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Clinicopathologic analysis of
angioimmunoblastic T-cell lymphoma
with or without *RHOA* G17V mutation using
formalin-fixed paraffin-embedded sections
(ホルマリン固定パラフィン包埋切片を用いた
血管免疫芽球性 T 細胞リンパ腫の *RHOA* G17V
変異の有無と臨床病理学的表現型の解析)

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先端医科学専攻

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- This doctoral thesis was written on the basis of the original article published in “the American Journal of Surgical Pathology”.
- I got permissions for the submission of the doctoral thesis to the institutional repository of Tokai University, in addition to reuse of part of my article in my doctoral thesis.

Summary

Angioimmunoblastic T-cell lymphoma (AITL) is an infrequent subtype of peripheral T-cell lymphoma derived from follicular helper T cells. Recently, a somatic G17V *RHOA* gene mutation has been reported. In this article, we examined the *RHOA* G17V mutation in 18 cases of AITL by 3 different techniques of Sanger sequencing, fully automated SNP genotyping, and deep sequencing, using routine diagnostic formalin-fixed paraffin-embedded tissue. The *RHOA* G17V mutation was detected in 10 cases (56%). Among the 10 mutated cases, 8 cases were detected by all 3 methods. The status of *RHOA* mutation was subsequently compared with the clinicopathologic characteristics of AITL. *RHOA*-mutated AITL (10 cases) was clinically characterized by high serum IL-2R and a poor ECOG performance status. By immunohistochemistry, expression of CD10, PD-1, CXCL13, and CCR4 and a wide distribution of CD21 (+) follicular dendritic cells were observed in *RHOA*-mutated cases. Among these, CCR4 expression and the CD21 (+) network in *RHOA*-mutated AITL cases were more extensive than in the *RHOA* mutation-negative AITL cases ($P < 0.05$). Thus, *RHOA*-mutated AITL cases are more characteristic of follicular helper T cells, and the presence of such a mutation is an important marker for AITL. Additionally, we examined 14 cases of peripheral T-cell lymphoma, nos (PTCL-NOS) and detected found *RHOA*-mutation in 3 cases of PTCL-NOS. These 3 cases showed Follicular helper T-cell phenotype, suggesting PTCL-NOS with AITL feature.

Background and Introduction

Angioimmunoblastic T-cell lymphoma (AITL) is an infrequent subtype of peripheral T-cell lymphoma (PTCL). AITL accounts for approximately 1% to 2% of malignant lymphoma and 15% to 20% of PTCL. Most patients are older than 50 years and have advanced disease at presentation, showing generalized lymphadenopathy with extranodal presentation. AITL is an aggressive lymphoma with an unfavorable prognosis, and the median survival time is <3 years, regardless of treatment type, including chemotherapy containing anthracycline. Unfavorable prognostic factors are reported to be age above 60 years, performance status (PS)>2, extranodal sites >1, the presence of B symptoms, low platelet count (prognostic index for AITL), elevated IgA and white blood cell levels, anemia, and thrombocytopenia (AITL prognostic index).

Almost all patients with AITL are diagnosed by excisional biopsy of the lymph node, which is characterized by a polymorphous infiltration of various hematopoietic cells, including clear T cells, proliferation of high endothelial venules, and hyperplasia of the follicular dendritic cell (FDC) meshwork. The neoplastic T cells are positive for the CD2, CD3, CD4, CD5, CD10, CXCL13, SAP, PD-1, and often BCL-6 antigens. This profile, as well as the molecular signature of AITL, suggests that the follicular helper T cells (TFH) may be a normal counterpart of AITL. Large-sized B cells positive for Epstein-Barr virus–encoded RNA (EBER) are observed scattered in most cases. Pathologic prognostic factors are reported to be a Ki-67 proliferation index >30% and transformed tumor cells >20%.

