High Frequency of t(14;18) Translocation in Salivary Gland Lymphomas from Sjögren's Syndrome Patients

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Summary

Sjögren's syndrome (SS) is a chronic autoimmune disorder characterized by lymphocytic infiltration of the salivary and lacrimal glands. These patients have a markedly increased frequency of developing non-Hodgkin's lymphoma in their salivary glands and cervical lymph nodes. Translocations of proto-oncogene bcl-2 t(14;18) were observed in five of seven SS-associated lymphomas by Southern blot analysis. Using primers specific for chromosomes 14 and 18, translocation of the proto-oncogene bcl-2 was detected by polymerase chain reaction (PCR) in all five lymphomas positive by Southern blot analysis. Among SS patients lacking clinical evidence of coexistent lymphoma, no bcl-2 translocations were detected in 50 consecutive salivary gland biopsies. Of particular interest, pre-lymphoma biopsies were available from the seven SS patients who subsequently developed lymphoma and these DNA samples lacked detectable t(14;18) translocations even though they exhibited oligoclonal rearrangements of their immunoglobulin genes. We conclude that the great sensitivity of PCR can help us in detecting early onset of lymphoma in SS patients and aid in understanding the transition from autoimmunity to lymphoma.

Patients with the autoimmune disease Sjögren's syndrome are at markedly increased risk of developing non-Hodgkin's B cell lymphoma involving the lacrimal and salivary glands (1). The steps involved in the transition from autoimmunity to B cell neoplasia are not well understood. It has been proposed that lymphomagenesis is a multistep process involving sequential activation of proto-oncogenes by translocations or mutations that alter normal cellular regulatory functions (2–4). Among B cell lymphomas, the most common translocation involves the bcl-2 oncogene in a t(14;18) translocation (Fig. 1) (5, 6). The breakpoints are remarkably focused on chromosome 18, where ~60% cluster at what is called major breakpoint region (mbr)[1] and up to 25% at minor cluster region (mcr) (7, 8) (Fig. 1). These translocations result in the deregulated expression of the bcl-2 gene and synthesis of inappropriately high levels of bcl-2 protein (9, 10). Gene transfer studies suggest a role for bcl-2 in cell survival, growth enhancement, and oncogenic transformation (11). DNA sequence analysis of bcl-2 translocations has suggested that these alterations may have occurred as a result of naturally occurring double-stranded DNA breaks with N-segment addition, repair, and ligation of the derivative 14th and 18th chromosomes (12). The particular location in the body where B cells undergo bcl-2 translocation remains unclear. Bakhshi et al. (12) suggested that bcl-2 translocation occurs in the bone marrow during the early stages of B cell development, while Cotter et al. (13) have demonstrated bcl-2 translocation at a later stage of B cell development after V-D-J joining. Our finding of bcl-2 translocations in salivary gland lymphomas of patients with Sjögren's syndrome provided an opportunity to examine their prelymphoma biopsies to see if such translocation preceded the emergence of overt lymphoma.

Sjögren's Syndrome (SS) is characterized by lymphocytic infiltration of salivary and lacrimal glands, hypergammaglobulinemia, and high levels of circulating autoantibodies including rheumatoid factor and antinuclear antibodies (14–16). Although the majority of lymphocytes infiltrating these glands are $CD4^+$ T cells (17), these patients develop B cell lymphomas (18) with a frequency >40-fold over age- and sex-matched controls (1). Further, the lymphomas arise almost exclusively in the salivary glands and cervical lymph nodes, which are the site of autoimmune inflammation in SS patients and are an unusual site of lymphoma presentation in patients lacking SS (19). Before developing lymphoma, SS patients have recurrent swelling of the salivary gland, which exhibits a "pre-lymphoma" or "myo-epithelial" lesion on biopsy (20, 21). Lymphocytes eluted from these pre-lymphoma biopsies are predominantly polyclonal T cells (17), and contain a small percentage of B cells that exhibit detectable clonal rearrangements of their heavy and light chain genes (21, 22). This has led to a discussion about whether these myoepithelial lesions are autoimmune or actually represent an early "lymphoma in situ" (23–25).

