Loss of 5-hydroxymethylcytosine is a frequent event in peripheral T-cell lymphomas

Mutations in the epigenetic regulators *TET2, IDH2,* and *DNMT3A* are frequent in peripheral T-cell lymphomas (PTCL), especially in those derived from T follicular helper (TFH) cells. However, the consequences of these alterations on overall levels of 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) are unknown. Here, using an immunohistochemical method to detect 5hmC and 5mC *in situ* in PTCL samples, we showed that PTCL tumor cells had lower 5hmC levels than normal T cells, independently of the presence of *TET2, IDH2,* or *DNMT3A* mutations. We observed the loss of 5hmC, not only in TFH-derived PTCL, but also in various nodal or extranodal PTCL entities, with the exception of hepatosplenic T-cell lymphomas. These results suggest that 5hmC loss is a general event in PTCL lymphomagenesis.

TET2, IDH2, and *DNMT3A* are three genes whose products are involved in the control of cytosine methylation and are frequently mutated in myeloid neoplasms. TET2 is a α-ketoglutarate-dependent dioxygenase involved in 5mC hydroxylation to form 5hmC, an epigenetic mark involved in cytosine demethylation.[1] Mutation of *IDH2* confers a neomorphic activity to this enzyme, leading to the production of D-2 hydroxyglutarate, an oncometabolite that is able to inhibit α-ketoglutarate-dependent dioxygenases, including TET2.[1] In acute myeloid leukemia, *TET2* and *IDH2* mutations are mutually exclusive, and are both associated with decreased 5hmC levels and a similar methylation profile[1]. DNMT3A is involved in *de novo* methylation and *DNMT3A* mutations affect its activity.[1,2] Although the consequences of these three mutations on DNA methylation levels are unclear, they are all expected to additively result in deregulated methylation and decreased 5hmC levels.

These three genes are frequently mutated in PTCL, especially in angioimmunoblastic T-cell lymphomas (AITL) in which *TET2* mutations are detected in up to 80% of cases, *IDH2* in 30%, and *DNMT3A* in 30%.[3-8] Although *TET2* and *DNMT3A* mutations can be found in several PTCL entities, they appear to be enriched in AITL and nodal PTCL with TFH phenotype, whereas *IDH2* mutations are only present in AITL.[6,7] Although somatic, *TET2* and *DNMT3A* mutations can be found not only in tumor cells, but also in CD34$^+$ hematopoietic progenitors or reactive cells, suggesting they may be an early event during lymphomagenesis,[3,6] whereas *IDH2* mutations likely occur as a second event.[9] Moreover, *IDH2* mutations frequently coexist with *TET2* mutations in AITL.[6,8,9] Altogether, at least one of these mutations is present in 80% of TFH-derived lymphomas, but their functional consequences on 5hmC/5mC are poorly understood.[10]

We explored the epigenetic consequences of these mutations by assessing 5hmC and 5mC levels in six reactive lymphoid tissues (2 lymph nodes and 4 tonsils), and a series of 71 PTCL with *TET2, IDH2* and *DNMT3A* sequencing data selected within the framework of a multicenter T-cell lymphoma consortium (Tenomic) (*see Online Supplementary Material*).

Due to the common paucity in tumor cells in AITL, we chose to evaluate 5hmC and 5mC in AITL using an established immunohistochemistry assay involving anti-5hmC and anti-5mC antibodies on de-paraffinized tissue sections which allows an *in situ* evaluation that can semi-quantitatively discriminate 5hmC and 5mC levels in tumor cells *versus* those from the surrounding environment. Indeed, a common, characteristic histopathological feature of AITL is the prominent tumor environment, with tumor cells usually representing only a minor component.

We first analyzed the distribution of 5hmC and 5mC in normal lymph nodes and tonsils, stained with anti-5hmC and anti-5mC antibodies (Figure 1 and *Online Supplementary Figure S1*). Cells in the interfollicular areas with a high T-cell content, were mostly positive for 5hmC, whereas almost all the germinal center cells were negative (Figure 1A). However, a few scattered cells were strongly positive for 5hmC. These positive cells were identified as follicular dendritic cells, macrophages and PD1-positive T cells, which are likely TFH cells given their phenotype and their location inside the germinal center (Figure 1B-D).

