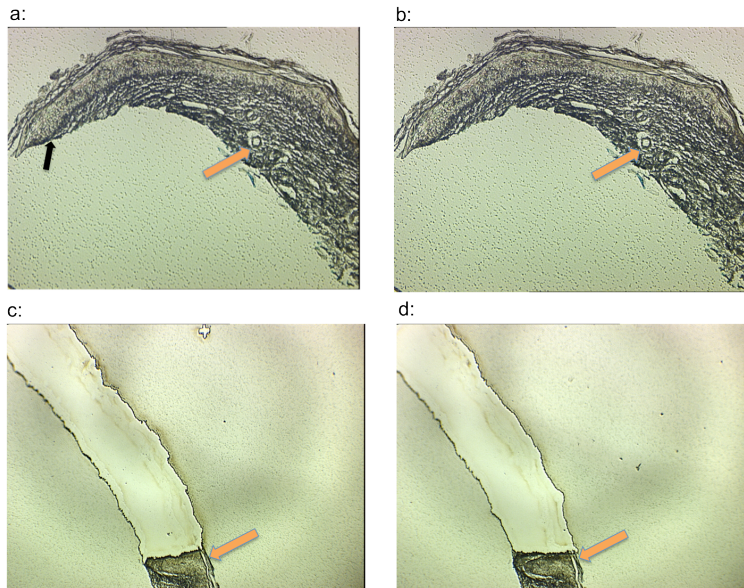


DNA Extraction

Two DNA extraction kits were used to extract DNA from the biopsy samples: QIAamp® DNA Micro kit and REPLI-g® FFPE Qiagen kit. Because the laser-dissected samples contained such a small amount of DNA, the REPLI-g® FFPE Qiagen kit was used not only to extract DNA from biopsies but also replicate whole genomic DNA. The amplified DNA could then serve as a sufficient template for PCR analysis. The QIAamp® DNA Micro simply extracts DNA and does not amplify genomic DNA. The REPLI-g® FFPE Qiagen kit was compared to the QIAamp® DNA Micro kit, the conventional DNA isolation kit used in the Yale Molecular Diagnostics Laboratory.

To ensure both kits compared the same amount of T-cell DNA for analysis, DNA extraction by both kits was performed on one biopsy specimen. Two sequential 6-micron thick slices were taken from one CTCL biopsy specimen. Anatomical landmarks such as vessels and hair follicles were located in both sequential slices and used to map laser dissection boundaries (Figure 3a-d). All tissue from the outer edges of each slice was dissected with the laser. Tissue was dissected up to an anatomical landmark located on both sequential biopsies. Dermis was then separated from epidermis at the epidermal-dermal junction. The epidermis was then removed. Both DNA extraction kits were used to extract DNA from epidermis and dermis from this one biopsy.

Figure 3: Histology of anatomical landmarks of the CTCL biopsy specimen used to capture similar amounts of T-cells for comparison of QIAamp® DNA Micro and REPLI-g® FFPE Qiagen kits. **a.** The epidermis and dermis of the 1st slice of CTCL biopsy specimen. The orange arrow points to a cross section of a vessel. The black arrow points to the epidermal-dermal junction **b.** The epidermis and dermis of the 2nd slice of CTCL biopsy specimen. The orange arrow points to a cross section of the same vessel seen in Figure 3a. **c.** The epidermis and dermis of the 1st slice of CTCL biopsy specimen has been removed up to the hair follicle (at the end of the orange arrow). **d.** The epidermis and dermis of the 2nd slice has been removed up to the same hair follicle (at the end of the orange arrow) also seen in the 1st slice.



The ligation enzyme, the FFPE enzyme, and REPLI-g Midi DNA polymerase from the REPLI-g® FFPE Qiagen kit were thawed on ice. The FFPE buffer and REPLI-g Midi reaction buffer were thawed at room temperature. Proteinase K and FFPE Lysis solution, 10x were kept at room temperature.

Tissue specimens were transferred from the laser capture dissection microcentrifuge tube to a stronger microcentrifuge tube. 10 μ l of FFPE Lysis solution (10x) and 90 μ l nuclease-free water were added to the tissue and incubated at 95°C for 10 min. Once the sample was at room temperature, 2 μ l of Proteinase K was added. The tissue was incubated at 60°C for 60 minutes and then at 95°C for 10 minutes. 10 μ l of lysed tissue was removed with care to avoid paraffin withdrawal and placed into a new microcentrifuge tube. The following reagents were mixed and added to the 10 μ l DNA sample: 8 μ l FFPE buffer, 1 μ l ligation enzyme, and 1 μ l FFPE enzyme. The mixture was incubated at 24°C for 30 minutes, at 95°C for 5

minutes, and then placed on ice. 29 μ l REPLI-g Midi reaction buffer and 1 μ l REPLI-g Midi DNA polymerase were added and incubated at 30°C for 2-8 hours. The sample was then incubated at 95°C for 10 min and stored at -20°C. The DNA concentration and purity was determined using a spectrophotometer. The final concentration of amplified DNA was diluted to 70 ng/ μ l with nuclease-free water.

On day one, 180 μ l of AE buffer from the QIAamp® DNA Micro kit was added to the microcentrifuge tube containing the sample and 40 μ l of AE buffer. 20 μ l of Protein Kinase was added to the tube, vortexed, and incubated overnight at 60°C. On day two, 200 μ l of Buffer AL was added to the tube, vortexed, and heated at 70°C for 10 minutes. 200 μ l of 100% ethanol was then added to the tube, vortexed, and incubated at room temperature for five minutes. The solution was briefly centrifuged and transferred to the QIAamp MinElute Column and centrifuged at 14,000 rpm for one minute.

The flow-through was discarded, and the column was transferred to a fresh 2 ml tube. 500 μ l of Buffer AW1 was added to the column and centrifuged at 14,000 rpm for one minute. The flow-through was discarded for a second time, and the column was transferred to another fresh 2 ml tube. 500 μ l of Buffer AW2 was added to the column and centrifuged again at 14,000 rpm for one minute. The flow-through was discarded for a third time, the column was transferred to another fresh 2 ml tube, and centrifuged at 14,000 rpm for three minutes to dry the column. The column was transferred to a fresh 1.5 ml tube and 20-40 μ l Buffer AE was added. The tube was incubated for five minutes at room temperature and then centrifuged

for one minute. The isolated template DNA concentration was measured using a spectrophotometer.

Polymerase Chain Reaction

PCR amplification of rearranged γ TCR genes was completed behind a plastic shield in a room in which PCR is the only experiment done. PCR was completed twice to ensure accurate results. Consensus primers (Table 1) in three multiplexes were used to amplify all potential γ TCR gene rearrangements (39). Non-fluorescently labeled PCR primers were used when amplified DNA was subjected to sequencing.

Table 1: γ TCR PCR primer sequences.

Primer Name	Primer Sequence
Vg1-8	5' FAM-ACC AGG AGG GGA AGG CCC CAC AG 3'
Vg9	5' FAM-AGA AAG GAA TCT GGC ATT CCG 3'
Vg10	5' FAM-AAT CCG CAG CTC GAC GCA GCA 3'
Vg11	5' FAM-GCT CAA GAT TGC TCA GGT GGG 3'
Jg12	5' ACC TGT GAC AAC AAG TGT TGT TC 3'
JgP12	5' AGT TAC TAT GAG CYT AGT CCC 3'

FAM = fluorescent label

Y= equal mixtures of T and C nucleotides

Each reaction was carried out in 200 μ l strip tubes using template DNA extracted from each biopsy specimen, 70 ng/ μ l of template DNA from tonsil cells, and 70 ng/ μ l template DNA from Jurkat cell lines. Jurkat cells are human leukemic

T-cell lines that were discovered in the 1980's by Steven Gillis and James Watson (48). Jurkat T-cells are clonal and possess V γ 1-8 and V γ 11 gene rearrangements; they are often used as positive controls when assessing malignancy.