If rare "lymphoma in situ" cells were present in a pre-

[1] *Abbreviations used in this paper:* mbr, major breakpoint region; mcr, minor cluster region; SS, Sjögren's Syndrome.

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lymphoma biopsy, they might contain the same karyotypic translocations that are detected in the subsequent lymphoma. This possibility is shown schematically in Fig. 2 *A*, where rare "lymphoma cells" subsequently undergo clonal expansion to become an overt lymphoma. To test this hypothesis, we examined pre-lymphoma biopsies from five SS patients whose subsequent lymphoma contained t(14;18) translocations detectable by PCR. The great sensitivity of PCR would make possible detection of rare cells bearing the translocation. Of importance, each of these pre-lymphoma SS biopsies previously has been shown to contain oligoclonal rearrangements of heavy and light chain Ig genes (22), and thus it is possible to determine whether bcl-2 translocation is detectable at the time of oligoclonal expansion of B-cells. An alternative hypothesis (Fig 2 *B*) is that the pre-lymphoma salivary gland does not contain B cells with karyotypic abnormalities and that oligoclonal B cell expansion precedes karyotypic translocation. Among the five SS pre-lymphoma biopsies, we did not find bcl-2 translocations. These results suggest that lymphoma develops in SS patients as a multistep process where oligoclonal B-cell expansion precedes t(14;18) translocation.

Materials and Methods

Patients with SS were seen at Scripps Clinic and Research Foundation (La Jolla, CA). All patients had definite SS with keratoconjunctivitis sicca, xerostomia, class 4–positive minor salivary gland biopsies, autoantibodies including rheumatoid factor and ANA (titers >640), and the presence of anti-SS-A/SS-B antibodies (15, 26). Among 200 SS patients followed for >5 yr, 14 developed non-Hodgkin's lymphoma involving cervical lymph node or salivary gland. The time interval between initial diagnosis of SS and the appearance of lymphoma was at least 3 yr. All lymphomas were B cell based on immunohistological study and Southern blot analysis of DNA for heavy and light chain rearrangement (22). 13 of 14 lymphomas were IgM-κ and one lymphoma was IgA-λ. In seven of these patients, previous biopsies of major salivary glands or lymph nodes had been performed and showed "reactive" changes but not overt lymphoma (17); therefore, these biopsies are referred to as "pre-lymphoma". Bone marrow aspirates from iliac crest were available from these seven patients. In two cases, combination chemotherapy was unsuccessful and patients died of septicemia; complete autopsy tissue was analyzed including thoracic and abdominal lymphoid tissues.

DNA was examined from other tissues including minor salivary gland biopsies from 50 consecutive SS patients who had no clinical evidence of lymphoma, 30 minor salivary gland biopsies from patients lacking autoimmune disease, 10 salivary gland biopsies from patients with benign adenoma or adenocarcinoma, 10 lymph node biopsies from patients with systemic lupus erythematous, 10 lymph node biopsies from patients with rheumatoid arthritis, 10 tonsillar lymph node biopsies from immunologically normal individuals, and seven lymph node biopsies containing "follicular" non-Hodgkin's lymphoma.

Genomic Southern Blot Analysis. Procedures for extraction of DNA from tissues and cell lines, Southern blot analysis of DNA, and radiolabeling of probes have been described (22, 27). High molecular weight DNA was digested with HindIII according to manufacturers instruction (New England Biolab, Beverly, MA). Chromosome 18–specific probes pFL-1 and pFL-2, detecting mbr and mcr, respectively (Fig. 1) (7, 28), were obtained from Dr. M. Cleary (Stanford University, Stanford, CA).

A B cell line lymphoma, SU-DHL6, containing known translocation t(14;18) involving mbr region was obtained from Dr. Allan Epstein (University of Southern California, Los Angeles, CA). Samples of lymphoma tissue from a patient with known translocation t(14;18) involving mcr region was obtained from Dr. M. Cleary (Stanford University, Stanford, CA).

