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1 *Leukemia & Lymphoma*

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4 **Clinical, histopathological, and molecular features of mucosa-associated**  
5 **lymphoid tissue (MALT) lymphoma carrying the**  
6 **t(X;14)(p11;q32)/GPR34-immunoglobulin heavy chain gene**

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20 *Running title:* MALT lymphoma with t(X;14)/GPR32-IGH

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1 Recurrent chromosomal translocations have been identified in MALT lymphoma,  
2 including t(11;18)(q21;q21), t(14;18)(q32;q21), t(1;14)(p22;q32), and  
3 t(3;14)(p14;q32).[1] The former three translocations lead to the generation of the  
4 BIRC3-MALT1 chimeric protein or deregulated expression of MALT1 or BCL10  
5 under the control of the juxtaposed immunoglobulin heavy chain gene (*IGH*),  
6 and finally activate the nuclear-factor  $\kappa$ B pathway.[1] t(X;14)(p11;q32) is the fifth  
7 translocation in MALT lymphoma involving the parotid gland or lung.[2, 3] The  
8 translocation results in the juxtaposition of the G protein-coupled receptor  
9 (GPCR) 34 (*GPR34*) gene at Xp11.4 to the *IGH* gene at 14q32, and  
10 t(X;14)-MALT lymphoma was found to have the highest *GPR34* mRNA levels  
11 among B-cell lymphoma subtypes.[2] However, it remains unclear whether  
12 t(X;14) characterizes a subgroup of MALT lymphoma that shows a particular  
13 cytomorphology and clinical behavior. We herein describe two cases of MALT  
14 lymphoma that carried the t(X;14).

15 The first case (case 1) was a 66-year-old female who presented with tumors  
16 within the left parotid gland. She had been diagnosed with Sjögren syndrome  
17 and immune thrombocytopenia 6 years earlier. Sjögren antibody SS-A/Ro index  
18 was 105 (reference value, <25.0), SS-B/La was 32 (<25.0), anti-nuclear antibody  
19 was  $\times 160$  (< $\times 40.0$ ), and rheumatoid factor (RF) was 16.9 IU/mL. Although no  
20 residual tumor was detected after superficial lobectomy of the parotid gland, she  
21 relapsed 6.5 years after the initial presentation; the disease involved the left  
22 palatine tonsil and ileocecum with regional lymph nodes (Supplementary Figure  
23 S1A). She received 6 cycles of chemotherapy consisting of rituximab,  
24 cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP),

1 leading to a complete response. The second cases (case 2) was a 66-year-old  
2 male who presented with symmetric enlargement of the bilateral parotid glands  
3 (Supplementary Figure S1B). He had been treated for rheumatoid arthritis with  
4 prednisolone and methotrexate followed by intravenous tocilizumab. His lactate  
5 dehydrogenase (LDH) level was 265 IU/L, soluble interleukin-2 receptor (IL-2R)  
6 was 2,037 U/mL, RF was 519.9 IU/mL, and matrix metalloproteinase-3 was  
7 218.7 ng/dL (reference range, 36.9 to 121). He was treated with 6 cycles of  
8 R-CHOP, excluding prednisolone because of seropositivity for the hepatitis B  
9 virus surface antigen, and achieved a complete response. Informed consent was  
10 obtained from the patients. This study was approved by the Institutional Review  
11 Board.

12 Examination of histopathological specimens obtained from the parotid gland  
13 disclosed salivary gland tissues diffusely infiltrated by CD20<sup>+</sup> lymphoma cells,  
14 and groups of these cells infiltrated into the glandular epithelium to generate the  
15 lymphoepithelial appearance (Supplementary Figure S2). The cells were  
16 medium to large in size with a moderate amount of cytoplasm, and CD79a<sup>+</sup>,  
17 BCL2<sup>+</sup>, CD5<sup>-</sup>, CD10<sup>-</sup>, and cyclin D1<sup>-</sup>. The Ki-67 proliferation index was  
18 approximately 50%. The cell surface immunoglobulins were  $\mu$ -heavy and  $\kappa$ -light  
19 chains, and the  $\delta$ -heavy chain was expressed at low levels (Supplementary  
20 Figures S3 and S4).