Table 1. Description of the PTCL cases included in the study.

	Number of cases	5hmC in tumor cells	5mC in tumor cells
AITL	30	0	30
Mutated AITL	14	0	14
WT AITL	16	0	16
PTCL-NOS[a]	10	0	10
ALK-ALCL[b]	3	0	3
ALK+ALCL	3	0	3
EATL	2	0	4
MEITL	16	0[c]	16
HSTL	3	3	3
ENKTCL	4	0	4
Total	71	3	52
Mutated cases	19	0	19
WT cases	52	3	33

"Mutated" designates cases with identified *TET2, IDH2*, and/or *DNMT3A* mutations in tumor tissues, WT designates the absence of *TET2, IDH2,* or *DNMT3A* mutations, PTCL-NOS: peripheral T-cell lymphoma not otherwise specified, ALK-ALCL: ALK-negative anaplastic large cell lymphoma, ALK+ALCL: ALK-positive anaplastic large cell lymphoma, EATL: enteropathy-associated T-cell lymphoma (type 1); MEITL: monomorphic epitheliotropic intestinal T-cell lymphoma; HSTL: hepatosplenic T-cell lymphoma, ENKTCL extranodal NK/T-cell lymphoma, nasal type. [a]including four *TET2, DNMT3A* and/or *IDH2* mutated PTCL-NOS. [b]including one *TET2* mutated ALK-ALCL. [c]including four cases in which a minority (<20%) of tumor cells presented down regulated but detectable levels of 5hmC.