Polymerase Chain Reaction. Amplification of DNA involving bcl-2 translocation was performed by PCR using a oligonucleotide primer specific for a consensus sequence of Ig heavy chain J segment and a primer specific for mbr or a mcr (29, 30). The PCR assay was performed with a DNA Thermal Cycler (Cetus Corp., Emeryville, CA), using 1 μg DNA, 100 pmol of each primer, 2.0 mM $MgCl_2$, 20 nmol of each dNTP, 2.5 U Taq polymerase (Cetus Corp.), and 35 cycles of amplification. One-tenth of the amplified product was electrophoretically separated in 1.6% agarose gel and transferred by alkaline blot method onto nylon membranes, which were hybridized with a ^{32}P end-labeled oligonucleotide probe at 42°C for 16 h. Autoradiography was performed for 4–24 h at −80°C using X-Omat AR film (Eastman Kodak Co., Rochester, NY) with a single intensifying screen.

Results

To demonstrate the sensitivity and specificity of the PCR reaction for bcl-2 t(14;18) translocations involving the mbr

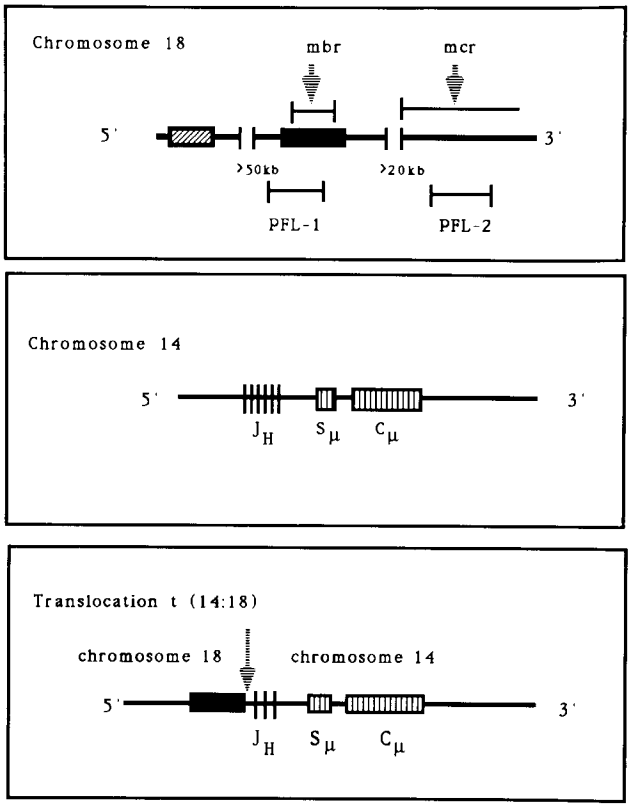

Figure 1. Schematic representation of chromosome 18 and 14. (*Top*) The location of the mbr and the mcr on chromosome 18. Solid boxes represent transcriptional units of the bcl-2 gene. pFL-1 and pFL-2 are chromosome 18–specific DNA probes. (*Middle*) The germline configuration of the Ig heavy chain allele on chromosome 14. (*Bottom*) The rearranged Ig heavy chain gene in a lymphoma with a t(14;18) translocation.