21 G-banding of metaphase spreads obtained from the specimens revealed the  
22 t(X;14)(p11;q32) in both cases, and fluorescence *in situ* hybridization using the  
23 *IGH* break-apart probe confirmed the translocation. Karyotypes according to the  
24 ISCN (2013) were 47,X,t(X;14)(p11;q32),+18,add(22)(p11)[9]/46,XX[1] in case 1

1 and 47,Y,t(X;14)(p11;q32),+21[8]/46,XY[2] in case 2 (Supplementary Figure S5).

2 To clone the t(X;14)(p11;q32) junction, we designed primers for the C $\alpha$   
3 constant gene of *IGH* and for the upstream sequence of the *GPR82* gene, which  
4 is another GPCR gene and is localized 27-kb downstream of *GPR34* (Figure 1A).  
5 Genomic DNA from the biopsies was subjected to long-distance polymerase  
6 chain reaction (LD-PCR) under the conditions for a long DNA target, resulting in  
7 the amplification of 3.6 and 6.0-kb of DNA, respectively (Figure 1B). We then  
8 cloned the LD-PCR products into the plasmid and performed Sanger sequencing  
9 (Figure 1C). Breakpoints were located at intron 1 (case 1) and the coding region  
10 of *GPR82* (case 2), and those at the *IGH* gene were within the switch region  
11 associated with *IGH* C $\alpha$ 2. As a result of translocation, *GPR34* and *IGH* C $\alpha$ 2  
12 were aligned in the same transcriptional orientation (Figure 1A).

13 We next performed reverse-transcriptase (RT-) PCR to investigate the mRNA  
14 levels of *GPR34* and *GPR82*, the expression of which delineates a unique  
15 molecular subset of marginal zone lymphoma.[2] As shown in Figure 1D, the  
16 level of *GPR34* mRNA in case 1 was significantly higher than those in various  
17 hematological tumor cell lines. On the other hand, the expression of *GPR82*  
18 mRNA was detected in case 1, while similar levels of RT-PCR products were  
19 generated in cell lines from B-cell precursor leukemia, follicular lymphoma, and  
20 Burkitt lymphoma. Since t(X;14) disrupted the genomic structure of *GPR82* and  
21 the primers were designed for exons 1 and 3 (Figure 1A), *GPR82* mRNA  
22 expression in case 1 theoretically represented the transcriptional activity of the  
23 non-translocated homologue at normal chromosome X.

24 We finally isolated the  $V_H-D_H-J_H$  sequences in the current two cases in

1 addition to the Mayo clinic case by the inverse PCR strategy to characterize the  
2 *IGH* repertoire in t(X;14)-MALT lymphoma (Supplementary Table SI). In the 3  
3 cases, DNA fragments including the entire length of the  $V_H$ - $D_H$ - $J_H$  segments were  
4 successfully amplified, and nucleotide sequencing revealed a single  $V_H$ - $D_H$ - $J_H$   
5 rearrangement in frame without stop codons. The closest germline  $V_H$ ,  $D_H$ , and  
6  $J_H$  genes were identified in each case and the sequence identities of the  $V_H$   
7 genes were 95.1%, 86.5%, and 96.5%, respectively, providing evidence for a  
8 somatic hypermutation (Table I). We then calculated the excess of replacement  
9 (R) mutations over silent (S) mutations in the complementarity determining  
10 regions (CDRs) and the scarcity of R mutations in the framework regions  
11 (FWRs). As shown in Table I, we found no preferential clustering of R mutations  
12 indicative of positive selection in CDRs, while a decrease in R mutations in  
13 FWRs was found in all cases and negative selection in the Mayo clinic case was  
14 significant with a *P* value of <0.05.[4, 5] The CDR3 sequences of the 3 cases  
15 were characterized by the GC-rich alignment, particularly in the N sequences,  
16 and the CDR3 length was 17 or 16 at the amino acid level (Supplementary Table  
17 SII). A database search revealed a similarity of the CDR3 sequence of cases 1  
18 with that of an IgM RF derived from healthy immunized donors (Supplementary  
19 Table SIII).