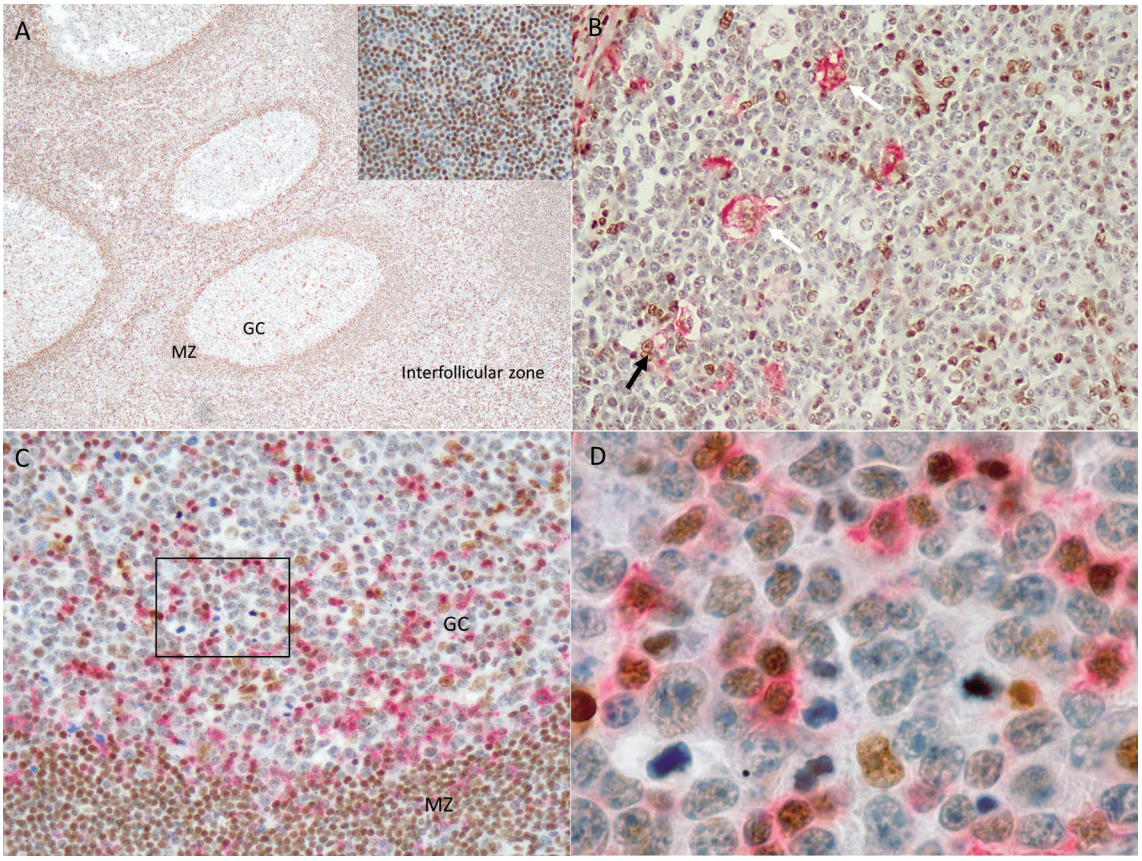

Figure 1. Characterization of cells positive for 5-hydroxymethylcytosine and 5-methylcytosine in a representative case of reactive tonsil with follicular hyperplasia. (A) 5hmC immunohistochemistry staining showing scattered positive cells within a germinal center (GC) contrasting with the positivity of most cells in the mantle zone (MZ) or interfollicular cells (inset). (B) Scattered 5hmC positive cells (brown) in the germinal center are CD163 positive (red, white arrow), or are binucleated as typical for follicular dendritic cells (black arrow). (C and D) Most cells stained for 5hmC (brown) in the germinal center are also PD1-positive (red) by double staining for PD1. Original magnification: objective X10 (A) and X40 (B, C) and X100 (D).

These results suggest that normal TFH cells have a high level of 5hmC. In contrast, 5mC staining showed a high level of 5mC in germinal centers and in the interfollicular zone (*Online Supplementary Figure S2*).

We then examined the levels of 5hmC and 5mC in AITL samples representative of various mutational patterns for *TET2, IDH2,* and *DNMT3A*. Staining for 5hmC showed a variable number of positive cells among samples, paralleling the variable content of reactive and tumor cells in AITL. 5hmC-positive cells were generally scattered with morphological features suggestive of cells from the microenvironment (Figure 2A,B). We confirmed the lack of detectable 5hmC in neoplastic cells by double immunohistochemical staining which showed the absence of 5hmC in PD1-positive AITL tumor cells (Figure 2C). We also confirmed the absence of detectable 5hmC in *IDH2*-mutated AITL tumor cells using an antibody specifically directed against the R172K mutant form of the IDH2 protein in IDH2R172K-mutated AITL samples[9] (Figure 2D). Among the 30 cases of AITL, nine carried *TET2* mutations, 12 *IDH2* mutations, six *DNMT3A* mutations, and 16 cases were wild-type for these three genes, five of them being sequenced by targeted deep sequencing at a mean coverage of 1219X to increase the sensitivity of the sequencing method (*see the Online Supplementary Methods*) and limit the risk of missing a mutation (Table 1 and *Online Supplementary Tables S1 and S2*). No 5hmC was observed in the neoplastic cells of any of the cases (Figure 2E-G). Together with the presence of 5hmC in normal TFH, these findings indicate the loss of 5hmC in AITL tumor cells regardless of the mutational status of these three epigenetic modifier genes. The presence of 5hmC-positive cells in the environment in every AITL case highlights the accuracy of using *in situ* methods such as immunohistochemistry or cell-sorted samples for studying epigenetic changes in AITL tumor cells.

This observation led us to extend our examination of 5hmC levels to other PTCL entities. As for AITL, there was no detectable 5hmC in the tumor cells of almost all investigated PTCL samples including ten PTCL-not otherwise specified, three ALK-positive anaplastic large cell lymphomas, three ALK-negative anaplastic large cell lymphomas, two enteropathy-associated T-cell lymphomas, 16 monomorphic epitheliotropic intestinal T-cell lymphomas and four extranodal NK/T cell lymphomas, nasal type (Table 1 and *Online Supplementary Tables S1, S2 and S3*) with the exception of three of three hepatosplenic T-cell lymphomas, in which neoplastic cells in the sinuses stained positively for 5hmC (*Online Supplementary Figure S3*). Detection of 5hmC in hepatosplenic T-cell lymphomas is intriguing but does not seem to be associated with the presence of a *SETD2* mutation or γδ origin, as *SETD2* and γδ T-cell receptor-positive monomorphic epitheliotropic intestinal T-cell lymphomas showed low levels of 5hmC (*Online Supplementary Table S3*). A variable proportion of reactive

