**IDH2**R172 mutations define a unique subgroup of patients with angioimmunoblastic T-cell lymphoma

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Running title: IDH2 mutations in AITL

Abstract: 218
Number of words in text: 3991
Number of tables/figures: 2/7
Number of supplemental tables/figures: 4/9
Supplemental section: one word file
Number of references: 52

Category: Lymphoid Neoplasm
Keywords: Angioimmunoblastic T cell lymphoma, IDH2 mutations, epigenetic regulation

Presented in part, at the 56th American Society of Hematology (ASH) annual meeting, San Francisco, CA, December 6th-9th, 2014
Key Points

- $IDH2^{R172}$ mutations define a unique subgroup with distinct $T_{FH}$-like gene expression signatures in AITL.
- $IDH2^{R172}$ mutations can induce DNA and repressive histone hypermethylation in AITL.
Abstract

Angioimmunoblastic T-cell lymphoma (AITL) is a common subtype of peripheral T-cell lymphoma (PTCL) with a poor prognosis. We performed targeted resequencing on 92 cases of PTCL and identified frequent mutations affecting TET2, DNMT3A, and isocitrate dehydrogenase 2 (IDH2). While IDH2 mutations are largely confined to AITL, mutations of the other two can be found in other types of PTCL although at lower frequencies. These findings indicate a key role of epigenetic regulation in the pathogenesis of AITL. However, the epigenetic alterations induced by these mutations and their role in AITL pathogenesis are still largely unknown. We correlated mutational status with gene expression and global DNA methylation changes in AITL. Strikingly, AITL cases with IDH2R172 mutations demonstrated a distinct gene expression signature characterized by down-regulation of genes associated with Th1 differentiation (e.g., STAT1 and IFNG) and a striking enrichment of an IL12-induced gene signature. Ectopic expression of IDH2R172K in the Jurkat cell line and CD4+ T cells led to markedly increased levels of 2-hydroxyglutarate, histone-3 lysine methylation, and 5-methylcytosine and a decrease of 5-hydroxymethylcytosine. Correspondingly, clinical samples with IDH2 mutations displayed a prominent increase in H3K27me3 and DNA hypermethylation of gene promoters. Integrative analysis of gene expression and promoter methylation revealed recurrently hypermethylated genes involved in TCR signaling and T cell differentiation that likely contribute to lymphomagenesis in AITL.
**Introduction**

Peripheral T-cell lymphoma (PTCL) is a heterogeneous group of generally aggressive lymphoid malignancies, accounting for 10-15% of all non-Hodgkin lymphomas (NHLs). Angioimmunoblastic T-cell lymphoma (AITL) represents 20-25% of all PTCLs and is recognized as a distinct entity. Currently used chemotherapy is rarely curative with a 5-year overall survival of less than 30%. Gene expression profiling (GEP) and pathological analysis suggest that the follicular helper T-cell (T<sub>FH</sub> cell) is the cell of origin for AITL.

We and others have identified frequent mutations affecting isocitrate dehydrogenase 2 (IDH2) at arginine-172 (R172), ten-eleven translocation 2 (TET2), DNA methyltransferase 3A (DNMT3A), CD28, and RHOA in AITL. IDH2, TET2, and DNMT3A mutations may all affect epigenetic modifications. TET2 and DNMT3A mutations seem to occur at an early stage of hematopoietic cell differentiation, as these mutations have been found in non-malignant hematopoietic cells of several PTCL cases and in normal elderly individuals. Loss-of-function mutations of DNMT3A have been observed in several hematological malignancies, leading to DNA hypomethylation. Mitochondrial IDH2 protein and cytosolic IDH1 protein are homologues. Surprisingly, unlike other malignancies, AITL harbors mutations only at IDH2<sup>R172</sup>, but not at IDH2<sup>R140</sup> or in IDH1. In the tricarboxylic acid cycle, IDH3 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG). Under anabolic conditions, IDH2 primarily catalyzes the reverse reaction, reductive carboxylation of α-KG to isocitrate. The IDH2<sup>R172</sup> mutation confers a neomorphic activity—namely, conversion of α-KG to the R-enantiomer of 2-hydroxyglutarate (R-2-HG), which, in normal cells, is maintained at low concentrations by 2-HG dehydrogenase. R-2-HG acts as a competitive antagonist of the α-KG-dependent dioxygenases, which notably include the TET family of 5-methylcytosine hydroxylases and jumonji-C domain-containing histone demethylases (JHDMs). Thus, mutant IDH2<sup>R172</sup> is predicted to impair DNA and histone demethylation and leads to abnormal regulation of gene transcription, which may promote lymphomagenesis.
The epigenetic alterations induced by \textit{IDH2}, \textit{TET2}, and \textit{DNMT3A} mutations and their roles in AITL pathogenesis are still largely unknown. The biochemical and functional consequences of \textit{IDH2}^{R172} mutations in T cells have not yet been elucidated. In this study, we performed targeted re-sequencing of these three epigenetic regulators in molecularly defined PTCL cases. We also analyzed the biochemical changes associated with \textit{IDH2}^{R172} mutations and investigated alterations in GEP, DNA methylation and histone modification to improve our understanding of their roles in the pathogenesis of AITL.
Materials and methods

