Detection of the tax gene of HTLV-I in labial salivary glands from patients with Sjögren’s syndrome and other diseases of the oral cavity

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Abstract

Objective
To confirm a possible association between Sjögren’s syndrome (SS) and the tax gene of human T lymphotropic virus type I (HTLV-I).

Methods
We studied by PCR labial salivary glands (LSG) from 50 patients with definite SS and from 58 controls including 32 patients with LSG involved by other inflammatory processes and 26 normal LSG. Antibodies to HTLV-I and antibodies to the Tax protein were searched for in serum.

Results
We detected the tax gene of HTLV-I in LSG from 15/50 (30%) of patients with SS but also in specimens from 9/32 (28%) patients with LSG involved by other inflammatory processes and 26 normal LSG. Antibodies to HTLV-I and antibodies to the Tax protein were searched for in serum.

Conclusion
Our observations raise the possibility that a very low number of copies of the tax gene may be harbored innocuously in cells within the oral cavity in some healthy individuals, but that this gene may play a role as a co-factor in the development of SS or other diseases of oral cavity.

Key words
Sjögren’s syndrome, retrovirus, HTLV-I, tax gene.

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**Introduction**

Sjögren’s syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltration of salivary and lacrimal glands, and systemic production of autoantibodies (1). The pathogenesis of this syndrome remains unclear but viral infections have been considered as a possible etiologic factor (2). An association between SS and the human T lymphotropic virus type I (HTLV-I) has been suggested. Some HTLV-I infected patients with tropical spastic paraparesis (TSP) develop features of SS (3); anti-HTLV-I antibodies are present in 23% of Japanese patients with SS from Nagasaki and in only 3% in the control group, not different from patients with lupus (6%) (4). Moreover, accumulation and expression of HTLV-I in salivary glands has been recently described in HTLV-I seropositive SS patients both in Japan (5) and in Martinique (6). Last, mice transgenic for the HTLV-I tax gene display a histopathological picture in the salivary and lacrimal glands resembling that of SS (7).

Using *in situ* hybridization and the polymerase chain reaction (PCR), we detected the tax gene of HTLV-I in epithelial cells of LSG biopsy specimens from 2 out of 9 patients with SS seronegative for HTLV-I, and from none of 9 control LSG biopsies (8). No HTLV-I gag, pol and env sequences could be amplified. Independently, Sumida et al. found the tax gene of HTLV-I in LSG from 4/15 Japanese SS patients seronegative for HTLV-I (9). The present investigation extends our preliminary study to 50 SS patients and 58 controls. In confirmation of our initial results, we detected the tax gene of HTLV-I in LSG from 15/50 SS patients seronegative for HTLV-I. However, although we could rarely detect the tax gene of HTLV-I in normal LSG, it was identified in LSG involved in a variety of other inflammatory processes.

**Materials and methods**

**Patients and controls**

Fifty patients (43 women and 7 men) with definite SS using Fox’s criteria (10) were studied, 39 with primary SS and 11 with SS secondary to rheumatoid arthritis (n=8), systemic lupus (n=2) or scleroderma (n=1). Rheumatoid factor, antinuclear antibodies and antibodies to SS-A and/or SS-B were detected in 89%, 76% and 55% of the sera, respectively. Four types of control LSG were studied: 19 labial extravasated cysts, 9 LSG from allografted patients with graft versus host (GVH) disease, 4 LSG involved by florid lesions of sarcoidosis and 26 normal LSG. This normal control group consisted of patients with sicca symptoms or systemic diseases and normal LSG biopsy (grade 0 or 1 of Chisholm) and patients with suspicion of amyloidosis and normal LSG biopsy. The mean age was 56 ± 13 years in the SS group and 52 ± 15 years in the control group.