The reaction mix consisted of 3.3 μ l 10X PCR Buffer II, 2.64 μ l MgCl₂ solution, 0.33 μ l of each 5mM nucleotide (dATP, dTTP, dCTP, and dGTP), 0.12 μ l AmpliTaq Gold Polymerase (all from Applied Biosystems), and 3 μ l 10 ng/ μ l template DNA or water. Primer sets Vg1-8, Vg9,10, and Vg11 were added to the reaction mix using 0.165 μ l of 100 μ M of each primer. Vg1-8 multiplex contained 22.125 μ l sterile water and the following primers: Vg1-8, Jg12, and JgP12. Vg9,10 multiplex contained 21.96 μ l sterile water and the following primers: Vg9, Vg10, Jg12, and JgP12. Vg11 multiplex contained 22.125 μ l sterile water and the following primers: Vg11, Jg12, and JgP12.

PCR amplification was carried out on a Biometra TGradient Thermal Cycler using the following conditions: 1 cycle at 94°C for 12 minutes, 40 cycles at 95°C for 1 minute, 60°C for 30 seconds, and 72°C for 30 seconds, followed by 1 cycle at 72°C for 30 minutes and then held at 4°C.

The sensitivity of using PCR and capillary electrophoresis to detect γ TCR gene rearrangements is approximately 92% (33, 38) compared to a sensitivity of only 59% using the southern blot technique (33). False negatives can result from sampling and technical error. Rarely, false negatives can result via analysis of lymphoid neoplasms with γ TCR genes that lack rearrangements (49), although >98% of lymphoid malignancies contain clonal gene rearrangements (30). False-

positive results can also be obtained when PCR amplifies a small sample.

Duplicating the PCR can ensure the perceived clonality is valid.

PCR Analysis

All patients' PCR products were analyzed with a 3130 ABI Prism capillary electrophoresis apparatus. 0.5 μ l ROX, 13 μ l Hidi, and 1 μ l template DNA sample were added to each well in a 96-well plate. Ms. Susan Bell and Ms. Vesna Meric loaded the samples and operated the capillary electrophoresis machine. Ms. Monica Talmor obtained the raw data from the machine. Dr. Jeffrey Sklar assisted with data interpretation.

Patient S1-S4's γ TCR PCR products were run on an 8% polyacrylamide gel. The gel was composed of the following: 42 g urea, 10 ml 10X TBE, 26.6 ml 30% Acrylamide Solution, approximately 100 ml distilled-deionized water, 35 μ l TEMED, and 700 μ l 10% APS. The PCR products were run at 296 volts for 2 hours. The gel was stained in 0.5 μ g/ml ethidium bromide in TE buffer for 20 minutes and viewed under UV light. The clonal bands were excised, transferred into separate tubes, incubated at 4° overnight in 20-40 μ l of autoclaved water, and subjected to sequencing.

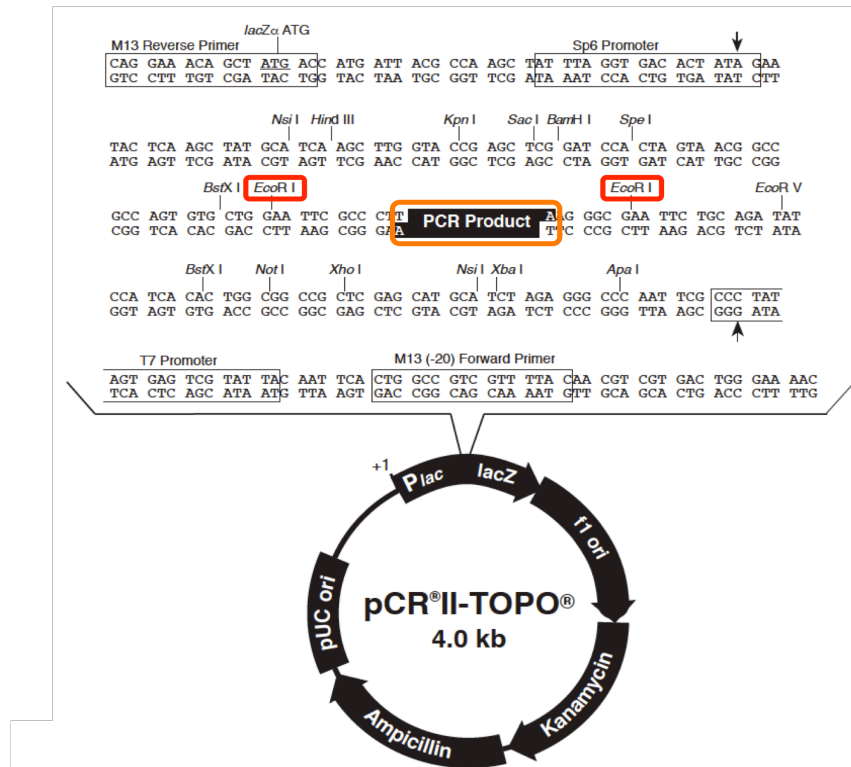
Sequencing

On day two, after overnight incubation in water, the samples were centrifuged and transformed into bacteria using the Invitrogen TOPO TA cloning kit.

4 μ l DNA gel solution, 1 μ l 1.2M NaCl, and 1 μ l vector were added to each tube. The mixture was incubated at room temperature for 40 minutes. 5 μ l of the solution was added to competent *Escherichia coli* (*E. coli*) bacterial colonies and incubated on ice for 30 minutes. The colonies were heat-shocked for one minute at 4°C and then transferred to ice for two minutes. 300 μ l of liquid agar was added to each tube and shook for one hour. 100 μ l of transformed bacteria were cultured overnight at 37°C on agar petri dishes with 80 μ l X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside).

X-gal is used in gene cloning to determine which bacterial colonies have incorporated the vector. The *lacZ* gene encodes β -galactosidase, an enzyme that cleaves X-gal into galactose and 5-bromo-4-chloro-3-hydroxyindole which is converted to a blue product. Bacterial colonies with a functional *lacZ* gene will synthesize β -galactosidase. *E. coli* do not have the *lacZ* gene, but the vector does. The PCR insertion site is located within the *lacZ* gene of the vector (Figure 4), therefore, a successful insertion of the PCR product into the bacterial vector will render the gene dysfunctional. The bacteria that uptake the vector that do not contain the PCR product insertion will grow blue colonies when incubated in the presence of X-gal because they have a functional *lacZ* gene. Bacterial colonies that have taken up the vector with the inserted PCR product do not have a functional *lacZ* gene, and therefore, do not cleave X-gal which produces a blue tint. Appropriately, these colonies are white and are used for further analysis.

Figure 4: Map of pCR®II-TOPO® and the sequence surrounding the TOPO® cloning site utilized in bacterial transformation. The EcoR1 restriction sites are circled in red, and the PCR product insertion site is circled in orange.