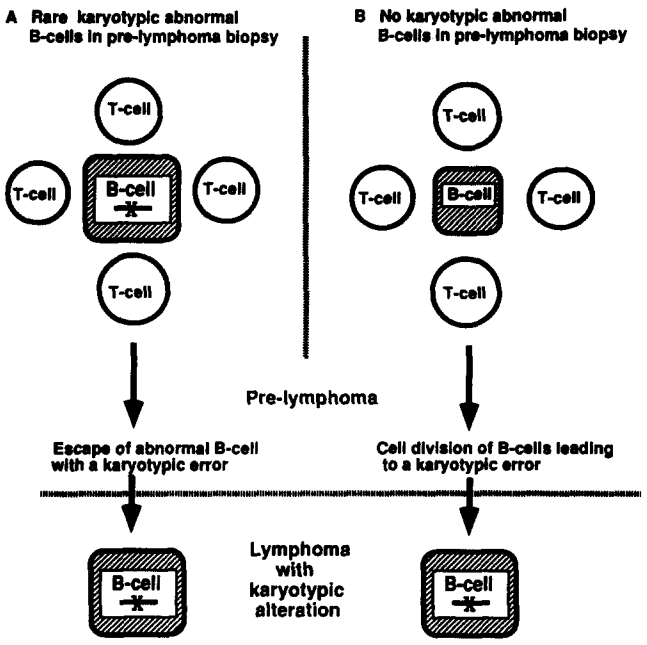

Figure 2. Schematic models for development of lymphoma in Sjögren's Syndrome (*A*) It is possible that rare B cells containing a bcl-2 translocation are present in the salivary gland biopsy of SS patients. An overt lymphoma results when this karyotypically abnormal B cell escapes T cell surveillance and clonally expands into a non-Hodgkin's lymphoma. (*B*) An alternate possibility is that karyotypically abnormal B cells are not present at a detectable level in SS biopsies, even though clonal expression of salivary gland B cells can be demonstrated by clonal Ig rearrangements. Persistent B cell division in the SS salivary gland may lead to increased chance of karyotypic error associated with lymphoma development.

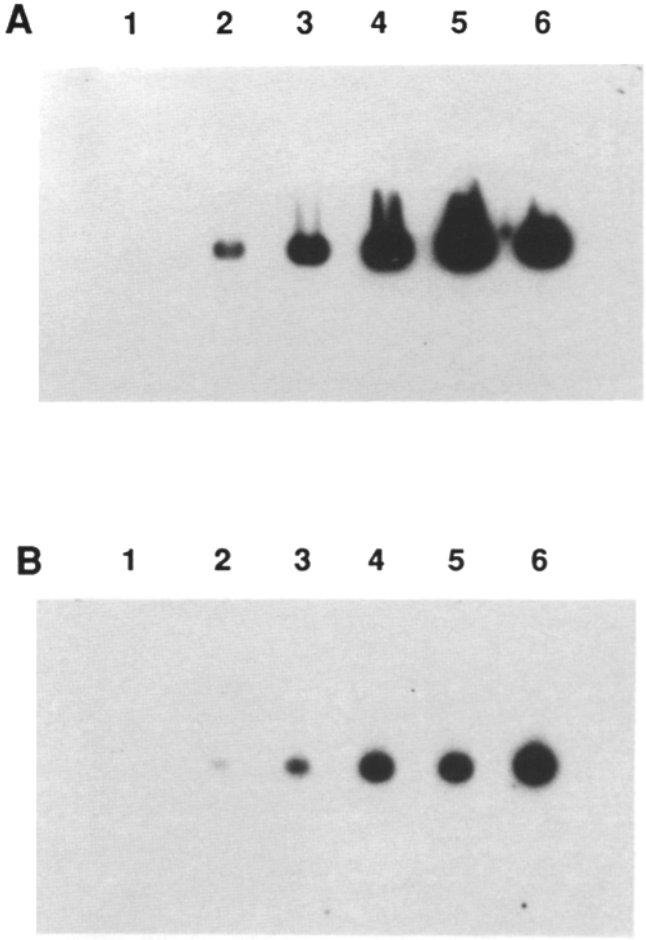

Figure 3. Southern blot analysis of PCR amplified t(14;18) translocations demonstrating the PCR sensitivity. 1 μg of DNA was amplified using oligonucleotide primers specific for mbr (*A*) or for mcr (*B*) and heavy chain J segment. After 35 cycles of PCR, the amplification product was separated on agarose gel and probed with ^{32}P-labeled probe to the mbr or mcr regions. (*A*) Lymphoma DNA containing mbr translocation. (Lane *6*) 0.1 μg of SU-DHL6 DNA was added to 1 μg of normal donor DNA. (Lanes *2–5*). Serial 10-fold dilutions of SU-DHL6 DNA were added to 1 μg of normal donor DNA before PCR amplification. Lane *1* contains DNA from a follicular lymphoma with a mcr translocation. The size of the mbr amplification product is 234 bp. (*B*) Serial dilutions of DNA from a lymphoma with a known mcr-breakpoint in normal donor DNA in lanes *2–6*. Lane *6* contains 0.1 μg lymphoma DNA mixed with 1 μg normal genomic DNA. All autoradiographs were exposed for 4 h. The size of the amplification product for this mcr lymphoma was 603 bp.

region, DNA from the lymphoma cell line SU-DHL-6 was mixed with normal genomic human DNA before PCR amplification. Using a 4-h autoradiogram (Fig. 3 *A*), 10^{-5} μg of SU-DHL-6 DNA easily could be detected in a mixture containing 1 μg of normal DNA. This corresponds to approximately one cell translocation per 10^5 of uninfected cells. Fig. 3 *B* shows a similar reconstitution experiment using lymphoma DNA that exhibits t(14;18) translocation involving mcr segment.