**Polymerase chain reaction**

Whole-cell lysate DNA from LSG was extracted from paraffin-embedded LSG sections from each patient as previously described (11) and was subsequently submitted to PCR amplification. The primer pairs (SK 43/44 and SK 54/55) used by Kwok et al. (12) amplified the tax and pol regions of the HTLV-I genome, respectively. The primers used to amplify the gag and env sequences were those described by Reddy et al. (13). In order to avoid false positive contamination we used dUTP and Uracil-N-glycosylase (Life Technologies, Cergy-Pontoise, France) to minimize carryover and we designed 2 new primer pairs in the pX region: 1) Fag 7: (5’-GTTCGGCC CGCTACATCGTA-3’) (7427-7448) and Fag 8: (5’-TCTGGGTGGGGAAGGGC GCCTACATCGTCA-3’) (7517-7497) and 3) Fag 3: (5’-TCTGGGTGGGGAAGG AGGGGAGTC-3’) (7564-7541), the numbering of the primers corresponded to those described by Seik et al. (14). These primers were chosen to generate a 138 bp fragment, amplified with 35 cycles, which partially overlaps the SK43-SK44 fragment and which is detectable with the SK45 probe. 2) a longer PCR fragment of 652 bp was generated with the primer pair Fag 1.26 (5’-CGCC TTTCAGCCCTTGTCTCCACT-3’) (6866-6889) and SK44 (14) (7517-7497) encompassing the 5′ portion of the pX gene 5′ from the tax region and was amplified with 40 cycles. After amplification, the samples were loaded on a 3% agarose gel and hybridized after transfer with the corresponding probe 5′ labelled with digoxigenin using a kit according to the manufacturer.
(Boehringer Mannheim, Meylan France). Each reaction was repeated 4 times. Positive and negative controls included SLB1 and lymphoid blood cells of a normal donor, respectively.

**Sequence analysis**

Purified DNA from LSG of two patients was sequenced. To sequence the 652 bp fragment, the Fag 1.26 and SK44 oligonucleotides were designed with an adaptation on their 5’ end, containing the Bam HI restriction site for cloning. After amplification, the PCR products were digested by Bam HI, ligated in plasmid Bluescript II KS (Ozyme, Montigny le Bretonneux, France) previously linearized by Bam HI and cloned in an E. coli DH5α strain (Ozyme). Sequencing was accomplished by amplification with the T7 sequencing kit (Pharmacia, Orsay, France).

**Antibodies to HTLV-I**

Antibodies to HTLV-I were evaluated by ELISA and Western blot using commercial kits (Dupont, Wilmington, Delaware, USA, and Diagnostics Biotechnology, Singapore). The strips were blotted with both native and recombinant viral proteins including a transmembrane glycoprotein (rgp21) and a membrane glycoprotein (rgp46).

**Antibodies to the Tax protein**

Recombinant full-length Tax-I antigen was used in Western blot assays essentially as described in detail elsewhere (15, 16). Briefly, recombinant full-length HTLV-I Tax protein was prepared by cloning PCR-amplified proviral DNA sequences spanning the entire Tax open reading frame from the prototypic HTLV-I infected cell line, C91PL (17), into the glutathione S-transferase (GST) fusion protein expression vector pGEX-2T (18). The recombinant GST-Tax-I fusion protein was expressed in E. coli BL21 cells and purified by chromatography using glutathione linked to Sepharose 4B and subsequent thrombin cleavage. The Tax antibody Western blot assay was performed using purified, recombinant Tax-I protein, which was resolved through 8.5% preparative polyacrylamide gels and electrophoretically transferred to nitrocellulose. The patients and control positive and negative sera were diluted at 1:10 and were studied blindly. The positive control antisera were obtained from the NIH AIDS Research and Reference Reagent Program and from a patient with TSP/HAM. For detection, goat anti-human IgA + IgG + IgM antibodies and goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) and the AP substrates NBT and X-Phosphate (Pierce Chemical Co., Rockford, IL) were used.

**Results**

**Detection of HTLV-I genes by PCR in patients with SS**

The HTLV-I gag, pol and env genes were never detected in LSG from SS patients. Using SK 43/44 and Fag 7/8 primers, the HTLV-I tax gene was detected in LSG from 15 out of 50 SS patients (30%) (Fig. 1). The frequency was the same in patients with primary SS: 12/39 (32%) and secondary SS: 3/11 (27%). Seven of these 15 Sjögren LSG specimens containing the tax gene of HTLV-I were subsequently tested with a third type of PCR using a different set of primers (Fag 1.26/SK 44) located within the pX gene 5’ from the tax gene amplifying 652 bp. Six out of the 7 tested specimens were positive. Among the 15 positive SS patients, 10 originated from France, 2 from Vietnam, 1 from Portugal, 1 from Morocco and 1 from Guadeloupe where HTLV-I is endemic. The 15 positive SS patients were not different from the 35 other SS patients negative for tax in terms of age, duration of SS, intensity of the salivary lymphoid infiltrate, or the presence of extra salivary symptoms, antinuclear antibodies (75% and 77%, respectively), anti-SS-A and/or anti-SS-B antibodies (58% and 54%, respectively) or rheuma-
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toid factor (82% and 92%, respectively).