Patient samples, T-cell line and normal CD4+ T cells

We included 90 molecularly defined PTCL cases with GEP and clinical outcome data as well as two pathologically diagnosed cases (Table 1). This study was approved by the Institutional Review Board of the University of Nebraska Medical Center and City of Hope Medical Center. Jurkat cells (Clone E6-1, ATCC® TIB-152™) were cultured in 10 mM HEPES-buffered RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37 ºC in a 5% CO2 atmosphere. Normal human CD4+ T cells were isolated from peripheral blood lymphocytes of healthy donors (Miltenyi Biotec) and were cultured in medium as mentioned above with IL-2 (30 U/ml) (R&D systems) and anti-CD3/CD28 Dynabeads (Invitrogen).

Targeted re-sequencing of TET2, DNMT3A and RHOA and mutational analysis of IDH2

We used TruSeq Custom Amplicon (TSCA) design (Illumina) to interrogate the entire coding regions of TET2, DNMT3A, and RHOA in genomic DNA. Twenty-five cycles of amplification were used for 250 ng genomic DNA per sample. We used the Mutascope algorithm for sequence analysis. Mutational analysis of the IDH2 gene was performed by bi-directional Sanger sequencing and pyrosequecing (supplementary methods) using primers that specifically amplify R140 or R172 regions.

Gene expression profiling (GEP) and pathway analysis

GEP was performed and reported in a previous study using HG-U133-plus 2.0 arrays (Affymetrix). BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html) was used for unsupervised hierarchical clustering and for determining significant differences in gene expression among pre-defined groups: IDH2/TET2 double-mutant, TET2 single-mutant and wild-type AITL using normalized log2-transformed signals. We used Ingenuity pathway analysis (IPA, Qiagen) to perform functional annotation. The GSEA algorithm (http://www.broad.mit.edu/gsea) was used to identify a leading edge set of the genes that best distinguished IDH2/TET2 double-mutant and TET2 single-mutant AITL.

Reduced Representation Bisulfite Sequencing (RRBS) for methylation analysis
RRBS was performed as described\textsuperscript{25} with some modifications. Alignment rates and coverage are shown in supplemental Figure 1. Full details are given in the supplemental methods.

**Histone trimethylation analysis by Immunohistochemistry**

We performed immunohistochemical staining on tissue microarray sections of AITL with 1:100 rabbit polyclonal anti-H3K27me3 (07-449, Millipore) and 1:400 rabbit polyclonal anti-H3K9me3 (ab8898, Abcam) as previously described.\textsuperscript{26} The stained sections were scanned by Ventana’s iScan Coreo slide scanner (Ventana Medical Systems) using a 40 x 0.25 objective and exported into ImageScope analysis software (Leica Microsystems) for quantification. The signal threshold for nuclei in the negative control was determined and applied to all scanned images. Areas above the signal threshold were automatically measured and divided into weak-positive, positive and strong-positive based on the signal intensities.

**Protein/ histone extraction and western blotting**

Whole cell lysates were prepared as previously described\textsuperscript{27}, and histones were isolated using a histone extraction kit (ab113476, Abcam). Protein was separated by 10% SDS-PAGE and transferred to PVDF membrane (Bio-Rad).\textsuperscript{27} After incubation with primary antibody (listed in supplemental Table 3) overnight at 4°C, the membrane was washed and incubated with anti-mouse IgG HPR-linked secondary antibody (7076S, Cell Signaling) at 1:10,000 or anti-rabbit IgG HPR-linked secondary antibody (7074S, Cell Signaling) at 1:10,000 according to the primary antibody used. A Western ECL substrate (170-5060, Bio-Rad) was used for detection of HRP.

**LC-MS/MS analysis of 2-HG, 5mC and 5hmC in genomic DNA**

2-HG was quantified using reverse-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as previously described.\textsuperscript{28} DNA hydrolysis was performed as described previously\textsuperscript{29} with slight modifications. 5mC and 5hmC levels were determined by mass spectrometry, and details are described in the supplemental methods.

**Statistical analyses**

Details are given in the supplemental methods.
Results

Mutations affecting epigenetic regulators are more frequent in AITL than other subtypes of PTCL

Consistent with our previous report, mutations in AITL were observed in IDH2 at R172, but not at R140, unlike in other cancer types. The overall frequency of IDH2R172 mutations in AITL was 32.8% (19/58), including cases from our previous cohort (Figure 1A). In contrast, the IDH2R172 mutation occurred in only 1 of 24 PTCL, not otherwise specified (PTCL-NOS) cases, which was molecularly classified into the TBX21 subgroup by GEP analysis. The most common mutant variants were arginine to serine or lysine, similar to the IDH2 mutational spectrum in AML. There was no difference in IDH2 mRNA expression levels between the IDH2R172 mutant and wild-type cases (supplemental Figure 2A). Unlike the findings in AML, where IDH2R172 mutations are associated with a poor prognosis, no association of IDH2R172 mutations with overall survival (OS) was observed (supplemental Figure 3A).