DNA from seven SS lymphomas was analyzed and a bcl-2 translocation involving the mbr region was detected in three of seven patients by PCR methods (Fig. 4, lanes *6*, *8*, and *10*). In comparison, lanes *1* and *2* are negative controls, and lane *3* contains DNA from a cell line (SU-DHL6) with known mbr t(14;18) translocation. In pre-lymphoma biopsies from each SS patient, mbr translocations were not detectable (Fig. 4, lanes *5*, *7*, and *9*).

DNA from SS lymphomas with breakpoints in the mcr of chromosome 18 is shown in Fig. 5, lanes *6*, *8*, and *10*. Again, the prelymphoma biopsies from SS patients did not demonstrate any detectable mcr translocation (Fig. 5, lanes *5*, *7*, and *9*). One patient (SS-1) showed translocations with both mbr and mcr breakpoints on chromosome 18 (Fig. 4, lane *6*; Fig. 5, lane *10*). To rule out the possibility that a negative result in some patient samples was due to inefficiency or complete failure of the PCR reaction, a single copy gene (HLA-DQα) was successfully amplified in all samples (data not shown).

To confirm the PCR results and to search for additional t(14;18) translocations that may not be detected by PCR, DNA from each of the lymphomas was digested with restriction enzyme HindIII and hybridized with PFL-1 and PFL-2 probes to mbr and mcr regions, respectively (Fig. 6). The results of restriction fragment length analysis using PFL-1 and PFL-2 probes were in correlation with the PCR results, and no additional SS lymphoma with bcl-2 translocation was detected. Fig. 6, *A* shows representative Southern blots from lymphomas with mcr translocation (lanes *4* and *6*) and the absence of

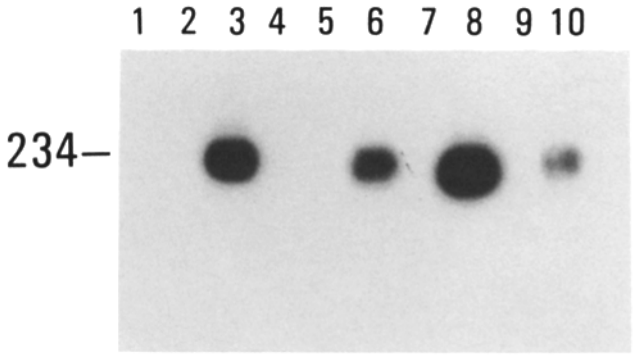

Figure 4. Southern blot analysis of PCR amplified mbr t(14;18) breakpoints in SS lymphomas. DNA from pre-lymphoma (PL) and lymphoma (L) salivary gland biopsies were subjected to PCR using a mbr-specific primer and a J_H primer. (Lane *1*) PCR reaction mixture without DNA template; (lane *2*) control DNA without t(14;18); (lane *3*) SU-DHL-6, cell line with mbr t(14;18); (lane *4*) patient DNA with known mcr t(14;18); (lane *5*) SS-1, PL; (lane *6*) SS-1, L; (lane *7*) SS-2, PL; (lane *8*) SS-2, L; (lane *9*) SS-3, PL; (lane *10*) SS-3, L.