Detection of HTLV-I genes by PCR in controls

Four kinds of control LSG were studied: 19 labial extravasated cysts, 9 LSG from allografted patients with graft versus host (GVH) disease, 4 LSG involved by florid lesions of sarcoidosis and 26 normal LSG from patients with sicca symptoms, other systemic diseases or suspicion of amyloidosis and normal LSG biopsy. HTLV-I tax DNA was detected in 5 out of 19 extravasated cysts (26%), 3 out of 9 (33%) LSG with GVH, 1 out of 4 (25%) LSG with florid sarcoidosis and only 1 out of 26 (4%) normal LSG. The only positive patient in the latter group had an unclassified chronic non erosive polyarthritis without any serum auto-antibodies and with grade 1 LSG.

Sequence analysis

The LSG samples of two SS patients (BAT and KER) were sequenced. Four different clones were sequenced for patient BAT and were identical and six different clones, also identical, were sequenced for patient KER. The 2 sequences were different (8 bp differences) but were 98 to 98.5% homologous to the established sequence of HTLV-1 (14) (Fig. 2).

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**Fig. 2.** Comparison of tax sequences from 2 Sjögren’s patients with the sequence of HTLV-I from Seiki et al. The 2 sequences were 8 bp different and 98% to 99% homologous to the Seiki sequence.
Antibodies to HTLV-I and to the Tax protein in patients with SS

In 30 SS patients (15 positive for tax DNA in their LSG and 15 negative for tax DNA in their LSG), we looked for the presence of HTLV-I antibodies by different ELISA and Western blots. ELISA and Western blot were negative in all patients. Using Western blot, we detected a reactivity with a single protein in 7 patients: env gp21 in 1 patient with tax in LSG and in 4 patients without tax in LSG, and gag p24 in 2 patients with tax in LSG.

The sera of 25 SS patients (10 proven positive for tax DNA in their LSG and 15 negative for tax DNA in LSG), were blindly analyzed for antibodies to the Tax protein. Anti-Tax antibodies were detected in 10/10, i.e. 100% of patients positive for tax DNA in their LSG and in 8/15 (53%) of patients negative for tax DNA in their LSG. Representative Western blots are illustrated in Figure 3. The rate of detection of anti-Tax antibodies in the serum of blood donors using the same technique was 8% (19).

Discussion

The present paper extends previous studies, which showed HTLV-I tax DNA in labial salivary glands of some SS patients by in situ hybridization and PCR (8) and presents a large number of controls, including 58 biopsies obtained from both normal individuals and patients with various inflammatory disorders of oral mucosa. The tax gene of HTLV-I was detected in LSG biopsy specimens from 15/50 (30%) patients with SS and also in 9/32 (28%) patients with LSG involved by other processes, such as GVH, extravasated cysts or sarcoidosis. Only 1 of 26 normal LSG biopsies (4%) turned out to be positive. These observations point out that the HTLV-I tax proviral sequence can be found in the salivary glands of individuals who do not have Sjögren’s syndrome.

Because it is always a question whether results like these could be due to PCR contamination, we amplified HTLV-I tax DNA by UNG PCR with 3 different primer sets: the SK 43/44 primers, the Fag7/8 primers and a third set of primers (Fag 1.26/SK44) located within the px gene but outside the tax plasmid used in our lab, which could have been a source of contamination. In addition, in situ hybridization with a tax probe in 6 LSG samples positive with PCR yielded a positive signal in 3 of them (2 SS patients and 1 patient with sarcoidosis) (8 and unpublished data). Last, the 8 bp differences in the 2 sequences argue against contamination.

Detection of tax DNA in salivary glands of HTLV-I seronegative patients with SS has been controversial. Our results are in accordance with those from 4 groups, 3 in Japan (5, 9, 20) and 1 in France (6) and contrast with data from 2 groups, 1 in England (21) and another in Japan (22) (Table I). The discrepancies between various PCR assays could be related to the sensitivity of the techniques. We previously determined that the frequency of salivary gland cells containing the HTLV-I tax gene may be up to $10^{-4}$ or less (23). This low frequency may explain the absence of detection of HTLV-I tax gene by investigators who used a PCR assay less sensitive than ours (21). By diluting our positive control plasmid in human DNA, we estimated that our PCR method with Fag 7/8 primers was sufficiently sensitive to detect up to 5 molecules of tax in a single PCR reaction (Fig. 2C). Last, a key point ext-