TET2 mutations were found in 82.1% (32/39) of AITL, 46.3% (19/41) of PTCL-NOS, and 33.3% (4/12) of ALK (-) ALCL (Figure 1A and supplemental Table 1). In PTCL-NOS, both the TBX21 (57%, 10/18) and GATA3 subgroups (41.7%, 5/12) harbored TET2 mutations. The TET2 mutations were distributed throughout the entire coding region; however, the majority of mutations were observed in the catalytic domains, including the Cys-rich and DSBH domains, with a few recurrently targeted codons (Figure 1B). We observed DNMT3A mutations at similar frequencies in AITL (38.5%, 15/39) and PTCL-NOS (36.6%, 15/41), but less frequently in ALK (-) ALCL (16.7%, 2/12). In PTCL-NOS, 33.3% (6/18) harbored DNMT3A mutations in the TBX21 subgroup and 25% (3/12) in the GATA3 subgroup (Figure 1A). Most DNMT3A mutations were clustered in the MTase-binding domain (Figure 1B), presumably leading to loss of function. Of note, the most frequent mutation site was residue Arg-882 (R882) located in the MTase-binding domain. This DNMT3AR882 mutant has been reported to inhibit wild-type DNMT3A activity by disrupting homotetramerization.

The majority (26/29) of RHOA mutations observed in AITL occurred at the same codon, resulting in a Gly17Val substitution in the GTP-binding domain. Consistent with previous reports, RHOA
mutations were very common in AITL (28/39), also present in PTCL-NOS (11/41), but absent in ALCL (0/12) (Figure 1A) and tended to co-occur with TET2 mutations (two-tailed Fisher’s exact test, $P = 0.012$) and IDH2 mutations (two-tailed Fisher’s exact test, $P = 0.003$). All IDH2/TET2 double-mutant AITL cases had a RHOA mutation.

Interestingly, IDH2 and TET2 mutations were not mutually exclusive, unlike in AML or glioblastoma, but showed significant ($P < 0.001$) co-occurrence in AITL (Figure 1A). Most IDH2 (68%) and DNMT3A (80%) mutant cases also had TET2 mutations.

**IDH2$^{R172}$ mutations define an AITL subgroup with a distinct gene expression signature**

To gain an overview of the functional consequences of the mutations affecting epigenetic regulators, we first performed unsupervised hierarchical clustering of 37 AITL samples with GEP data and known mutational status of IDH2, TET2, and DNMT3A. Interestingly, most IDH2$^{R172}$ mutant cases formed a single cluster in unsupervised hierarchical clustering analysis (Figure 2A). We used Fisher’s exact test to determine the significance of the tendency of neighboring cases on the dendrogram to have identical mutation status. The fraction of IDH2$^{R172}$ mutant cases that had mutant neighbors was significantly higher than the fraction of IDH2 wild-type cases that had mutant neighbors ($P = 0.011$). In contrast, neither TET2 mutant cases ($P = 0.073$) nor DNMT3A mutant cases ($P = 0.727$) showed significant clustering (Figure 2A), indicating that IDH2$^{R172}$ mutations are associated with a unique gene expression profile in AITL.

To identify the genes that are differentially expressed between IDH2 wild-type and mutant groups, we performed supervised hierarchical clustering using a univariate permutation test for the significance of individual genes ($P = 0.001$). Among the 344 differentially expressed genes, approximately 40% (136/344) were downregulated in IDH2$^{R172}$ mutant cases compared to IDH2 wild-type cases, an observation distinct from AML (supplemental Figure 4A). Only a small subset of genes was downregulated in IDH2 mutant cases in both AITL and AML (supplemental Figure 4B), suggesting different functional consequences of IDH2 mutations in these two malignancies.
Since mutant IDH2 generates 2-HG and consequently can inhibit TET2 enzyme activity, IDH2 mutation is not expected to co-occur with TET2 mutation; nevertheless, most IDH2R172 mutant cases in AITL also harbor TET2 mutation (30/36). Therefore, we performed class comparison among IDH2/TET2 double-mutant, TET2 single-mutant, and wild-type AITL cases to identify the differences among these groups and found 1035 differentially expressed genes. The tumor cell content of TET2 single-mutant AITL and IDH2/TET2 double-mutant AITL was similar (supplemental Figure 5). Wild-type cases and TET2 single-mutant cases are similar, but markedly different from IDH2/TET2 double-mutant cases. Double-mutant cases showed upregulation of T<sub>FH</sub>-associated genes (IL21 and ICOS), and downregulation of genes associated with T<sub>H1</sub> (IL2, STAT1, CXCR3), T<sub>H2</sub> (IL10RA) and T<sub>H17</sub> (IL17RA), indicating a more T<sub>FH</sub> cell-like phenotype (Figure 2B and supplemental Figure 6). To gain further biological insight, we performed Ingenuity pathway analysis (IPA) and identified several important clusters of genes associated with proliferation, apoptosis, cell cycle, and T-cell development and differentiation. Pathways involved in T helper cell differentiation, IL-17 signaling (IL17RA, AKT3, CXCL10, and CCL2), and Interferon (IFN) signaling (IFNG, STAT1, IFITM3, IRF1, and IFIT3) were dysregulated in IDH2/TET2 double-mutant cases (Figure 3A and supplemental Figure 7). Strikingly, IPA upstream analysis predicted activation of the VEGF pathway and inhibition of IFN signaling in the double-mutant group (Figure 3B). This observation was further supported by GSEA using the Broad Institute and lymphoid signature databases, which showed significant enrichment of IFN response genes (P < 0.01) in the TET2 single-mutant group. However, the double-mutant cases showed enrichment of EZH2 targets, MYC targets, FOXP3 targets, downregulated FOXO3 target genes, and genes associated with proliferation and cell cycle transitions or in apoptosis due to CDKN1 via TP53 (supplemental Table 2 and Figure 3C).

