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**ROQUIN/RC3H1 Alterations Are Not Found in Angioimmunoblastic T-Cell Lymphoma**

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**Abstract**

Angioimmunoblastic T-cell Lymphoma (AITL) is one of the most frequent T-cell lymphoma entities. Follicular helper T lymphocytes (TFH) are recognized as the normal cellular counterpart of the neoplastic component. Despite a clonal T-cell feature and few described recurrent cytogenetic abnormalities, a driving oncogenic event has not been identified so far. It has been recently reported that in mice, heterozygous inactivation of Roquin/Rc3h1, a RING type E3 ubiquitine ligase, recapitulates many of the clinical, histological, and cellular features associated with human AITL. In this study we explored whether ROQUIN alterations could be an initial event in the human AITL oncogenic process. Using microarray and RT-PCR analyses, we investigated the levels of ROQUIN transcripts in TFH tumor cells purified from AITL (n = 8) and reactive tonsils (n = 12) and found similar levels of ROQUIN expression in both. Moreover, we also demonstrated that ROQUIN protein was expressed by AITL TFH (PD1+) cells. We then analysed ROQUIN coding sequence in 12 tumor cell-rich AITL samples and found no mutation in any of the samples. Finally, we analysed the expression of MiR101, a putative partner of ROQUIN involved in the modulation of ICOS expression and found similar levels of expression in tumor and reactive TFH. Altogether, this study shows that neither alteration of ROQUIN gene nor deregulation of miR101 expression is likely to be a frequent recurrent event in AITL.

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**Introduction**

Angioimmunoblastic T-cell Lymphoma (AITL) is a distinct T-cell lymphoma entity [1] originally described as a dysimmune condition [2]. It usually manifests with generalized lymphadenopathy, hepatomegaly, splenomegaly, fever, sweats, and skin rash and is frequently associated with clinical and biological autoimmune manifestations [3]. A clonal T-Cell Receptor gene rearrangement is detected in around 80% of the cases [4,5], and few recurrent cytogenetic abnormalities have been reported [reviewed in [6]]. Recently, we have reported mutations in isocitrate dehydrogenases 2 (IDH2) [7] and Ten-Eleven Translocation 2 (TET2) [8] genes in AITL, two genes involved in epigenetic gene regulation, but to date, no driving oncogenic event has been identified. We and others have shown that Follicular Helper T (THF) cells are the normal cellular counterpart of the neoplastic component of AITL [9–12]. THF cells constitute a specialized subset of T cells which allows the selection of high-affinity B lymphocytes within germinal centers and provide helper function for antibody production [13]. Human THF cells express high levels of BCL6, PD1, ICOS, the chemokine CXCL13 and its receptor (CXCR5) and secrete the cytokine IL-21 [14–18].

Recently, a mouse model has been proposed for AITL [19]. It recapitulates many of the clinical and pathological features associated with AITL, including lymphadenopathy, hypergammaglobulinemia and accumulation/expansion of clonal THF cells. This phenotype is specifically linked to heterozygous Roquin/Rc3h1 point mutation (sanroque allele) in T cells [20]. Roquin, a RING-type E3 ubiquitin ligase family member, has been previously identified as a regulator of autoimmune responses in mice [20]. We thus hypothesized that in human, ROQUIN/RC3H1 alterations could occur as an initial event of the AITL oncogenic process, leading to THF accumulation or proliferation prone to subsequent transforming events.

**Material and Methods**

The present study was approved by the institutional review board “Comité de Protection des Personnes, Créteil, France” (CPP 09–008). Written consent was obtained from patients with lymphoma. Reactive human tonsils were collected from children...
undergoing routine tonsillectomy. Oral information was given to parents. A consent form attesting the oral consent was signed by the surgeon and given to the research team with tonsils.

Cell samples and AITL tissues

Normal cell subsets were isolated from reactive human tonsils. Briefly, mononuclear cells were isolated by mechanical disruption followed by Ficoll-hypaque density gradient centrifugation. T<sub>FH</sub> cells were purified after depletion of CD19, CD8, CD14 and CD16-positive cells with magnetic beads (Milteny Biotec, Paris, France), by cell sorting of CD4<sup>+</sup>-FITC, CXCR5-PE and ICOS-PC7 triple-positive cells on Mo-Flo legacy (Beckman Coulter, Villepinte, France). Tonsil CD4<sup>+</sup>, CD8<sup>+</sup> T-cells and B-cells were purified by positive selection with antibodies directed against CD4, CD8, and CD19 respectively (Milteny Biotec, Paris, France).