| Table I. Results of different studies in the literature which looked for the proportion of HTLV-I seronegative SS patients or controls harboring DNA of the tax gene of HTLV-I by PCR in labial salivary glands. |
|-----------------------------|-----------------|-------------------|
|                            | Patients with SS or dryness | Controls | Patients with other diseases of the oral cavity |
| Sumida et al. (9), Japan    | 4/14             | 0/14 | |
| Rigby et al. (21), England  | 0/36             | 0/15 | |
| Yamano et al. (22), Japan   | 0/15             | 0/15 | |
| Ohyama et al. (5), Japan    | 5/20             | 0/15 | |
| Mizokami et al. (20), Japan | 3/17             | 0/15 | |
| Tangy et al. (6), Martinique (France) | 5/12 | 0/22 | |
| France                      | 2/12             | 0/22 | |
| Present study : France      | 15/50            | 1/26 | 9/32 |
| Total                       | 34/176 (20%)     |       |       |
plaining the discrepancies between different laboratories may be the method of DNA extraction: in patients with mycosis fungoides, the tax gene of HTLV-I was detected in PBMC only if Southern hybridization was carried out on whole-cell lysates of PBMC rather than on DNA extracted by the classical phenol-chloroform method, suggesting that the tax sequence might be present in cell nuclei as episomes (24).

Three hypotheses can be proposed to explain the presence of tax in LSG. First, tax may be a new endogenous retroviral sequence. However, the 652 bp pX region which has been sequenced in two patients was 98 to 99% homologous to the pX region of HTLV-I itself, whereas homologies between endogenous retroviral sequences and the corresponding exogenous retroviruses are usually less than 80%. Finally, the low number of copies of tax in LSG rules out the possibility of an endogenous retroviral sequence. The second hypothesis could be an infection with a defective HTLV-I virus. Tax DNA has also been detected in the circulating mononuclear cells and the skin-infiltrating lymphocytes of a series of patients with mycosis fungoides, who did not have antibodies to HTLV-I (15). The presence in 2 patients of the 5’ part of the 3’LTR (data not shown) makes the hypothesis of a defective retrovirus possible. The third hypothesis is based on the possibility that a new retrovirus, or a known sialotropic virus, integrated the tax gene of HTLV-I. Interestingly, a putative retroviral agent infecting the salivary glands of patients with SS was identified by culturing extracts from lip biopsy specimens with lymphoblatsoid cells (25). Recently, a novel exogenous pol-like sequence has been identified in salivary glands of patients with and without SS (26).

Whatever the origin of the tax sequence in the salivary glands may be, we provide evidence here that it is not only found in Sjögren’s syndrome. Indeed, we found HTLV-I tax in conditions as different from SS as sarcoidosis, GVH disease and extravasated LSG cysts. However, this sequence was only rarely detected in histologically normal LSG. These data could indicate a defective virus located in cells inhabiting the oral cavity. Under normal circumstances, it may not be detectable because of a very low number of copies, but it may become detectable under inflammatory conditions, whatever their etiology. In the light of the high prevalence of SS in HTLV-I infected patients in regions of the world where HTLV-I is endemic (4-6), this hypothesis does not preclude the possibility that Tax may also be a co-factor for the development of SS in non endemic areas. The higher rate of detection of serum anti-Tax antibodies in Sjögren’s patients (72%) versus blood donors using the same technique (8%) (19), argue in favor of this hypothesis. The protein Tax could play a role in pathogenesis by transactivation of cellular as well as other viral promoters (including the promoters of the IL2 and IL2-RI genes), through the induced expression of the pleiotropic transcription factor NF-kB (27). Tax may also induce apoptosis mediated by ICE-protéases (28). Interestingly, ICE-protease activity is increased in salivary glands of lymphocytes of the nonobese (NOD) mouse which is a model for SS (29). Moreover, in human disease, increased apoptosis of epithelial cells (30) and increased protease activation during apoptosis leading to the expression of neo-antigen such as the 120 kD fragment of α-fodrin (31) are both potentially relevant with the physiopathology of the disease. Further studies looking for the presence of tax mRNA or Tax protein in LSG and for a cellular response to the Tax protein are warranted to support this hypothesis of a possible pathogenic role of Tax in SS or in other diseases of oral cavity.

References