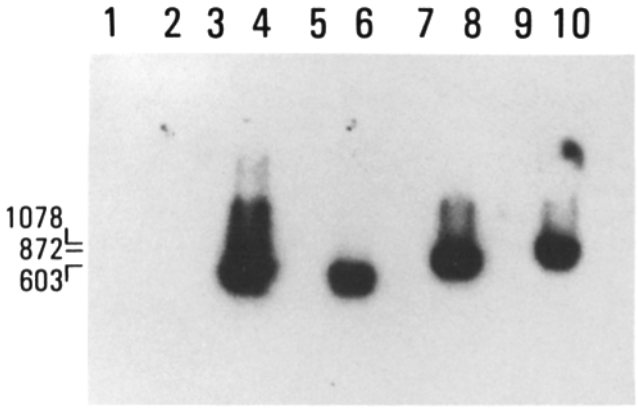

Figure 5. Southern blot analysis of PCR-amplified mcr t(14;18) breakpoints in SS lymphomas. DNA from pre-lymphoma [PL] and lymphoma [L] salivary gland biopsies were subjected to PCR using a mcr-specific primer in conjunction with a J_H primer. (Lanes *1* and *2*) Negative controls as in Fig. 3; (lane *3*) SU-DHL-6, cell line with mbr t(14;18); (lane *4*) non-SS Lymphoma patient DNA with known mcr t(14;18) translocation; (lane *5*) SS-4, PL; (lane *6*) SS-4, L; (lane *7*) SS-5, PL; (lane *8*) SS-5, L; (lane *9*) SS-1, PL; (lane *10*) SS-1, L.

translocation in the pre-lymphoma biopsies (lanes *3* and *5*). Similarly, Fig. 6 *B* shows mcr translocation in a lymphoma from a SS patient (lane *4*). Of particular interest, patient SS-1, who exhibited both mcr and mbr translocations using PCR, had detectable bcl-2 rearrangement on Southern blot only with mcr probe pFL-2 (Fig. 6 *A*, lane *6*).

Finally, DNA from salivary gland and lymph node tissues of normals, patients with autoimmune disease, and patients with solid tumors was studied. These included minor salivary gland biopsies from 50 SS patients lacking lymphoma, 10 lymph nodes from patients with rheumatoid arthritis or systemic lupus erythematous, and 10 adenocarcinoma salivary gland tumors. These DNA samples did not exhibit detectable bcl-2 translocation by PCR analysis.

Discussion

Patients with SS have increased risk of developing lymphoma involving the cervical lymph nodes and salivary glands (1). We now show that five of seven of these tumors had bcl-2 t(14;18) translocations. From a clinical and histologic point of view, it is often difficult to distinguish "pseudolymphoma" (pre-lymphoma) in SS patients from definite non-Hodgkin's lymphoma (21, 22, 31). Therefore, analysis of bcl-2 translocations in tissue biopsies will aid diagnosis. Since only a small amount of tissue is required for PCR analysis, diagnostic samples may be obtained by percutaneous biopsy or fine needle aspiration. This is in contrast to the current need for an open surgical biopsy to remove enlarged salivary glands