(http://www.genomex.com/vector_maps/pcriitopo_map.pdf)

On day three, two small white bacterial colonies with the same inserted clonal PCR products were selected for further analysis. The colonies were cultured overnight at 37°C in liquid LB agar enhanced with Ampicillin. On day four, the tubes were centrifuged at 3,000 RPM for 10 minutes. The supernatant was discarded, and 250 µl each of Buffer P1 and Buffer P2 mix were added. The tubes were shaken at room temperature for 10 minutes and then centrifuged. The contents were transferred to a column and centrifuged at 7,600 RMP for one minute. 700 µl of Buffer PB was added and again centrifuged at 7,600 RPM for one minute. 700 µl of

Buffer PE was added and centrifuged at 7,600 RPM for two minutes. The contents were transferred to a column, eluted with 140 µl of water, and centrifuged for 10 minutes.

The bacterial DNA was digested and analyzed on a gel to determine which colonies were successfully transformed. 6.5 µl DNA from each colony was digested with 1 µl 10X buffer, 0.5 µl EcoR1 endonuclease, and 2 µl deionized water. The tubes were incubated in 70°C water bath for 1 hour. The digested DNA was then subjected to electrophoresis in a 1.75% agarose gel at 120 volts for 25 minutes.

The DNA from the bacteria known to possess the PCR products was sent to W. M. Keck Facility at Yale University for sequencing. Sequencing alignment and similarity assessment was determined using the Basic Local Alignment Search Tool (BLAST) program.

Results:

Analysis of Epidermal and Dermal Clonal T-cells

A total of six biopsies (L1-L6) were used to study clonality in the epidermis and dermis of patients with CTCL using laser capture microdissection. Four patients were male and two were female whose ages ranged from 32-79 years and averaged 57.5 years (Table 2). Five patients were diagnosed with CTCL and one, the control, was given the histological diagnosis of squamous cell carcinoma (SCC). Various sizes of epidermal and dermal sections were successfully isolated in all biopsy specimens.

Table 2: Patient demographics.

Biopsy ID	Age	Gender	Diagnosis	Biopsy Date	DNA Concentration (ng/ μ l)
<i>Laser Micro-dissection</i>					<i>Epidermis/ Dermis</i>
L1	55	M	CTCL	9/8/2009	2.1 / 5.5
L2	54	M	CTCL	3/18/2008	4.8 / 1.8
L3	52	M	CTCL	10/19/2006	1.3 / 3.5
L4	32	F	CTCL	1/12/2006	1.6 / 0.4
L5	73	F	CTCL	10/20/2006	0 / 3.0
L6	79	M	SCC	1/19/2010	-
<i>Sequencing</i>					<i>Whole Biopsy</i>
S1	70	M	CTCL	2/22/2010	15.5
S2	60	M	CTCL	5/5/2010	22.3
S3	58	F	CTCL	5/27/2010	16.5
S4	60	M	CTCL	6/8/2010	30

SCC = Squamous cell carcinoma

DNA extraction and amplification using REPLI-g® FFPE Qiagen kit yielded 2830 and 3286 ng/ μ l of DNA in the epidermis and dermis, respectively. However, when the PCR products of the amplified DNA from the REPLI-g® FFPE Qiagen kit were analyzed on capillary electrophoresis, no polyclonal or clonal PCR products were detected. The conventional DNA extraction method was therefore used for the remaining experiments. DNA extraction using the QIAamp® DNA Micro kit yielded

on average 0-5.5 ng/ μ l of DNA from laser microdissected tissue and 15.5-30 ng/ μ l of DNA from whole biopsy specimens (Table 2).

Vg1-8 primer multiplex is known to yield 225-255 base pair PCR products; Vg910 primer multiplex yields 175-205 base pair PCR products; Vg11 primer multiplex yields 175-205 base pair PCR products (39). The Vg1-8 rearrangement PCR products ranged from 222.77- 251.58 base pairs ,and the Vg910 rearrangement PCR products ranged from 170.94-201.22 base pairs. Finally, the Vg11 gene rearrangements produced PCR fragments ranging from 184.31-186.47 base pairs.

The epidermal and dermal clonality results of five patients with CTCL and one with squamous cell carcinoma (SCC) are listed in Table 3. All five CTCL specimens revealed clonality confirmed by repeat PCR. Patient L1 exhibited two V γ 1-8 and two V γ 9,10 clonal rearrangements in both the epidermis and dermis. The clones in the epidermis and dermis had the same number of base pairs. Patient L2 had one epidermal V γ 1-8 clone and two V γ 9,10 clones within the epidermis and dermis. The epidermal and dermal V γ 9,10 clones had the same number of base pairs. Patient L3 had one epidermal and one dermal V γ 9,10 clone, but they differed in the number of base pairs. Patient L4 displayed one dermal V γ 1-8 and two V γ 9,10 clonal rearrangements, and patient L5 failed to produce any PCR products even after repeated PCR.

Table 3: Laser capture microdissection epidermal and dermal clonality results.

Biopsy ID	No. Base Pairs of Epidermal V γ 1-8 Clones	No. Base Pairs of Dermal V γ 1-8 Clones	No. Base Pairs of Epidermal V γ 9,10 Clones	No. Base Pairs of Dermal V γ 9,10 Clones	No. Base Pairs of Epidermal V γ 11 Clones	No. Base Pairs of Dermal V γ 11 Clones
L1	239 & 240	239 & 240	188 & 189	188 & 189	-	-
L2	240	-	188 & 189	188 & 189	-	-
L3	-	-	195	189	-	-
L4	-	240	-	195 & 200	-	-
L5	∅	∅	∅	∅	∅	∅
L6	251	-	-	-	-	-

∅ = no clonal or polyclonal PCR products detected
 - = no clonal PCR products detected

Unexpectedly, the SCC biopsy (L6), the control, displayed one V γ 1-8 rearrangement clone in the epidermis. Polyclonal T-cells with V γ 1-8, V γ 9,10, and V γ 11 rearrangements were scattered throughout the epidermis and dermis.

Analysis Supernumerary Clonal γ TCR Gene Rearrangements

A total of four biopsies (S1-S4) were used to study the oligoclonal γ TCR clonality because they were previously found to have multiple clonal PCR products. Three patients were male and one was female whose ages ranged from fifty-eight to seventy years and averaged sixty-two years (Table 2). Patient S1 had three clones, patient S2 had four clones, patient S3 had seven clones, and patient S4 had three clones (Table 4).