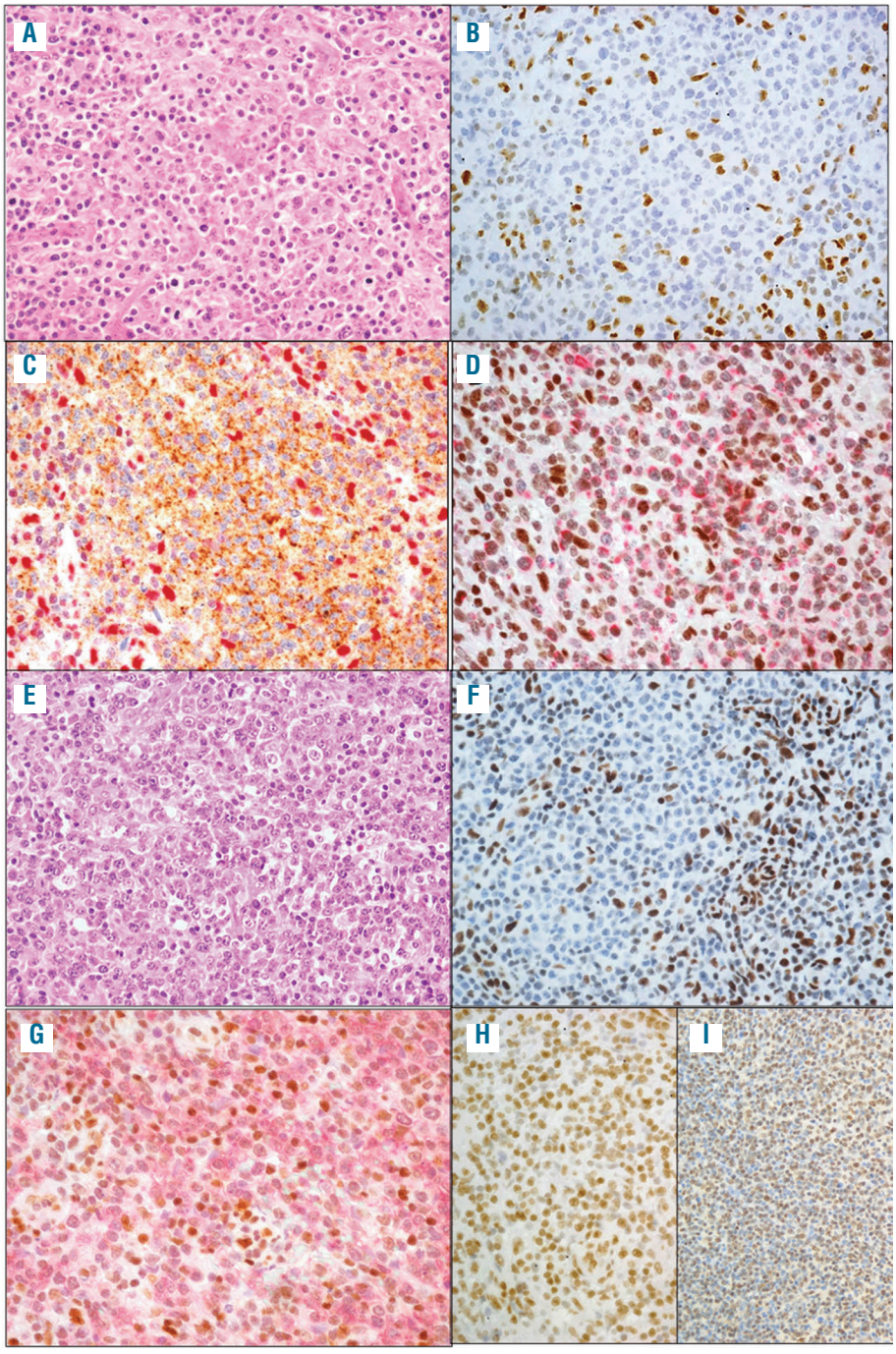

Figure 2. Characterization of cells positive for 5-hydroxymethylcytosine and 5-methylcytosine in mutated or wild-type angioimmunoblastic T-cell lymphoma. (A-D) *TET2, IDH2,* and *DNMT3A* mutated AITL (case 397) (A) Hematoxylin and eosin stain (HES), (B) 5hmC immunohistochemistry staining showing a few scattered cells with nuclear staining suggestive of cells of the environment. (C) Double immunostaining showing that the PD1-positive cells with membrane staining (brown) do not have detectable levels of 5hmC in their nuclei (red). (D) In this case with a *IDH2*R172K mutation, neoplastic cells with granular cytoplasmic staining for the *IDH2*R172K mutant (red) lack 5hmC (brown). (E-G) *TET2*, *IDH2*, and *DNMT3A* WT AITL (case 007) (E) Hematoxylin and eosin stain (HES). Similar observations were made in a representative case of WT AITL with (F) scattered cells positive for 5hmC staining, (G) lack of detectable 5hmC (brown) in neoplastic PD1-positive lymphocytes (red). (H and I) 5mC is found in the nuclei of virtually all cells in cases 397 and 007. Original magnification: objective X40.

cells were strongly stained by the 5hmC antibody in all PTCL samples, and served as internal positive controls.

In contrast, virtually all cells, including neoplastic cells, of all PTCL samples stained for 5mC. We failed to demonstrate any variation in the level of 5mC among samples including any dependency on the PTCL entity, within the limit of the sensitivity of the method (Figure 2H,I).