20 In line with the general concept of the development of MALT lymphoma, both  
21 patients had chronic autoimmune disorders and showed serological evidence of  
22 each underlying disease at presentation. By comparison with the typical MALT  
23 lymphoma cytomorphology, lymphoma cells in the current two cases were larger  
24 and had rounder nuclear contours, more vesicular chromatin, and more

1 conspicuous nucleoli, representing the features of diffuse large B-cell lymphoma  
2 (DLBCL). Nevertheless, marginal zone infiltrates of reactive follicles and  
3 lymphoepithelial lesions were in agreement with the histopathological criteria of  
4 MALT lymphoma. On the other hand, the palatine tonsil and ileocecum involved  
5 in case 1 at relapse are unusual sites of involvement of MALT lymphoma, but  
6 preferentially affected by DLBCL. In case 2, the marked enlargement of both  
7 parotid glands and elevated LDH and sIL-2R levels initially indicated aggressive  
8 lymphoma. Therefore, the current two cases appear to be characterized by  
9 intermediate features in cytomorphology and clinical behavior between  
10 low-grade MALT lymphoma and DLBCL.

11 *GPR34* is the first GPCR gene that is affected by chromosomal translocation  
12 in B-cell tumors. Since potent enhancer complexes are localized downstream of  
13 the two *IGH C $\alpha$*  genes,[6] and since t(X;14) does not interrupt the coding region  
14 of *GPR34*, t(X;14) theoretically promotes the expression of *GPR34*, and we  
15 confirmed the strong expression of *GPR34* mRNA in case 1. GPCRs, including  
16 class A *GPR34*, are integral membrane proteins containing 7 putative  
17 transmembrane domains and mediate signals to the interior of the cell via the  
18 activation of heterotrimeric G proteins. A large number of GPCRs are  
19 overexpressed in various cancer types, and contribute to tumor cell growth when  
20 activated by circulating or locally produced ligands.[7] Lysophosphatidylserine,  
21 which is an endogenous lipid mediator generated by the hydrolysis of the  
22 membrane phospholipid phosphatidylserine, has been proposed to be the ligand  
23 of *GPR34*.[8] Since the current two cases developed in the setting of chronic  
24 autoimmune disorders, the potential link between the underlying inflammatory

1 disease and occurrence of lymphoma may be attributed to ligands being steadily  
2 generated in salivary gland tissues affected by chronic inflammation, which, in  
3 turn, stimulate GPR34-overexpressing cells resulting from t(X;14), thereby  
4 leading to the development of florid lymphoma.

5 We showed that  $V_H$  genes were variable among the 3 cases. Of the 3  $V_H$   
6 genes,  $V_H1-2$  has been reported to be preferentially used in splenic marginal  
7 zone lymphoma,[9] while its usage is infrequent in MALT lymphoma that  
8 develops in the stomach, lung, and parotid gland.[10-12] In contrast,  $V_H1-69$  is  
9 involved in not only cases of B-cell chronic lymphocytic leukemia, but also  
10 salivary gland MALT lymphoma at a frequency of 8 of 14 (57%).[13, 14]  $V_H3-7$   
11 appears to be preferentially used in gastric MALT lymphoma.[10-12] On the  
12 other hand, a statistical analysis of the distribution of R and S mutations within  
13 CDRs or FWRs provided no significant evidence for antigen selection, whereas  
14 negative selection for R mutations in FWRs in order to maintain the configuration  
15 of the IGH molecule appeared to have been operating. Taken together with the  
16 long CDR3 length, t(X;14)-MALT lymphoma may arise from B cells that  
17 recognize self-antigens.[15]