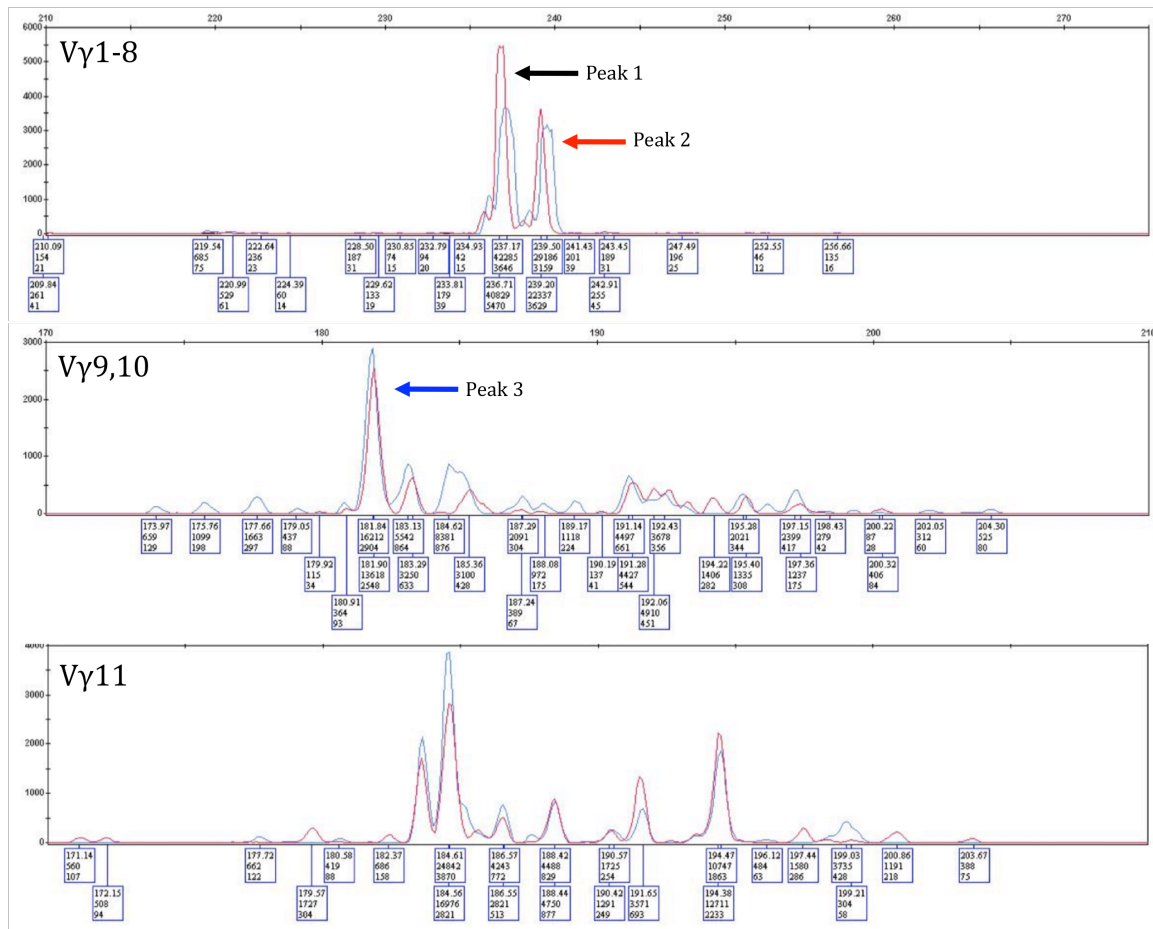
Table 4: Number of supernumerary clonal γ TCR gene rearrangements in patients with CTCL.

Patient	No.	No.	No.
	V γ 1-8 Clones	V γ 9,10 Clones	V γ 11 Clones
S1	2	1	0
S2	4	0	0
S3	5	1	1
S4	1	1	1

Capillary electrophoresis of patient S1's γ TCR PCR products revealed three clonal peaks (peaks one, two, and three) amidst polyclonal peaks (Figure 5). Peaks one and two had V γ 1-8 TCR gene rearrangements and estimated PCR fragment sizes of 237 and 239 base pairs, respectively. Peak three, a V γ 9,10 TCR gene rearrangement, had an estimated size of 181 base pairs. Peak one was 72.9 times taller than the tallest polyclonal peak. Peak two was 48.4 times taller than the tallest polyclonal peak. Peak three was 3.3 times taller than the tallest polyclonal peak. V γ 11 TCR gene rearrangements revealed only polyclonal PCR products.

Figure 5: Capillary electrophoresis of γ TCR gene rearrangement PCR products of patient S1. The x-axis represents PCR product size in number of base pairs. The y-axis represents the DNA concentration. The arrows point to clonal rearrangements

(peaks 1-3). $V\gamma 1-8$ has two dominant clones, $V\gamma 9,10$ has one dominant clone, and $V\gamma 11$ has no dominant clone.



Patient S2 had four $V\gamma 1-8$ TCR, one $V\gamma 9,10$, and no $V\gamma 11$ TCR clonal gene rearrangements. The $V\gamma 1-8$ TCR PCR products were 223, 231, 239, and 243 base pairs, and the $V\gamma 9,10$ TCR PCR product was 199 base pairs. All clonal peaks were at least three times taller than the tallest polyclonal peak. There were no $V\gamma 11$ TCR gene rearrangements.

Patient S3 had five $V\gamma 1-8$ TCR, one $V\gamma 9,10$, and one $V\gamma 11$ TCR clonal gene rearrangements. The $V\gamma 1-8$ TCR PCR products were 236, 238, 240, 241, and 247

base pairs. The V γ 9,10 TCR PCR product was 189 base pairs, and the V γ 11 PCR clone was 183 base pairs. All clonal peaks were at least three times taller than the tallest polyclonal peak.

Patient S4 had one V γ 1-8 TCR, one V γ 9,10, and one V γ 11 TCR clonal gene rearrangements. The V γ 1-8 TCR PCR product was 231 base pairs, the V γ 9,10 TCR PCR product was 175 base pairs, and the V γ 11 PCR product was 195 base pairs. All clonal peaks were at least three times taller than the tallest polyclonal peak.

The γ TCR PCR products were subjected to polyacrylamide gel electrophoresis (Figure 6). Patient S1's V γ 1-8 PCR products revealed two distinct clonal bands approximately 250 base pairs in size amongst a smear of polyclonal DNA; the V γ 9,10 PCR products revealed one distinct clonal band approximately 200 base pairs in size amongst a smear of polyclonal DNA (Figure 6a). Although patient S2, S3, and S4s' clonal γ TCR DNA bands were visible, they were not strong or sharp enough to excise for sequencing (Figure 6b), therefore, further characterization was prohibited. Only patient S1's clonal PCR products were further characterized.

Figure 6: Polyacrylamide gel electrophoresis of γ TCR PCR products of patients with supernumerary clonal γ TCR gene rearrangements. **a.** Patient S1's results. The red box represents two V γ 1-8 clonal rearrangements, and the orange box represents one V γ 9,10 clonal rearrangement. **b.** Patients S2-4s' results. The red boxes represent V γ 1-8 products, the orange boxes represent V γ 9,10 products, and the yellow boxes represent V γ 11 products. The clonal DNA bands are not separated

enough to extract and sequence. The tonsil lane has contamination with the size standard and Jurkat DNA. The white arrow points to primer dimers.