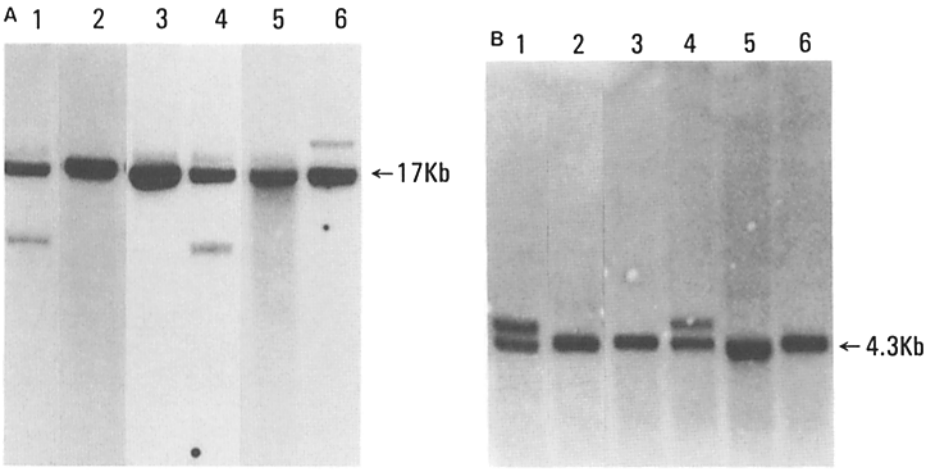

Figure 6. Southern blot analysis of t(14;18) translocations. Genomic DNA from SS patients and from lymphomas with known t(14;18) translocations were digested with HindIII, electrophoretically separated in 0.7% agarose, transferred to nylon filters, and subsequently hybridized with the mbr-specific probe pFL-1 (*A*) or the mcr-specific probe pFL-2 (*B*). The germline HindIII bands containing pFL-1 and pFL-2, respectively, corresponds to 4.3 and 17 kb. (*A*) Detection of mcr t(14;18). (Lane *1*) DNA with known mcr t(14;18); (lane *2*) control DNA; (lane *3*) SS-5, pre-lymphoma biopsy; (lane *4*) SS-5, lymphoma; (lane *5*) SS-1, pre-lymphoma biopsy; (lane *6*) SS-1, lymphoma. (*B*) Detection of mbr t(14;18). (Lane *1*) SU-DHL-6; (lane *2*) Control DNA; (lane *3*) SS-2, pre-lymphoma biopsy; (lane *4*) SS-2, lymphoma; (lane *5*) SS-1, pre-lymphoma biopsy; (lane *6*) SS-1, lymphoma.

and the associated risk of damage to the facial nerves. A negative result (i.e., no detectable translocation) would not eliminate the diagnosis of lymphoma, but a positive finding would alert the internist and surgeon to the increased chance of lymphoma. It has recently been reported that patients with B cell lymphomas with t(14;18) translocations have a poorer response to therapy (32). Therefore, detection of bcl-2 translocation at the earliest stage would give more opportunities for clinical intervention to prevent lymphoma progression.

The finding of bcl-2 translocation in SS lymphomas also helps clarify the steps in transition from autoimmunity to lymphoproliferation. At the stage of pre-lymphoma, the majority of lymphocytes infiltrating the salivary gland are polyclonal T cells (17). However, 15–20% of these salivary gland lymphocytes are B cells that express follicular B cell–associated antigens (33), exhibit oligoclonal rearrangements of their Ig genes (22), and have an increased proportion of cells undergoing DNA replication (17). The present study demonstrates that these pre-lymphoma B cells lack detectable bcl-2 translocations. We propose that these B cells undergo cell division as a result of stimulation by activated T cells, their growth factors, and/or autoantigen. As a result of the cell division within the salivary gland, the B cells have increased opportunity for bcl-2 translocation and resulting lymphoma. Other factors that may contribute to lymphomagenesis in SS patients may include the high levels of growth factors produced in the salivary gland (34) and Epstein-Barr virus that has latency at this site (35, 36).

The lymphomas in SS patients occur predominantly in the salivary glands and therefore we favor the occurrence of the bcl-2 translocations at this peripheral site. This is in contrast to the hypothesis of Bakhshi et al. (12), who suggested that bcl-2 translocations could occur only among pre-B cells in the bone marrow. Although we can not rule out that the translocation first occurred in the marrow and the neoplastic cells subsequently migrated to the salivary glands, this possibility seems less likely since lymphomatous cells were not detected at other lymphoid sites such as abdominal lymph nodes, spleen, or bone marrow in SS lymphoma patients where autopsy was performed. Also, it remains possible that rare circulating pre-B cells underwent bcl-2 translocation within the microenvironment of the salivary gland. However, Cotter et al. (13) have demonstrated bcl-2 translocations among mature B cells in tissue culture, and our results suggest that a similar process has occurred in vivo at an inflammatory site.

Surprisingly, one patient's lymphoma contained both an mbr and an mcr translocation using PCR. Based on Southern blot analysis, the proportion of cells that contained the mcr translocation was much higher than the proportion of cells with the mbr translocation. It is not yet possible to determine whether the mbr translocation occurred in a cell with a prior mcr alteration or on a distinct B cell. However, DNA sequence analysis of these bcl-2 translocations is in progress to clarify the basis of this unusual finding.

In conclusion, our results demonstrate the presence of bcl-2 translocations in non-Hodgkin's lymphoma occurring in SS patients. These translocations were not detected in the pre-lymphoma biopsies from the same patients. Thus, detection of blc-2 translocations may aid in improved early detection of lymphoma and improved treatment of SS patients. Although the function of the bcl-2 gene product is not yet clear (37, 38), the translocation juxtaposes the bcl-2 gene with Ig heavy chain locus, increases B cell survival, and may lead to increased chance of neoplastic transformation (11, 28, 29, 39, 40).

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