18

## 19 **Acknowledgments**

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22

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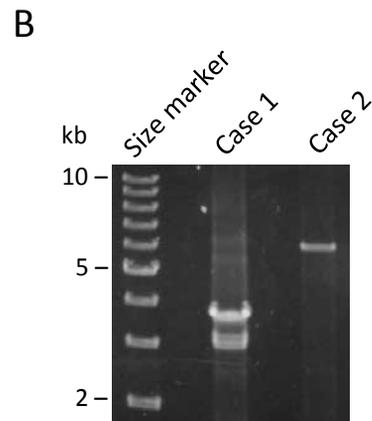
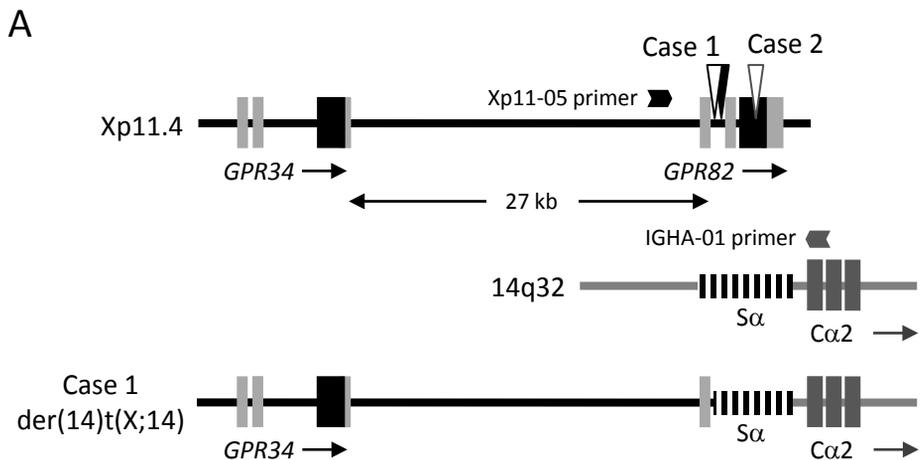
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- 19
- 20

1 **Figure legends**

2

3 **Figure 1.** Molecular cloning of the t(X;14)(p11;q32)/*GPR34-IGH* junction. (A)  
4 Schematic presentation of the anatomy of t(X;14)(p11;q32). The coding exons of  
5 *GPR34* and *GPR82* are intron-less (rectangles filled with black). Breakpoints in  
6 case 1 and case 2 (open triangle) as well as that in the Mayo clinic case (closed  
7 triangle) [2] are distributed within the *GPR82* gene. (B) Ethidium bromide-stained  
8 gel electrophoresis of LD-PCR encompassing the t(X14)(p11;q32) junction. The  
9 positions of the primers for LD-PCR are indicated in A. (C) Nucleotide  
10 sequences of the t(X;14)(p11;q32) junction in case 1 and case 2. Vertical lines  
11 indicate nucleotide identity. (D) RT-PCR for the expression of *GPR34* and  
12 *GPR82* mRNA. The hematological tumor cell lines used were: ME-1, acute  
13 myeloid leukemia; CCRF-CEM, T-cell acute lymphoblastic leukemia; NALM-20  
14 and NALM-6, B-cell precursor leukemia; SMZ-1, peripheral T-cell lymphoma;  
15 Karpas299, anaplastic large cell lymphoma; KM-H2 and L428, Hodgkin  
16 lymphoma; FL-318 and FL-218, follicular lymphoma; and Ramos and Raji,  
17 Burkitt lymphoma. RT-PCR for *GAPDH* is shown in the *bottom* to confirm that  
18 similar amounts of mRNA were loaded. The sequences of the primers for LD-  
19 and RT-PCR are described in Supplementary Table SI.