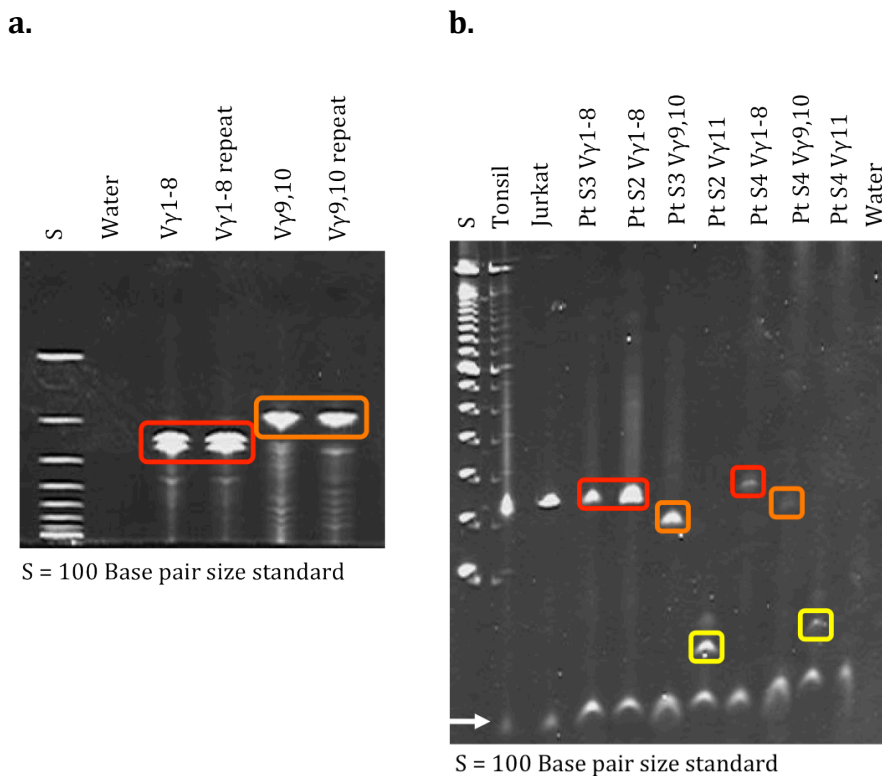
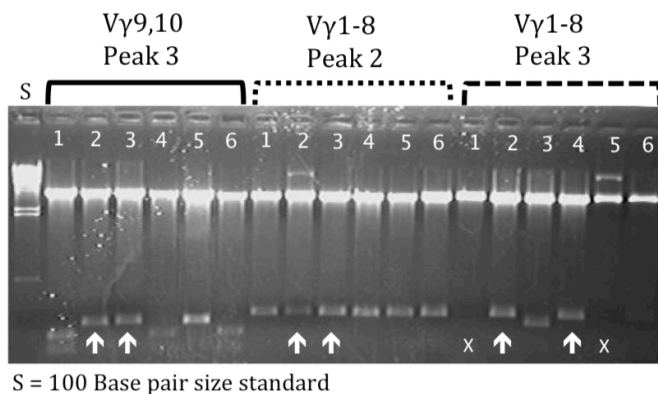


Figure 7 displays the success of the transformed bacterial colonies with patient S1's PCR products. Successfully transformed colonies displayed 2 bands (the vector and the spliced out PCR product) after exposure to EcoR1. Two bacterial colonies with amplified vector and PCR product DNA from each clonal DNA of patient S1 were selected for sequencing.

Figure 7. Agarose gel electrophoresis of the DNA of bacterial colonies containing clonal γ TCR PCR products of patient S1. These results determine the success of the transformation. Successfully transformed colonies display two bands (the vector and the spliced out PCR product) after exposure to EcoR1 endonuclease. The

unsuccessfully transformed colonies only showed one band (denoted by the X's) because the spliced out DNA product does not contain a PCR product, and therefore, is too small to create a visible band. The arrows point to the clones selected for sequencing.



The DNA sequences of patient S1's peaks one, two, and three were successfully determined (Table 5). The V γ 1-8 rearrangement DNA sequences of peaks one (234 base pairs) and two (237 base pairs) were 86% similar (Figure 8a). Peak three, the V γ 9,10 sequence (184 base pairs) was 19% similar to the 237 base pair V γ 1-8 sequence (Figure 8b) and 17% similar to the 234 base pair V γ 1-8 sequence (Figure 8c).

Table 5: Nucleotide sequences of the clonal V γ TCR PCR products of patient S1.

Sample	Primer Sequence	No.	*Estimated
		Base Pairs	No. Base Pairs

V γ 1-8 Peak 1	5' ACC AGG AGG GGA AGG CCC CAC AGC ATC TTC CAT ACT ATG ACC CCT ACT ACT CCA GGG TTG TGT TGG AAT CAA GAA TCA GTA GAG GAA AGT ATT TTA CTT ATG CAA GCA TGA GGA GGA GCT GGA AAT TGA TAC TGC AAA ATC TAA TTG AAA ATG ATT CTG GAT CTA TTA CTG TGC CAC CTG GGA CAG GCG TCT GGG AAA CTC TTT GGC AGT GGA ACA ACA CTT GTT GTC ACA GGT 3'	234	237
V γ 1-8 Peak 2	5' ACC AGG AAG GGG AAG GCC CCA CAG CGT CTT CTG TAC TAT GAC TCC TAC AAC TCC AGG GTT GTG TTG GAA TCA GGA ATC AGT CGA GAA AAG TAT CAT ACT TAT GCA AGC ACA GGG AAG AGC CTT AAA TTT ATA CTG GAA AAT CTA ATT GAA CGT GAC TCT GGG GTC TAT TAC TGT GCC ACC TGG GAT AGT TAT TAT AAG AAA CTC TTT GGC AGT GGA ACA ACA CTT GTT GTC ACA GGT 3'	237	239
V γ 9,10 Peak 3	5' AGA AAG GAA TCT GGC ATT CCG TCA GGC AAA TTT GAG GTG GAT AGG ATA CCT GAA ACG TCT ACA TCC ACT CTC ACC ATT CAC AAT GTA GAG AAA CAG GAC ATA GCT ACC TAC TAC TGT GCC TTG TGG GAG CCG AGG GGA TTA TAA GAA ACT CTT TGG CAG TGG AAC AAC ACT TGT TGT CAC AGG T 3'	184	181

*Estimation determined by capillary electrophoresis.
(Primer sequences are bolded)

Figure 8: Alignment of the similar sequences of the clonal V γ TCR PCR products.

The horizontal lines between the bases indicate similarity between sequences and spaces represent dissimilarity. The numbers specify the number of base pairs at that point in the sequence. **a:** The black arrows points to the sequence of the first

clonal V γ 1-8 peak, and the red arrow points to the sequence of the second V γ 1-8 clonal peak. The sequences are 86% similar. **b:** The black arrow points to the sequence of the first V γ 1-8 clonal peak, and the blue arrow points to the sequence of the V γ 9,10 clonal peak. The sequences are 19% similar. **c:** The red arrow points to the sequence of the second V γ 1-8 clonal peak, and the blue arrow points to the sequence of the V γ 9,10 clonal peak. The sequences are 17% similar.

a:

→	1	ACCAGG-AGGGGAAGGCCCCACAGCATCTTCCATACTATGACCCCTACTACTCCAGGGTT	59
→	1	ACCAGGAAGGGGAAGGCCCCACAGCGTCTTCTGTACTATGACTCCTACAACCTCCAGGGTT	60
→	60	GTGTTGGAATCAAGAATCAGTAGAGGAAAGTATTTTACTTATGCAAGCATGA-GGAGGAG	118
→	61	GTGTTGGAATCAGGAATCAGTTCGAGAAAAGTATCATACTTATGCAAGCA-CAGGGAAGAG	119
→	119	-CTGGAAATTGATACTGC AAAATCTAATTGAAAATGATTCT-GGATCTATTACTGTGCCA	176
→	120	CCT-TAAATTTATACTGGAAAATCTAATTGAACGTGACTCTGGGGTCTATTACTGTGCCA	178
→	177	CCTGGGACAG-GCGTCTGGGAAACTCTTTGGCAGTGGAACAACACTTGTGTGCACAGGT	234
→	179	CCTGGGATAGTTATTATAAGAAAATCTTTGGCAGTGGAACAACACTTGTGTGCACAGGT	237

b:

→	195	GAAACTCTTTGGCAGTGGAACAACACTTGTGTGCACAGGT	234
→	145	GAAACTCTTTGGCAGTGGAACAACACTTGTGTGCACAGGT	184

c:

→	191	ATTATAAGAAACTCTTTGGCAGTGGAACAACACTTGTGTGCACAGGT	237
→	138	ATTATAAGAAACTCTTTGGCAGTGGAACAACACTTGTGTGCACAGGT	184

Discussion:

Epidermal and Dermal Clonal T-cells

Lymphomas are clonal malignancies of lymphatic cells of the immune system. Only 5-7% of all lymphomas arise from T-cells (30), and just 2% of all lymphomas are cutaneous in nature (50). Interestingly, although the majority of lymphomas

and leukemias arise from B-cells, lymphomas presenting in the skin typically consist of T-cells (17). CTCL's are malignancies of CD4+ helper T-cells that acquire cutaneous lymphoid antigen (CLA) on their surfaces. CLA functions as a skin-homing receptor guiding the malignant cells to the skin early in the disease process (51). As mentioned previously, skin-homing T-cells migrate to the epidermis during early stages of CTCL. Elucidating the migration patterns of clonal T-cells in CTCL may help to decipher the pathophysiology of this complicated disease. Furthermore, studying T-cell migration patterns during different stages of CTCL may lead to the development methods which permit earlier detection.

Only one study has been published analyzing T-cell micro-compartmentalization in CTCL; Gellrich *et al* used laser capture microdissection, γ TCR PCR, and sequencing to study the clonality of individual T-cells in the epidermis and dermis of five patients with CTCL of various stages (22). They showed that in early stages of CTCL, the majority of the epidermal T-cells are clonal. As the disease progresses, the clonal T-cells migrate from the epidermis to the dermis. Although this study verified malignant T-cell epidermotropism in early stages of CTCL, only five patients were analyzed. We sought to perform a similar study with a greater number of participants to evaluate the reliability and generalizability of their findings.

Normal, non-inflamed skin does not have intraepidermal lymphocytes. For some unknown reason, malignant CTCL T-cells have the unique ability to traverse the epidermal-dermal junction during early stages of disease while the polyclonal normal T-cells remain confined to the dermis. It is unknown whether the malignant

T-cells are attracted to the epidermis by an unknown antigen or if normal T-cells are inhibited from entering the epidermis by an unidentified mechanism which malignant T-cells somehow escape.

An analysis of γ TCR clonality in the epidermis and dermis of CTCL specimens was performed with the result that most patients displayed epidermal and dermal clonality. Patient L2 exhibited V γ 1-8 clonality in the epidermis without dermal involvement; however, two V γ 9,10 clones were both in the epidermis and dermis. Since the epidermis and dermis of patient L2 displayed two different clones with the same number of base pairs, the four clones probably represent two sets of malignant T-cells that have begun to metastasize. Interestingly, since the V γ 1-8 clonal T-cells in this patient seem to be isolated to the epidermis, and the V γ 9,10 clonal T-cells appear to be losing affinity for the epidermis and migrating to the dermis, this may indicate that V γ 9,10 clonal T-cells are likely to become metastatic sooner than V γ 1-8 clonal T-cells.

Patients L1 and L3 both exhibited epidermal and dermal clonality. These biopsies were probably taken during an intermediate stage of disease progression when the malignant T-cells lost affiliation for the epidermis and traveled to the dermis. Only one CTCL biopsy, L4, showed isolated clonal T-cell dermal infiltration without epidermal involvement, suggesting that the sample was taken during a late stage of disease.

Although clinical correlation to the CTCL biopsies analyzed in this study was not possible, a seminal finding of this study is that it is possible to detect clonality in epidermis alone. Furthermore, the same clonal population was detected in the

epidermis and dermis of some patients. The PCR test for clonality has variable detection rates based upon the stage of CTCL (52, 53). Specifically, the identification of γ TCR PCR clones is associated with an increase in histopathological score system developed by Guitart *et al* (54). Dominant clones were found in only 51% of samples with a score of less than 5 (early disease) and 92% of samples with a score equal to or greater than 5 (late disease) (55). It is possible there are not enough T-cell clones during early stages of the disease to ensure proper detection. However, since the epidermal-dermal junction seems to act as a filter for malignant T-cells, testing for clonality in the epidermis only may enhance the ability to detect malignancy, especially in early cases when the concentration of malignant cells is low. PCR and electrophoresis can detect a dominant clone as long as the clonal population is at least 0.1-1.0% of the total cell population (9); therefore, if the polyclonal T-cells in the dermis are removed and the clonal T-cells in the epidermis are isolated and analyzed for γ TCR clonality, they will encompass a larger percentage of the total T-cell population.

Interestingly, no $V\gamma 11$ clonal rearrangements were detected. However, polyclonal $V\gamma 11$ clonal rearrangements were detected in some patients. Although the admittedly small sample size of four limits our ability to draw conclusions, it is possible malignant T-cells have a preference for $V\gamma 1-8$ and $V\gamma 9,10$ rearrangements. Conversely, the $V\gamma 1-8$ primer multiplex can amplify eight possible V genes, and the $V\gamma 9,10$ primer multiplex can amplify two possible variable genes. The $V\gamma 11$ primer multiplex, on the other hand, can only amplify one possible V gene. Statistically, T-cells are less likely to have the $V\gamma 11$ rearrangement. Alternatively, the Molecular

Diagnostics Laboratory at Yale School of Medicine has reported difficulty in PCR amplification of the V γ 11 gene rearrangement. However, since polyclonal V γ 11 rearrangements were detected in some biopsy samples, this indicates the V γ 11 PCR was indeed successful.

Unexpectedly, the biopsy of a patient with SCC displayed γ TCR clonality in the epidermis. An inflammatory response is often detected in SCC (56), but previous studies have found this response to be polyclonal (nonmalignant) in nature (33). However, a case report of a patient with SCC was found to have an atypical lymphocytic infiltrate consistent with Lyp (a CTCL variant) was published (57). This report along with our clonality results suggest there may be an association with a subset of SCCs and CTCL or its variants.

There are myriad technical difficulties associated with laser capture microdissection. Obtaining equal sections of epidermal and dermal dissected tissue is a technical challenge, while appropriate preservative solutions must be chosen in advance to avoid combustion secondary to the heat generated by the laser. Furthermore, since laser capture was performed using unstained biopsy specimens, it is unknown whether or not dermal projections into the epidermis contaminated the laser-captured epidermal tissue. Conversely, it is not known whether or not epidermal projections into the dermis contaminated the dermal tissue. These potential confounds may produce unreliable results. Another problem encountered was the failure of some biopsies to produce any PCR products. Patient L5's biopsy did not reveal any clonal or polyclonal T-cell DNA. However, this biopsy was excised and processed in 2006. It is possible this archival specimen underwent DNA

degradation as tissue stored for more than five years has been shown have significantly reduced PCR amplification (58).

The QIAamp® DNA Micro kit was ultimately used for DNA isolation of laser capture microdissected biopsies. Although the DNA extraction using the REPLI-g® FFPE Qiagen kit yielded 2830 and 3286 ng/ μ l of DNA, an average of 1,112 times the concentration of DNA extracted by the QIAamp® DNA Micro kit, the products failed to produce viable DNA. PCR products were not detected on capillary electrophoresis. The high concentration of DNA detected by spectrometry was likely due to individual nucleotides and not whole genomic DNA.