Although mutations presenting in various hematological malignancies, such as TET2, IDH2, and DNMT3A, have been described in AITL, the molecular pathogenesis specific to AITL remains unknown. Recently, the *RHOA* G17V mutation has been detected in AITL by means of whole-exome sequencing, Sanger sequencing, and quantitative allele-specific polymerase chain reaction (PCR). This mutation appears to be specific to T-cell lymphoma

and has been identified in 68% of AITL and 17% of cases of PTCL, not otherwise specified, but is absent in B-cell lymphoma. *RHOA* protein has GTPase activity and regulates the signal transduction pathway linking plasma membrane receptors to the assembly of focal adhesions and actin stress fibers. *RHOA* mutations functionally interfere with the binding of Rho-binding domain, resulting in an inactivating effect on GTPase activity. *RHOA* mutation also has an effect on cell proliferation and invasion. G17V mutant acts in a dominant-negative manner, similar to the T19N mutant, to promote cell proliferation. The effect of increased cell proliferation is due to the phosphorylation of the AKT kinase, which is regulated by the *RHOA*-ROCK-PTEN pathway. Cells expressing inactive *RHOA* such as G17V have increased AKT phosphorylation in comparison with wild-type *RHOA*. Furthermore, Jurkat and H9 (human cutaneous T-lymphocyte lymphoma) cells expressing inactive *RHOA* (G17V or T19N) have 60% more invasive capability than those expressing wild-type *RHOA* or G14V *RHOA*. *RHOA* also regulates the expression and function of the Nav1.5 channel in cancer cells, such as breast carcinoma-enhancing growth and metastatic dissemination.

In this project, we aimed to identify the *RHOA* G17V mutation in a series of AITL cases using routine diagnostic formalin-fixed paraffin-embedded tissue samples by Sanger sequencing, fully automated SNP genotyping, and deep sequencing. The status of *RHOA* mutation was then compared with the clinicopathologic characteristics of AITL. We demonstrated that *RHOA*-mutated AITL cases had overexpression of CCR4 and a wide distribution of FDC meshwork.

Discussion

In this project, we identified the *RHOA* G17V mutation by 3 different techniques: Sanger sequencing, fully automated SNP genotyping, and deep sequencing. In our homogenous series of 18 cases from a single institution, we demonstrated that the *RHOA* G17V and G17L

mutation was present in 56% of AITL cases. The rate of *RHOA* mutation in AITL has been previously reported to be 53% to 68%, which is consistent with our results. The G17V *RHOA* mutation is a recurrent mutation in AITL that can be detected using formalin-fixed paraffin-embedded tissue.

Among the 3 methods that we used in this paper to detect *RHOA* mutation, Sanger sequencing is the most conventional and well-used method. Next-generation sequencing (NGS) such as deep sequencing is one of the most sensitive and useful tools. This NGS approach allows researchers to detect a small population that represents as little as 1% of the sample, although NGS is time consuming and costly in comparison with the other methods, and the required equipments and facilities are limited. In contrast, fully automated SNP genotyping using the QP method, i-densy IS5320, is an easy and simple method. It takes only within an hour even using formalin-fixed paraffin-embedded sections, therefore it may be useful. One case (case 7) with a *RHOA* G17L (as predicted amino acid) mutation was classified as a variation of *RHOA* mutation, because the mutation was not silent. Because the QProbe for identifying the mutation was constructed to detect a G/T change at the *RHOA* c.50 position, the SNP genotyping was unable to detect this new mutation. Another case (case 14) showed *RHOA* mutation by deep sequencing but not by Sanger sequencing or fully automated SNP genotyping. Although the reason is not obvious it may be related to the sensitivity of the techniques and the necessary percentage cutoff of mutation-positive cells in the sample. Sanger sequencing and fully automated SNP genotyping are estimated to be effective in more than approximately 10% cutoff and 3% cutoff (in instruction manual), respectively. Ondrejka et al reported 3 discrepant cases between deep and Sanger sequences in *RHOA* mutation among 27 cases of AITL and suggested different sensitivity of the 2 methods. Therefore, it may be difficult to detect all the cases of <10%, for which deep sequencing is more effective. Thus, we identified a *RHOA* mutation in 10 of the 18 AITL

cases (56%).

In this series, we found 2 unusual cases. Although histology of the lymph node of case 9 corresponded to a typical AITL, it showed low expression of markers of TFH cells as well as no proliferation of FDC. *RHOA* mutation was not found in this case. Therefore, it is possible that a diagnosis of this case is PTCL with clear T cells. Another case (No. 7) had all features of AITL except the presence of EBER (+) cells. This case had the 2 consecutive G/T mutations resulting in the predicted G17L. Relationship between these 2 phenomena is, however, unknown.