TET2 or *IDH2* mutations are expected to impair 5hmC formation in mutated AITL. We did indeed demonstrate the lack of 5hmC in the neoplastic T cells of most PTCL, but the lack of detectable 5hmC was not restricted to *TET2-, IDH2-*, or *DNMT3A*-mutated AITL. However, this immunohistochemical assay was recently used in acute myeloid leukemia samples, in which TET2 mutated samples displayed lower 5hmC levels than wild-type samples,[11] in adult T-cell leukemia/lymphoma, in which aggressive forms showed lower 5hmC levels than indolent forms[12] and in B-cell lymphomas, in which the level of 5hmC depends on the cell of origin.[13] These three independent reports suggest that immunohistochemistry is a reliable method for evaluating 5hmC levels in hematologic malignancies.

Although it is a step toward cytosine demethylation, the exact function of 5hmC in normal and malignant biology is still elusive. Loss of 5hmC occurs in several types of cancer in which it is associated with tumor progression[12,14] or a

proliferative state.[15] In PTCL, however, loss of 5hmC was essentially constant irrespective of the proliferation index (*Online Supplementary Figure S4*). The mechanism for the decrease of 5hmC levels in PTCL in the absence of genetic alterations in the *TET2, DNMT3A*, and *IDH2* epigenetic modifiers needs to be clarified, focusing particularly on whether it could involve undetected genomic anomalies in these genes, metabolic causes inhibiting TET function, TET expression down-regulation, *TET1* or *TET3* alterations or yet other mechanisms.[1]

Regardless of the mechanism, our findings highlight the high frequency of epigenetic changes in PTCL, which may not be restricted to patients bearing mutations in *TET2, DNMT3A*, or *IDH2*. Whether these epigenetic anomalies could be specifically targeted, using hypomethylating agents or other drugs, and which patients could benefit from these treatments, need to be determined in further studies.