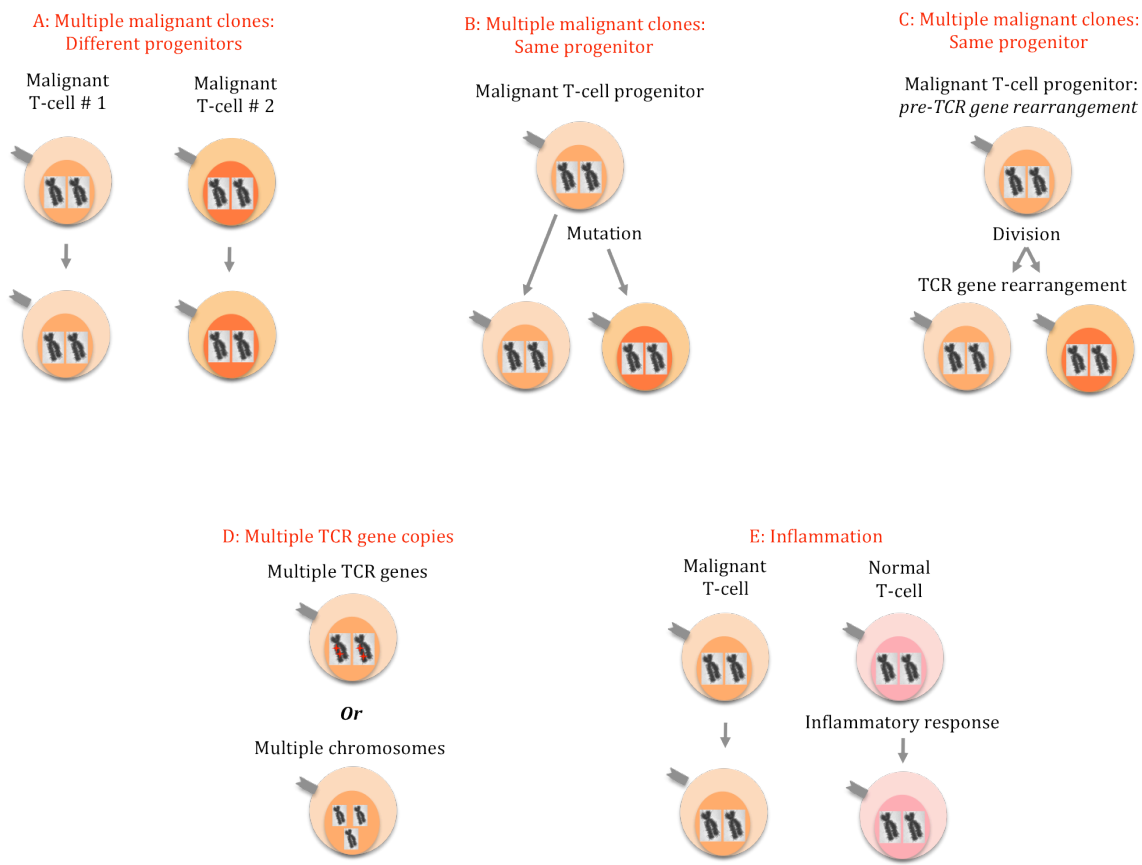
Supernumerary Clonal γ TCR Gene Rearrangements

CTCL is generally considered a monoclonal T-cell neoplasm (59). Detection of one dominant monoclonal γ TCR gene rearrangement sequence is expected if one allele has undergone rearrangement and the other has not or if both alleles have undergone the exact same gene rearrangement. Two dominant monoclonal γ TCR gene sequences are found if biallelic gene rearrangement occurs when both alleles have undergone separate γ TCR gene rearrangement in the original tumor progenitor cell. In this scenario, although two clonal sequences are detected, the neoplasm is still monoclonal. However, we detected supernumerary (more than two) clonal sequences in a minority of patients with CTCL. Although supernumerary clonality in CTCL is underreported, oligoclonality in angioimmunoblastic T-cell lymphoma has been described (60). Based on our

results and evidence of other oligoclonal T-cell lymphomas, we propose that CTCL can be a polyclonal neoplasm.

The supernumerary peak phenomenon can be explained by many theories. The first involves our hypothesis that CTCL can be a polyclonal neoplasm as it may involve more than one malignant T-cell clone. If true, the fundamental question is whether CTCL T-cells arise from multiple, separate T-cells responding to a common stimulus (Figure 9a) or whether the clones are daughter cells of a common malignant T-cell progenitor.

Figure 9: Possible theories explaining the supernumerary clonal γ TCR phenomenon in CTCL.



Our γ TCR clonality results show that some supernumerary clonal sequences differ in size only by a few base pairs. The most parsimonious explanation for these results is that multiple malignant T-cell populations arise from a common malignant progenitor; as the disease progresses, the primary malignant T-cell population could develop mutations (insertions or deletions) due to its genetic instability thereby producing a second malignant population with only a slightly different PCR sequence (Figure 9b). To test this hypothesis, we sequenced the clonal PCR products in one patient. One would expect the genetic sequences between the clones with almost the same number of base pairs to be quite similar. However, upon sequencing three monoclonal PCR products of one CTCL patient, we found this not to be the case. The sequences of the two $V\gamma$ 1-8 rearrangements were 86% similar. The $V\gamma$ 9,10 sequence was 17% similar to the 234 base pair $V\gamma$ 1-8 sequence and 19% similar to the 237 base pair $V\gamma$ 1-8 sequence. Given this degree of dissimilarity in genetic sequences, the explanation that a second malignant T-cell population is generated from a parent cell as a result of a simple mutation is unlikely. Nonetheless, this sequencing data from a single patient does not rule out the theory that CTCL is a polyclonal T-cell malignancy.

The supernumerary clonal γ TCR gene rearrangement phenomenon may still be explained by the theory that more than one T-cell clone is present in these patients' CTCL lesions, however, the details become more complex. Combining our sequencing results with this theory, one can imagine a malignant progenitor cell in which both γ TCR alleles had not yet undergone rearrangement. The malignant progenitor T-cell in germline configuration would divide, replicate, and undergo

mono- or bi-allelic gene rearrangement therefore producing multiple malignant T-cell populations (Figure 9c). In this scenario, the malignant T-cells all arise from the same progenitor cell and are presumably stimulated by the same antigen, but differ in their γ TCR sequences.

Prior publications have indicated a minority of CTCL T-cells do not have rearranged γ TCR genes. Specifically, less than 2% of lymphoid malignancies do not contain clonal γ TCR gene rearrangements (30) and reports of γ TCR germline configurations in CTCL biopsies have been published (61). These non-rearranged T-cells could represent the malignant progenitor T-cells that have not undergone γ TCR gene rearrangement. Furthermore, Ralfkiaer *et al* reported β TCR germline (nonrearranged) gene configuration in four patients with histologically verified CTCL and in patients with clinically suspicious CTCL but histologically nondiagnostic lesions. Conversely, they reported that aggressive variants or advanced stages of CTCL revealed β TCR clonal rearrangements (62). This suggests that CTCL T-cells undergo TCR rearrangement as the disease progresses and supports the theory that the malignant progenitor T-cell is one in which both γ TCR alleles have not yet undergone rearrangement. However, the fact that rearranged β TCR clonality is not detectable during early stages of CTCL may simply indicate the malignant population is too small to be detected (59).