We also observed a striking correlation of GEP of IDH2/TET2 double-mutant cases with that of primary CD4<sup>+</sup> T cells treated with IL-12 (Figure 2B). IL-12 is a major driver of T<sub>FH</sub> cell differentiation, and it can induce naïve CD4<sup>+</sup> T cells to differentiate into T<sub>FH</sub>-like cells in vitro. We analyzed 6245 genes that were highly expressed (top quartile) in primary CD4<sup>+</sup> T cells either after 48h IL-12 treatment and/or no
treatment. Among these 6245 genes, the differences in GEP between individual double-mutant cases and the median tumor value strongly correlated with the changes in GEP induced in normal CD4+ T-cells by IL-12 (Figure 3D).

To investigate the similarity between double-mutant AITL and primary T\textsubscript{FH} cells, we utilized transcriptome sequencing data of T\textsubscript{FH} cells (n = 3), naïve CD4+ T cells (n = 4), and non-T\textsubscript{FH} effector cells (CD4+CD45RA−TCR\textbeta−PD-1\textsuperscript{lo}CXCR5\textsuperscript{lo}PSGL-1\textsuperscript{hi}) (n = 3) from a published dataset.\textsuperscript{37} By identifying genes with significantly different expression (P ≤ 0.05, one-sided Student’s t test) between T\textsubscript{FH} cells and both naïve CD4+ T cells and non-T\textsubscript{FH} effector cells, we defined 1045 “T\textsubscript{FH}-up genes” and 699 “T\textsubscript{FH}-down genes”. Similar numbers of “T\textsubscript{FH}-up genes” were upregulated (comparing individual cases to the median expression level) in all three mutation groups, but significantly more genes were downregulated in IDH2/TET2 double-mutant AITL than wild-type and TET2 single-mutant cases (Figure 3E). This suggests that mutant IDH2 is associated with the repression of non-T\textsubscript{FH} differentiation genes, presumably through epigenetic mechanisms.

**IDH2\textsuperscript{R172} mutations are associated with DNA hypermethylation in AITL and T cells**

To investigate the effect of IDH2\textsuperscript{R172} mutations on the T-cell methylome, we transduced Jurkat cells and primary CD4+ T cells with vectors expressing IDH2\textsuperscript{R172K} or wild-type IDH2. As anticipated, ectopic expression of mutant IDH2 resulted in increased intracellular 2-HG in Jurkat cells (Figure 7A). Remarkably, a global increase in 5-methylcytosine (5mC) and a global decrease in 5-hydroxymethylcytosine (5hmC) were observed in the DNA of Jurkat cells expressing the IDH2\textsuperscript{R172K} mutant compared with cells expressing wild-type IDH2 or empty vector (Figure 4A and B), indicating that IDH2\textsuperscript{R172K} mutation leads to epigenetic changes in DNA through 2-HG. Similar results for 2-HG, 5mC, and 5hmC were observed in primary CD4+ T cells (Figure 4C-E).

As an AITL-specific DNA methylation pattern is not known, we conducted RRBS to investigate the genome-wide alterations in DNA methylation in 18 AITL cases with relatively high tumor content, and compared them with T-cell samples isolated from peripheral blood of four donors. Most of AITL cases with high tumor content harbor TET2 mutations (17/18), and 10 out of 18 cases harbor IDH2\textsuperscript{R172}
mutations. Since AITL tumors have a considerable number and variety of non-malignant cells, we included three tonsils to serve as additional controls to filter out differences resulting from cell type-specific methylation of non-malignant cells.