Neoplastic T<sub>FH</sub> cells were isolated from cryopreserved mononuclear cell suspensions of AITL lymph node biopsies, through a one-step CD4<sup>+</sup>-FITC, CXCR5-PE and ICOS-PC7 cell sorting.

Twelve AITL tumor frozen tissue samples were selected on the basis of high tumor cell content. After complete immunostaining for T<sub>FH</sub> markers including PD1, ICOS and CXCL13, a semi-quantitative evaluation of tumor cells was performed as previously described [21] and cases with more than 50% tumor cells were selected for ROQUIN sequence analyses (Figure 1).

Immunohistochemistry

For in situ evaluation of ROQUIN expression, deparaffinised tissue sections of 8 AITL samples were stained with a polyclonal antibody (Novius biologicals NBP1-89590, Cambridge, United Kingdom) using a Vectastain immunoperoxidase method (Vector Labs, Peterborough, UK) and revealed with Diaminobenzidine (DAB). Specificity of the antibody was validated using NIH3T3 transfected with human full length ROQUIN cDNA (data not shown). The distribution and phenotypic characteristics of ROQUIN-positive cells in vivo was explored by double immuno-stainings for ROQUIN and either PAX5 (as a B-cell marker) and PD1 (as T<sub>FH</sub> marker). Briefly, cases were first stained for ROQUIN using an immunoperoxidase method (Vectastain), then for PAX5 ((DakoCytomation, Glostrup, Denmark) or PD1 (Figure 3).

Figure 1. Illustrative case of AITL rich in tumor cells. Diffuse proliferation of large neoplastic cells surrounded by inflammatory cells (plasma cells, eosinophils) and vascular hyperplasia (hematoxylin-eosin, original magnification ×20) (A). Numerous neoplastic cells highlighted by positivity for T<sub>FH</sub> markers CXCL13 (B), ICOS (C) and PD1(D) (immunoperoxidase, original magnification ×20). doi:10.1371/journal.pone.0064536.g001

Figure 2. ROQUIN expression in human reactive and tumoral lymphoid samples. Levels of ROQUIN transcripts determined by gene expression profiling of 17 AITL tumor tissue samples and 2 AITL cell suspensions enriched in tumor cells (≥50%) samples as previously reported [10]. (probe-set 228996_at): ROQUIN transcripts level is slightly higher in enriched tumor cell sample (P = 0.0067 unpaired t-test) (A). ROQUIN mRNA levels determined by quantitative RT-PCR in reactive tonsils: total extract (n = 1), CD4<sup>+</sup> (n = 2), CD8<sup>+</sup> (n = 2), or CD19<sup>+</sup> (n = 2) lymphocytes. Results were normalized by HPRT and compared to reactive purified T<sub>FH</sub> cells as calibrator: reactive CD4<sup>-</sup>CD8<sup>-</sup>- and CD19<sup>-</sup>-positive subsets display heterogeneous levels of ROQUIN mRNA (B). ROQUIN mRNA levels ([228996_at probeset] in purified reactive (n = 12) and neoplastic T<sub>FH</sub> cells (n = 8). T<sub>FH</sub> cells were purified from 12 reactive tonsils and 8 AITL lymph nodes. RNA was extracted and whole genome expression was analysed on HG-U133 plus 2.0 Affymetrix GeneChip arrays. Similar levels of ROQUIN transcript are observed (C).

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Figure 3. Immunohistochemical detection of ROQUIN in AITL. Among the many cells showing a cytoplasmic granular staining for ROQUIN (brown), a few are PAX5-positive large cells (B-immunoblasts) (pink arrow) whereas most of them are small to medium-sized PAX5-negative lymphoid cells forming small aggregates, corresponding to neoplastic cells of AITL (black arrows) (A). In addition, these aggregates of medium-sized ROQUIN-positive cells (brown, granular staining) co-expressed the T<sub>FH</sub>-associated marker PD1 (red, membrane staining) (B). Double immunohistochemistry, original magnification ×250. doi:10.1371/journal.pone.0064536.g003
The level of ICOS mRNA expression is maintained even in the presence of ROQUIN transcripts both in human reactive and tumor T\(_{FH}\) cells (A). Level of miR101 (has-miR-101) is low and similar in both tumor and reactive T\(_{FH}\) cells (p = 0.8, unpaired t-test, NS) (B).