François Lemonnier,[1,2] Elsa Poullot,[2,3*] Aurélie Dupuy,[1,2] Lucile Couronné,[4] Nadine Martin,[1,2] Laurianne Scourzic,[4] Virginie Fataccioli,[1,2,3] Julie Bruneau,[5] Rob A Cairns,[6] Tak W. Mak,[6] Olivier A. Bernard,[4] Laurence de Leval[7] and Philippe Gaulard[1,2,3]*

[1]INSERMU955 équipe 9, Institut Mondor de Recherche Biomédicale, Créteil, France; [2]Université Paris-Est, Faculté de Médecine, Créteil, France; [3]Département de Pathologie, Groupe Henri-Mondor Albert-Chenevier, AP-HP, Créteil, France; [4]INSERMU1170, Institut Gustave Roussy, Université Paris-Sud, Villejuif, France; [5]Département de Pathologie, Hôpital Necker Enfants Malades, APHP, Paris, France; [6]Campbell Family Institute for Breast Cancer Research, Princess Margaret Hospital, Toronto, Canada and [7]Institute of Pathology, Lausanne University Hospital, Lausanne, Switzerland

**These authors contributed equally to this paper*

Acknowledgments: the authors thank all members of the TENOMIC program. FL received a grant from the Institut National du Cancer (INCa).

Funding: this study was supported by a grant from the Association pour la Recherche contre le Cancer (ARC), the grant N. DEQ20160334875 "Equipe FRM 2016" from Fondation pour la Recherche Médicale and the Leukemia Lymphoma Society (LLS).

Correspondence: philippe.gaulard@aphp.fr
doi:10.3324/haematol.2017.167973

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Scourzic L, Mouly E, Bernard OA. TET proteins and the control of cytosine demethylation in cancer. Genome Med. 2015;7(1):9.
2. Scourzic L, Couronné L, Pedersen MT, et al. DNMT3AR882H mutant and Tet2 inactivation cooperate in the deregulation of DNA methylation control to induce lymphoid malignancies in mice. Leukemia. 2016;30(6):1388–1398.
3. Quivoron C, Couronné L, Della Valle V, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. Cancer Cell. 2011; 20(1):25–38.
4. Lemonnier F, Couronné L, Parrens M, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. Blood. 2012;120(7):1466–1469.
5. Couronné L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. N Engl J Med. 2012;366(1):95–96.
6. Sakata-Yanagimoto M, Enami T, Yoshida K, et al. Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. Nat Genet. 2014; 46(2):171–175.
7. Cairns RA, Iqbal J, Lemonnier F, et al. IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. Blood. 2012;119(8):1901–1903.
8. Odejide O, Weigert O, Lane AA, et al. A targeted mutational landscape of angioimmunoblastic T-cell lymphoma. Blood. 2014; 123(9):1293–1296.
9. Lemonnier F, Cairns RA, Inoue S, et al. The IDH2 R172K mutation associated with angioimmunoblastic T-cell lymphoma produces 2HG in T cells and impacts lymphoid development. Proc Natl Acad Sci. 2016;201617929.
10. Wang C, McKeithan TW, Gong Q, et al. IDH2R172 mutations define a unique subgroup of patients with angioimmunoblastic T-cell lymphoma. Blood. 2015;126(15):1741–1752.
11. Magotra M, Sakhdari A, Lee PJ, et al. Immunohistochemical loss of 5-hydroxymethylcytosine expression in acute myeloid leukaemia: relationship to somatic gene mutations affecting epigenetic pathways. Histopathology. 2016;69(6):1055–1065.
12. Marçais A, Waast L, Bruneau J, et al. Adult T cell leukemia aggressivenness correlates with loss of both 5-hydroxymethylcytosine and TET2 expression. Oncotarget. 2016;8(32):52256-52268.
13. Matsuda I, Imai Y, Hirota S. Distinct global DNA methylation status in B-cell lymphomas: immunohistochemical study of 5-methylcytosine and 5-hydroxymethylcytosine. J Clin Exp Hematop. 2014; 54(1):67–73.
14. Lian CG, Xu Y, Ceol C, et al. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell 2012;150(6):1135–1146.
15. Jin S-G, Jiang Y, Qiu R, et al. 5-Hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with IDH1 mutations. Cancer Res 2011;71(24):7360–7365.