Studies in several cancers have shown that gain-of-function mutations of IDH2 are associated with a DNA hypermethylation phenotype,\(^{18}\) possibly through inhibition of TET2. Compared to normal tissues, IDH2 mutant AITL showed global alterations of the DNA methylation landscape including hypermethylation of regulatory regions, such as promoters and CpG islands (CGIs) (Figure 4F) that are known to be correlated with downregulation of gene transcription while gene body and intergenic regions are hypomethylated.\(^{38}\) CpG island shores (CGSs) are ± 2 kb regions flanking CGI, and CGI and CGS are normally unmethylated for most genes. We classified promoters into CGI-associated promoters, CGS-associated promoters, and non-CGI/CGS-associated promoters. We observed global hypermethylation of CGI-associated promoters and global hypomethylation of non-CGI/CGS-associated promoters in both IDH2 mutant and wild-type AITL (Figure 4G). We observed that IDH2 wild-type AITL had hypermethylation of many genes that are normally not expressed in CD4\(^+\) T cells. However, among genes normally expressed in CD4\(^+\) T-cells, a significantly higher number were hypermethylated in the IDH2 mutant group (Figure 4H), indicating a more significant functional effect of IDH2\(^{R172}\) mutations in AITL. Additionally, using RRBS and GEP data, we identified genes most significantly hypermethylated in promoter regions and downregulated in IDH2 mutant compared with IDH2 wild-type AITL, including a subset of helper T-cell differentiation genes (SMAD7 and SMURF2) (Table 2).

To identify genes whose expression is likely downregulated by promoter methylation, we selected differentially methylated promoters in AITL cases compared with normal tissues. A total of 46 hypermethylated genes and nine hypomethylated genes in AITL were identified having strong correlation (R < -0.4) with transcriptional changes (Figure 5). The epigenetically downregulated genes included negative regulators of TCR signaling (PTPN7, SIT1, and DGKA), positive regulators of T\(_{H1}\) activity (MATK and PHF11), and other cell surface marker genes (CD7 and IL10RA) (supplementary Figure 8).

One of the most striking findings from GSEA analysis of the genes differentially expressed among wild-type, TET2 single-mutant, and IDH2/TET2 double-mutant groups was the enrichment of genes
associated with the repressive mark histone 3 trimethyllysine-27 (H3K27me3) (Figure 6A). We analyzed genes with both H3K4me3 and H3K27me3 in human embryonic stem cells (hESCs) ("poised" genes) as these genes are particularly prone to repression by H3K27me3. Indeed, hESC-poised genes expressed in CD4+ T cells are downregulated in AITL with IDH2\textsuperscript{R172} mutations (Figure 6B). Initially H3K27me3-repressed genes are prone to cancer-specific promoter DNA hypermethylation, definitively silencing them.\textsuperscript{39} Thus, we compared DNA methylation between hESC-poised genes and non-hESC poised genes and found that poised genes are significantly more likely to be hypermethylated than non-poised genes (Figure 6C).

**IDH2\textsuperscript{R172} mutations are associated with enhanced H3K27me3 in AITL and T cells**

To determine whether the mutant IDH2\textsuperscript{R172} can alter histone lysine methylation in vitro, we ectopically expressed mutant IDH2\textsuperscript{R172K}, wild-type IDH2, and empty vector in Jurkat cells (Figure 7A). H3K27me3, H3K9me3, and H3K4me3 were significantly increased in Jurkat cells expressing IDH2\textsuperscript{R172K} (Figure 7B). Immunohistochemical staining revealed significantly enhanced H3K27me3 in the IDH2\textsuperscript{R172} mutant AITL cases group (n = 3) (average 42.2% positive cells) compared to cases in the IDH2 wild-type group (n = 3) (average 16.2% positive cells) (Figure 7C). Strongly positive areas averaged only 2.9% in the IDH2 wild-type cases, but constituted 18.8% in the IDH2\textsuperscript{R172} mutant cases (Figure 7D). Overall, these data demonstrate increased H3K27me3 both in vitro and in tumor samples in agreement with our GEP findings and strongly suggest that IDH2\textsuperscript{R172K} mutations can dysregulate the post-translational modification of histones in T cells.
Discussion

The aberrant DNA and histone methylation identified in various malignancies suggests an important role for these epigenetic changes in tumorigenesis, although the profiles vary among tumors, indicating a possible dependency on cellular context. Mutations of epigenetic regulators (IDH2R172, TET2, and DNMT3A) are common in AITL, emphasizing the importance of aberrant epigenetic alterations in lymphomagenesis. Given the presence of TET2 and DNMT3A mutations in other subtypes of PTCL, but not IDH2R172 mutations, it is highly likely that IDH2R172 mutations play a unique role in the pathogenesis of AITL. Indeed, unsupervised hierarchical clustering identified a distinct GEP associated with IDH2R172 mutations in AITL. The changes in GEP in IDH2R172 mutant cases likely resulted in part from promoter hypermethylation, but also from disruption of histone lysine demethylation.