Alternatively, the supernumerary clones may not represent clonal T-cell populations; they may arise from extra copies of the rearranged γ TCR gene within each malignant T-cell (Figure 9d). CTCL T-cells have been shown to have chromosomal abnormalities (63-65). Either each cell contains multiple

chromosomes with the γ TCR gene or they may contain multiple copies of the γ TCR gene on one chromosome. This explanation is less likely as up to six clones have been detected which indicates six copies of differently rearranged γ TCR genes exist in one cell. It is difficult to imagine a mechanism responsible for creating such a high number of copies of different gene sequences in one cell. Furthermore, the clones have been reported to change from one PCR product size to another based on the date and location the biopsy was taken (53). This observation suggests the first biopsy analyzed contains a certain population of malignant cells, and the second biopsy contains another population of malignant cells. It is unlikely that the number of γ TCR genes is changing within each cell.

A fifth theory with broad support is that the extra peaks represent a benign, T-cell inflammatory response often seen in malignancy (Figure 9e). Benign dermatoses are known to display T-cell clonality. The normal T-cell population in these conditions has responded appropriately to antigen stimulation, undergone division, and become the dominant T-cell clone amongst the other normal polyclonal inflammatory T-cells. Although clonal T-cell populations have been detected in benign conditions, evidence shows these patients with reactive T-cells might be at a greater risk of developing CTCL (33, 59). These clonal T-cells in benign dermatoses may have malignant properties and detection of clonality may be a marker for future malignancy. Furthermore, patients with *clonal dermatitis* with progression to CTCL have the same clone present in both the CTCL and dermatitis lesions (59). Of 103 patients diagnosed with non-CTCL, 18% had detectable clonality in a skin biopsy, however, 27% of these patients were eventually

diagnosed with CTCL (66). Furthermore, several studies have shown that CTCL-associated diseases arise as subclonal derivatives of the original tumor (25, 67). Patients with T-cell clonality detected in benign disease warrant close follow-up to ensure the patient does not develop CTCL or one of its variants.

This inflammation theory represents an implausible explanation for the observed monoclonality because a monoclonal peak indicates the corresponding T-cell population has greatly outnumbered the surrounding T-cell populations. A normal T-cell inflammatory response mimicking the degree of cellular concentration seen in malignancy is unlikely. These hyper-reactive cells could arguably be labeled as tumor cells because patients with reactive T-cells in dermatoses might be at a greater risk of developing CTCL (as mentioned previously). Furthermore, biopsy specimens that are γ TCR PCR negative have more moderate to severe inflammation compared to PCR positive CTCL specimens (52). If the supernumerary clonal peaks were a result of inflammation, one would expect the specimens with moderate to severe histologically visible inflammation to display oligoclonal peaks.

It is also possible the extra peaks result from a technical issue inherent to the capillary electrophoresis process. The device utilized in this process detects base pair number and fluorescent intensity associated with each PCR product size. If, by chance, two different non-malignant T-cell populations have the same number of nucleotides in the rearranged γ TCR gene, the tall peak may be recorded and mistaken for a clonal population. However, this is less unlikely given that the device reports PCR product size to the hundredth decimal.

Another technical issue that may be responsible for creating the extra clonal peaks is inherent to PCR. Sometimes, for unknown reasons during PCR cycling one template DNA strand is overamplified relative to all other sequences, resulting in a monoclonal peak on electrophoresis. This can be ruled out by duplicating each PCR reaction; if the monoclonality is genuine, it should be reproducible.

Future Studies

Regardless of the mechanism responsible for creating the multiple clonal γ TCR gene PCR products, additional studies are needed to clarify their significance. Laser capture microdissection is a technology that may assist with deciphering this mystery. Since the malignant T-cells are isolated to the epidermis in early stages of CTCL and the non-malignant T-cells are isolated to the dermis, dissecting the T-cells from the epidermis in patch stage CTCL and assessing γ TCR clonality may clarify whether or not the clones represent malignant T-cells. If there are supernumerary clones in the epidermis and not in the dermis, presumably the clones are not a result of a benign inflammatory response and are likely due to malignancy.

One could further study the theory that malignant CTCL T-cells arise from a progenitor cell in which both γ TCR alleles had not yet undergone rearrangement by using laser capture microdissection to isolate single T-cells which appear to be microscopically atypical and presumed to be malignant. Sequencing the γ TCR genes of these T-cells to determine if they are in the germline configuration might help to identify the progenitor cell. Additionally, since female X chromosomes undergo random X-inactivation in each cell, determining the X-inactivation patterns in

malignant T-cell might determine if the malignant cells arise from a common progenitor. If the supernumerary clonal malignant CTCL T-cells in women all have the same X-inactivation pattern, one could argue they all arose from a common progenitor.

The δ TCR gene rearranges before the γ TCR (27, 28). Studying the δ TCR clones in patients with supernumerary clonal γ TCR genes may also establish whether or not multiple clones arise from the same progenitor. If the CTCL patients with supernumerary clonal γ TCR gene rearrangements display the same δ TCR gene rearrangement, this would indicate the multiple clones had arisen from one progenitor cell after δ TCR gene rearrangement. However, if the malignant cells are $\alpha\beta$ T-cells, the δ gene may be deleted, and this experiment would not provide any useful data.

The multiple γ TCR gene copies theory is unlikely, but needs to be ruled out. Fluorescent in situ hybridization (FISH) with probes hybridizing to DNA flanking the γ TCR gene would determine the number of γ TCR genes within each cell. It would be difficult to design probes large enough to directly anneal to the γ TCR gene because each DNA sequence is different as a result of rearrangement. Therefore, using probes flanking the gene is more feasible.

The specificity of PCR analysis in the diagnosis of CTCL is often achieved by performing PCR on biopsy specimens taken from two anatomically distinct sites. Multiple lesions from the same patient typically have identical monoclonal T-cell PCR products (61). However, there have been reports of the dominant clones changing from one clone to another (53). Identical γ TCR gene rearrangements in

two distinct biopsy sites were found in approximately 80% of CTCL patients; of the patients with inflammatory diseases, none had an identical clone in both biopsy specimens (68). Comparing the clonality of multiple biopsy sites of patients with supernumerary peaks might help to elucidate whether or not the additional clones are due to inflammation. If inflammation is indeed the cause of additional clonal γ TCR gene rearrangements, the clones would differ at each biopsy site.

Conclusion

CTCL is widely considered to be a monoclonal malignancy amongst the medical community. However, our research shows that CTCL biopsies can display oligoclonality. Elucidating the origin of the supernumerary monoclonal peaks in the γ TCR gene rearrangement PCR test to detect CTCL is of vital importance because these additional clones may prove to be clinically relevant and yield some insight into the etiology of this disease. Furthermore, an understanding of this oligoclonality is of great value in providing accurate interpretation of the results of a test used routinely in the diagnosis of CTCL. This last point is of particular importance, given that clinicians use the positive or negative results of this test to aid in the differentiating benign disease from cancer, a diagnosis which carries great weight. The results of this study, rather than providing clear answers to these questions, underscores the point that the objective diagnosis of CTCL via such tests is not as straightforward as initially hoped.

Our research demonstrates that the supernumerary clonal γ TCR gene rearrangements detected in CTCL do not represent clonal daughter T-cell

populations derived from initial mutated progenitor T-cell populations. However, the question still remains as to whether CTCL arises out of multiple sets of T-cells or from a single progenitor T-cell. If these extra monoclonal peaks are indeed due to multiple malignant T-cell populations arising from a progenitor cell that has failed to undergo gene rearrangement, then this will transform the way clinicians think about and perhaps even approach the treatment of CTCL. Nonetheless, deciphering the supernumerary monoclonal peaks in CTCL promises to shed light on what has proven to be an elusive and ambiguous disease entity.

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