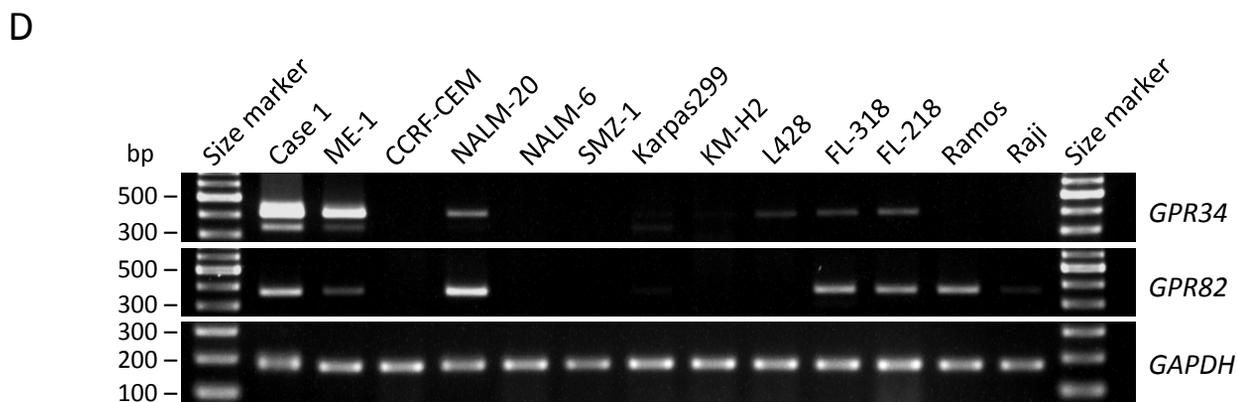
20



**C**

Xp11.4 GTTTTTAGTATACTATGCATTATCTGCACAAGGTAGGAGCTACTGCTTTTCTTTAACAGCATCATAAAAG  
 Case 1 GTTTTTAGTATACTATGCATTATCTGCACCAACTGGGCTGGGCTAGATTGGGCTGGGCTGGACTGGGCTG  
 Salpha2 GACTGGGCTGGGCTGGGCTGGGCTGGGCTGAGCTGGGCTGGGCTAGATTGGGCTGGGCTGGACTGGGCTG

Xp11.4 AGTATTTTTAAACCCATTTTTTATGTTCTACACCAAAGAGATAACTGTCAGCAATTGAATTATTTAATAG  
 Case 2 AGTATTTTTAAACCCATTTTTTATGTTCTACACCGGTGAGCTGGACTGGGCCGGGCTGGGCTGAGCTGAG  
 Salpha2 CTGGACTGGGCTAGGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGGACTGGGCCGGGCTGGGCTGAGCTGAG



**Table I. Somatic mutation analysis of the *IGH* gene in MALT lymphomas with t(X;14)(p11;q32)**

| Case             | <i>V<sub>H</sub></i> gene | % identity | Observed mutations |   |     |    | Expected |      |      |      | Focused test*                |        |                   |         |
|------------------|---------------------------|------------|--------------------|---|-----|----|----------|------|------|------|------------------------------|--------|-------------------|---------|
|                  |                           |            | CDR                |   | FWR |    | CDR      |      | FWR  |      | Selection value ( $\Sigma$ ) |        | <i>P</i> -value** |         |
|                  |                           |            | R                  | S | R   | S  | R        | S    | R    | S    | CDR                          | FWR    | CDR               | FWR     |
| Case 1           | <i>V<sub>H</sub>3-7</i>   | 95.1%      | 4                  | 1 | 5   | 4  | 0.2      | 0.05 | 0.56 | 0.2  | 0.00553                      | -0.823 | 0.492             | -0.0872 |
| Case 2           | <i>V<sub>H</sub>1-2</i>   | 86.5%      | 5                  | 2 | 20  | 12 | 0.14     | 0.05 | 0.61 | 0.2  | -0.38                        | -0.547 | -0.227            | -0.0571 |
| Mayo clinic case | <i>V<sub>H</sub>1-69</i>  | 96.5%      | 2                  | 1 | 2   | 5  | 0.17     | 0.06 | 0.57 | 0.21 | -0.543                       | -1.77  | -0.240            | -0.0059 |

Abbreviations: R, replacement; S, silent; CDR, complementarity determining region; FWR, framework region.

\* $\Sigma$  and *P* values were calculated using the Focused-Z test to detect selection.[4, 5]

\*\*A negative sign indicates negative selection. *P* values < 0.05 were considered to be significant.