One of the most interesting characteristics in the IDH2/TET2 double-mutant group is enrichment of the T_{FH}-like phenotype and downregulation of features of other types of helper T-cells. IL-6, IL-12, and IL-23 have been shown to induce IL-21 expression in human naïve CD4⁺ T cells. IL-12 not only can induce IL-21 production but also can induce other T_{FH} phenotypes, including upregulation of CXCR5, ICOS, and BCL6 and the capacity to facilitate B cell differentiation into antibody-secreting cells. Treatment of normal human CD4⁺ T cells by IL-12 for 48 hours induced a steady increase of IL-21 and CXCR5 (supplemental Figure 9). The GEP of IDH2/TET2 double-mutant AITL was highly enriched in the signature of CD4⁺ T cells stimulated by IL-12, suggesting a more polarized T_{FH} phenotype. In addition, IDH2/TET2 double-mutant AITL showed similarity of gene expression with primary T_{FH} cells. These observations suggest that limiting the plasticity of differentiation to other T_{H} lineages is an important consequence of IDH2R172 mutations. Of the signaling pathways attributable to differentiation of helper T-cells, IFN signaling (STAT1, IRF1, and IFIT3) and IL-17 signaling (IL17RA, CXCL10, and CCL2) were downregulated in the IDH2/TET2 double-mutant group. Conversely, TGF-β signaling, which contributes to the differentiation of T_{FH} cells, was enriched. In keeping with this observation, we identified a subset of helper T-cell differentiation genes (SMAD7 and SMURF2) that were hypermethylated in patients with an IDH2R172 mutation (Table 2).
Unlike in AML, IDH2 and TET2 mutations are not mutually exclusive in AITL. Despite the significant differences between IDH2/TET2 double-mutant AITL and normal tissues, and between TET2 single-mutant AITL and normal tissues, there is only a moderate difference in genome-wide methylation between IDH2/TET2 double-mutant and TET2 single-mutant AITL, which seems insufficient to explain the much greater differences on GEP. However, GEP includes secondary changes from genetic and epigenetic alterations. The significant changes in gene expression in the IDH2R172 mutant group may also represent consequences of histone modification.

Mutant IDH2 inhibits various histone demethylases. Nearly all histone demethylation is brought about by the Jumonji family of histone demethylases, with 27 distinct enzymes in humans. H3K4, H3K9, H3K27, and H3K79 methylation has been shown to be elevated in glioma cells expressing mutant IDH1/2, both in vitro and in vivo. Consistent with this finding, we observed similar alterations in vitro and significantly elevated levels of H3K27me3 in AITL cases with IDH2R172 mutation, suggesting that 2-HG produced by IDH2 mutants indeed interferes with histone demethylases. In ESCs, bivalent chromatin modification is thought to silence genes controlling cell lineage through the repressive histone mark H3K27me3, while rending them “poised” for subsequent activation during differentiation through H3K4me3. Histone lysine demethylases (KDM) 6A/B are responsible for demethylating H3K27me3. Inactivating mutations of KDM6A have frequently been identified in hematopoietic malignancies and solid tumors. Genes that are poised in ESCs are especially likely to be persistently “turned off” by H3K27me3 due to inhibition of KDM6A/B by mutant IDH2. In the context of AITL, our results suggest that IDH2R172 mutation further promotes lymphomagenesis by altering gene expression through histone lysine methylation. This hypothesis is supported by the observation that hESC-poised genes were significantly downregulated in the IDH2R172 mutant group. Aberrant repression by H3K27me3 may be induced, albeit less readily, in other genes that are downregulated in IDH2R172 mutant AITL. In both IDH2R172 mutant and wild-type cases, genes with little or no expression in T-cells are especially likely to undergo promoter DNA hypermethylation. Thus, some of the genes aberrantly repressed by H3K27me3 may later become hypermethylated in their promoters, locking in their transcriptional silencing. Thus, loss of function of TET2 and histone demethylases may cooperate in epigenetic dysregulation.
In AML, both R140 and R172 neomorphic mutants of IDH2 were identified. In contrast, AITL harbors mutations only at IDH2\textsuperscript{R172}, but not at IDH1\textsuperscript{R132} or IDH2\textsuperscript{R140}. This exclusivity is highly interesting, but the reason is currently not clear. Studies by others have shown that mutations differ quantitatively in 2-HG production\textsuperscript{33,46}; this raises the possibility that different tumor types may favor certain ranges of 2-HG concentrations. In previous studies\textsuperscript{33,46}, IDH2\textsuperscript{R140} was shown to be a weaker 2-HG-producing mutant than IDH2\textsuperscript{R172}. In addition, IDH2\textsuperscript{R172K} mutant 10T mesenchymal progenitor cells, but not IDH2\textsuperscript{R140Q} mutant cells, can grow as xenografts\textsuperscript{33}, indicating different biological consequences resulting from the two mutants. In AML, IDH2\textsuperscript{R140} mutant AML cells frequently have normal cytogenetics and NPM1 mutations, but IDH2\textsuperscript{R172} is frequently the only mutation detected\textsuperscript{47}, again suggesting biological differences. Understanding the mechanistic difference between the IDH2\textsuperscript{R140} and IDH2\textsuperscript{R172} mutants is certainly important and should be investigated further in the future.