**Results and Discussion**

The levels of ROQUIN transcripts are similar in neoplastic and reactive T\(_{FH}\) cells

The analyses of ROQUIN probesets in our previously published transcriptomic dataset [12] disclosed the presence of ROQUIN transcripts in 17/17 AITL tissue samples with a slightly higher transcriptomic dataset [12] or for microRNA gene expression profiling on Agilent Human v3 miRNA microarray (G4471A, Agilent, Santa Clara CA). Analyses of gene expression profiles focused on probesets matching to ROQUIN (228996_at), ICOS (210439_at), and on miR101 (has-miR-101). The levels of ROQUIN and ICOS transcripts in reactive and neoplastic T\(_{FH}\) were determined by TaqMan quantitative reverse-transcriptase PCR (qRT-PCR; Applied Biosystems) on Light Cycler 480, after normalization to HPRT mRNA, and compared to purified reactive T\(_{FH}\) according to the 2\(^{-}\text{ΔΔCT}\) method.

**Sequence analyses**

ROQUIN cDNA was amplified by PCR in 3 fragments encompassing the coding sequence. Direct sequencing of PCR products was performed for the first two 5’ fragments. A cloning phase was necessary for exons 16 to 19 sequencing due to alternative splicing. Sequences were obtained from 12 AITL samples with a high tumor load as well as normal CD4\(^{+}\)/CXCR5\(^{+}\)/ICOS\(^{+}\) sorted T\(_{FH}\) cells obtained from reactive tonsils (n = 12) and AITL (n = 8) on Affymetrix microarray. Similar levels of ROQUIN transcripts were observed in T\(_{FH}\) purified either from reactive tonsils or from AITL lymph nodes (Figure 2C), thus excluding the hypothesis of a ROQUIN extinction by promoter alteration or gene expression dysregulation in AITL.

**ROQUIN protein is expressed by AITL tumor cells**

In situ evaluation of the pattern of ROQUIN expression was performed by immunohistochemistry. In all eight AITL cases investigated, numerous cells showing a granular cytoplasmic staining were observed. These comprised scattered large cells resembling B-blasts, smaller lymphocytes and many small to medium-sized atypical cells suggestive of the neoplastic cell component, as well as endothelial cells (Figure 3A). Double immunostainings performed in 4 cases demonstrated that most ROQUIN-positive cells were PAX5-negative and that many of them expressed PD1, therefore sharing the characteristic morphological and phenotypic features of neoplastic T\(_{FH}\) cells (Figure 3B). Furthermore, the observed granular cytoplasmic staining is compatible with Roquin localization in P bodies or stress granules as reported in the mouse [23,24].

**ROQUIN coding sequence is not mutated in human AITL**

We next investigated the presence of missense mutations in ROQUIN coding sequence. The 3402 bp ROQUIN coding sequence was obtained from 12 AITL samples with a high tumor load as well as normal CD4\(^{+}\) T cells sorted from 2 reactive tonsils. In contrast to Sanroque mice that develop a T\(_{FH}\) cell lymphoproliferative disorder with several symptoms of AITL including auto-immune manifestations and organomegaly as a result of Roquin mutations [19], no mutation was found in any of the AITL patients.

ICOS and miR101 expression are similarly expressed in reactive and AITL T\(_{FH}\) cells

Physiologically, in mice, Roquin limits ICOS expression by promoting the degradation of ICOS mRNA in a dose-dependent manner [24,25]. In sanroque mice, mutated Roquin is unable to promote ICOS mRNA degradation, resulting in the overexpression of the protein. Here, we show that the level of ICOS mRNA expression is maintained even in the presence of ROQUIN transcripts both in human reactive and tumor T\(_{FH}\) cells.
shown here that neither alteration of pathways deregulated by recurrent abnormality in AITL. Expanding knowledge on the deregulation of miR101 expression is likely to be a frequent accordance with recent finding in mouse showing that BCL6 could repress inhibitors of specific Tfh expressing gene including miR101 [29].

Conclusion

Altogether, by comparing reactive and AITL Tfh cells, we have shown here that neither alteration of ROQUIN gene nor deregulation of miR101 expression is likely to be a frequent recurrent abnormality in AITL. Expanding knowledge on the pathways deregulated by Roquin mutation in Sanroque mice might uncover other molecules of potential relevance to AITL pathophysiology.

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Author Contributions

Conceived and designed the experiments: MHDL TA MT LiL. AdR KT PA. Performed the experiments: TA MT LiL. AdR KT PA CA NM. Analyzed the data: TA MHL1 MT AdR NM PG. Contributed reagents/materials/analysis tools: KT PA AdR. Wrote the paper: MHL1 TA PG LiL.

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