We next aimed to identify the clinicopathologic differences between *RHOA*-negative AITL cases and *RHOA*-mutated AITL cases. Interestingly, *RHOA*-mutated AITL cases were more characteristic of TFH cells than *RHOA*-negative AITL cases. We investigated the phenotypes of the T cells in AITL by both flow cytometry and immunohistochemical techniques. Flow cytometry detected a positive fraction of CD10, a marker for TFH cells, and a higher number of CD25 (+) cells were found in many of the *RHOA*-mutated cases. When results of flow cytometry and immunohistochemistry were combined, it was found that CD10 was expressed in 8 of 10 cases with *RHOA*-mutated cases. In immunohistochemistry, although programmed cell death 1 (PDCD1, also known as PD-1) was expressed in many of the AITL cases irrespective of the mutational status, higher expression of CD10, chemokine (C-X-C motif) ligand 13 (CXCL13), and CCR4 was observed in *RHOA*-mutated cases. Moreover, a prominent, expanded CD21 (+) FDC network was often observed in *RHOA*-mutated cases compared with *RHOA*-negative cases ($P = 0.009$). These findings support the idea that AITL is a neoplasm derived from TFH cells, is clinically characterized by significant immune deregulation, and that the pathobiology may be partially associated with a defect in the mechanisms of immune homeostasis and tolerance. The greater expansion of FDCs and increased expression of TFH cell markers suggest that the *RHOA* mutational status may be

able to discriminate between the 2 subtypes of AITL with different biological and clinical features.

Interestingly, chemokine (C-C motif) receptor 4 (CCR4) positivity was observed in 7 of the 9 *RHOA*-mutated cases and in 3 of the 9 *RHOA*-negative cases. CCR4 expression was significantly higher in *RHOA*-mutated cases ($P = 0.046$). CCR4 is primarily expressed in regulatory T cells and helper T cells, where it functions to induce homing of these leukocytes to sites of immune inflammation. CCR4 is highly expressed by aggressive PTCLs, particularly adult T-cell leukemia/lymphoma (ATL) and cutaneous T-cell lymphomas. The expression of CCR4 is important because this marker can be targeted by glycol-engineered therapeutic antibodies, such as mogamulizumab, a drug already approved for the treatment of relapsed or refractory CCR4-positive ATL, CCR4-positive PTCL, and CCR4-positive cutaneous T-cell lymphoma. For ATL, the use of anti-CCR4 humanized monoclonal antibody may be a suitable choice for those who are ineligible for hematopoietic stem cell transplantation and/or in combination with mLSG15.

The expression of CCR4 was previously reported to be positive in 34.8% of AITL patients by Ishida and Ueda, which is in concordance and within our series' range of expression. Of note, we did not observe any differences in survival between the CCR4 (+) and CCR4 (-) AITL cases; therefore, the usefulness of anti-CCR4 in AITL remains to be established in a larger series.

Rho-specific inhibitor, for example, Rhosin, has been identified for *RHOA*-targeted therapy. Rhosin contains 2 aromatic rings tethered by a linker, and it binds to the surface area sandwiching Trp58 of RhoA with a submicromolar K_d and effectively inhibits GEF-catalyzed RhoA activation. By suppressing RhoA or RhoC activity Rhosin inhibits mammary sphere formation by breast cancer cells, suppresses invasion of mammary epithelial cells, and induces neurite outgrowth of PC12 cells in synergy with NGF. As *RHOA*

mutation is present in most AITL cases, it will be useful to identify *RHOA* mutation for judging adaptation of such treatment.

AITL is a distinct type of lymphoma with a postulated cell of origin of TFH cells. The pathogenesis and model of development of AITL is thought to be a multistep process. According to Sakata-Yanagimoto and colleagues, TET2 mutations coexisted in all cases of AITL and PTCL, not otherwise specified, with *RHOA* G17V mutation. The *RHOA* G17V mutation was a specific event in tumor cells. In contrast, TET2 and DNMT3A mutations seemed to have taken place in the non-neoplastic hematopoietic stem cells. Therefore, TET2 and/or DNMT3A mutation may occur before *RHOA* mutation and thus be a prerequisite for subsequent *RHOA* G17V and development of an AITL.

Conclusion

The *RHOA* mutation was detected using formalin-fixed paraffin-embedded tissue in 56% of the AITL cases. AITL cases with the *RHOA* mutation are characterized by the expansion of CD21 (+) FDCs, CCR4 (+) T cells, and CD25 (+) cells and are associated with higher sIL-2R levels and an unfavorable PS.