Both IDH2\textsuperscript{R172} and TET2 mutations can lead to promoter hypermethylation, giving a strong rationale for considering the use of hypomethylating agents in AITL treatment. TET2-mutated myelodysplastic syndrome patients have been shown to response to hypomethylating agents.\textsuperscript{48} Moreover, a case report using 5-azacytidine to treat a patient with chronic myelomonocytic leukemia (CMML), who also had TET2-mutant AITL, led to a complete remission of both the AITL and CMML.\textsuperscript{49} VEGF-A has been showed to be over-expressed in malignant cells and endothelial cells from AITL and associated with an adverse prognosis\textsuperscript{50}, suggesting that it is another potential therapeutic target. A small molecule inhibitor targeting mutant IDH2, AG-221, has also shown promising results in treating IDH2-mutant AML in animal models and in clinical trials,\textsuperscript{51,52} suggesting that inhibition of mutant IDH2 could be a useful therapeutic strategy that specifically targets an oncogenic mutation in AITL. However, since most IDH2-mutant cases also have TET2 mutations, it is possible that combination with a DNA hypomethylating agent or anti-angiogenic drugs could improve efficacy.
Acknowledgments

We would like to acknowledge helpful discussions with Dr. Katharine Yen and her help in measuring the 2-HG levels at Agios Pharmaceuticals (Cambridge, MA). We are grateful for Rhishikesh Thakare and Dr. Yazen Alnouti’s help in measuring global 5hmC and 5mC, and Victoria B Smith and Dr. Charles A Kuszynski’s technical assistance with Fluorescence Activated Cell Sorting at University of Nebraska Medical Center. We thank Dr. David L Klinkebiel and Dr. James Eudy for constructive discussions, Dr. Francoise Berger for contribution to sample collection and characterization and Dr. Xin Wang for support and suggestions. This work was supported in part by the Lymphoma Research Foundation (to J.I.), translational research program of Leukemia and Lymphoma Society (6129-14, to J.I.), UNMC Clinical-Translational Research Scholars Program, NCI Eppley Cancer Center Support Grant (P30CA036727), City of Hope Cancer Center Support Grant (P30 CA033572) and Specialized Programs of Research Excellence (1 P50 CA 136411-01 01A1 PP-4, to W.C.C.). C.W. and B.J. were recipients of Chinese Scholarship Council funding.

Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Table 1. Summary of the samples and techniques utilized

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Targeted resequencing of TET2 and DNMT3A</th>
<th>Sanger sequencing of IDH2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GEP</th>
<th>RRBS</th>
</tr>
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<tbody>
<tr>
<td>Tumor specimens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AITL</td>
<td>39</td>
<td>58</td>
<td>36</td>
<td>18</td>
</tr>
<tr>
<td>PTCL-NOS</td>
<td>41</td>
<td>17</td>
<td>NA</td>
<td>NA</td>
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<td>ALK(-) ALCL</td>
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<td>NA</td>
<td>NA</td>
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<tr>
<td>Primary CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
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<td>NA</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Tonsils</td>
<td>NA</td>
<td>NA</td>
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</table>

Abbreviations: AITL= angioimmunoblastic T-cell lymphoma; PTCL-NOS= peripheral T-cell lymphoma, not otherwise specified; ALCL= anaplastic large cell lymphoma; GEP= gene expression profiling; RRBS = reduced representation bisulfite sequencing; NA= not applicable

<sup>a</sup>Low tumor content cases were confirmed by pyrosequencing; 39 out of 58 AITL cases have corresponding targeted resequencing data. <sup>b</sup>Treated with IL-12 for 0, 2, 8, 24, 48 hours. <sup>c</sup>Two sets were freshly isolated from donor PBMC, and the other two were cultured with anti-CD3/CD28 beads for two weeks before DNA extraction.
Table 2. Top 46 genes most significantly hypermethylated in promoter regions and downregulated in IDH2 mutant AITL

<table>
<thead>
<tr>
<th>Gene</th>
<th>EntrezID</th>
<th>Downregulation (IDH2 WT &gt; IDH2 mutant)</th>
<th>Hypermethylation (IDH2 mutant &gt; normal tissue)</th>
<th>Correlation between gene expression and methylation</th>
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<td>ABL1</td>
<td>25</td>
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<td>P = 0.017</td>
<td>R = -0.308</td>
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<td>AFF1</td>
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<td>R = -0.559</td>
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<td>347902</td>
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<td>P = 0.065</td>
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<td>ANTXR2</td>
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<td>55911</td>
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<td>924</td>
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<td>P-value (RNA)</td>
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Figure Legends

Figure 1. Mutational profiles of IDH2, TET2, DNMT3A, and RHOA in patients with peripheral T-cell lymphomas
(A) Co-occurrence of IDH2R172, TET2, DNMT3, and RHOA mutations in patients with AITL (n = 39), subgroups of PTCL-NOS (n = 41), and ALK (-) ALCL (n = 12). White color indicates wild-type cases. Grey color indicates cases with variant allele frequency (VAF) of at least 3% but lower than 10%. Black color indicates cases with VAF of at least 10%. (B) Mutations occur in functional domains of the TET2 and DNMT3A proteins. Missense mutations, nonsense mutations, and insertion/deletions are indicated.

Figure 2. IDH2R172 mutations define a unique subgroup of AITL with distinct gene expression
(A) Dendrogram of unsupervised hierarchical clustering of 37 AITL cases with mutational status of IDH2R172, TET2, and DNMT3A. The branches indicate Pearson's correlation among the samples. Fisher's exact test was performed to determine the significance of the tendency of neighboring cases on the dendrogram to have identical mutation status. (B) Supervised heat map of samples across 1035 genes differentially expressed among IDH2/TET2 double-mutant (n = 12), TET2 single-mutant (n = 18), and wild-type (n = 6) AITL (univariate permutation test, P < 0.01). The profile on the left shows gene expression in primary CD4+ T cells treated with IL-12 for 0h, 2h, 8h, 24h, and 48h. Each column represents a case. Green and red color scale indicates row z-score. Selected differentially expressed genes are listed at the right side of the profile.

Figure 3. Functional annotation of genes differentially expressed between IDH2/TET2 double-mutant AITL and TET2 single-mutant AITL
(A) Selected top IPA canonical pathways differing between IDH2/TET2 double-mutant AITL and TET2 single-mutant AITL. (B) IPA upstream analysis to predicted activation state of annotated proteins. “Vegf” and “Interferon alpha” are groups of proteins instead of single molecules. Activation z-score > 3 was considered as significant. (C) Apoptosis pathway identified by GSEA analysis significantly altered in
**IDH2/TET2** double-mutant AITL compared with **TET2** single-mutant AITL. The normalized enrichment score (NES), *P* value, and FDR value are indicated. (D) Correlations between (1) the differences in GEP between each individual AITL case and the median tumor value with (2) the changes in GEP induced in normal CD4+ T-cells by IL-12 (48 h). (E) Comparison of the number of “T<sub>FH</sub>-up genes” and “T<sub>FH</sub>-down genes” (see text) in AITL. Data are shown as mean ± SEM. *P* value was calculated using the two-tailed Student's t test. * indicates *P* < 0.05, ** indicates *P* < 0.01, *** indicates *P* < 0.001 and **** indicates *P* < 0.0001.

**Figure 4. IDH2<sup>R172</sup> mutations are associated with DNA hypermethylation in AITL and T cells**

(A and B) Simultaneous detection of 5mC and 5hmC in transduced Jurkat cells by the LC-MS/MS method. (C and D) Simultaneous detection of 5mC and 5hmC in transduced human CD4+ T cells by the LC-MS/MS method. (E) LC-MS/MS showing alteration of intracellular 2-HG in corresponding groups (*n* = 4 each). (F) RRBS showing alterations of methylation level among normal tissues (*n* = 7, three tonsils and four CD4+ T-cell samples), IDH2 wild-type AITL (*n* = 8), and IDH2<sup>R172</sup> mutant AITL (*n* = 10) in different genomic regions. Promoters are ± 1kb regions relative to transcription start site (TSS). Gene bodies are regions of transcribed sequences excluding promoter regions. Intergenic regions are the sequences other than promoters and gene bodies. (G) RRBS showing alterations of methylation level in CpG island (CGI), CpG island shore (CGS), and non-CGI-CGS regions. (H) Number of hypermethylated genes in IDH2 wild-type AITL (*n* = 8), and IDH2<sup>R172</sup> mutant AITL (*n* = 10) compared with normal tissues. Red indicates genes only hypermethylated in IDH2<sup>R172</sup>-mutant AITL. Green indicates genes only hypermethylated in IDH2 wild-type AITL. Blue indicates genes hypermethylated in both IDH2<sup>R172</sup> mutant and IDH2 wild-type AITL. Genes with expression data were divided into deciles based on the mean gene expression level of five normal CD4+ T cell samples (10 = highest). Data are shown as mean ± SEM. *P* value was calculated using the two-tailed Student's t test. * indicates *P* < 0.05, ** indicates *P* < 0.01, *** indicates *P* < 0.001 and **** indicates *P* < 0.0001.
Figure 5. RRBS methylation analysis (left) and gene expression analysis (right) of 46 hypermethylated and 9 hypomethylated genes between 18 AITL and 7 normal samples

Gene selection criteria include: (a) Promoters of the selected genes must be significantly hyper- or hypomethylated compared with normal tissue (three tonsils and four CD4+ T-cell samples) ($P \leq 0.05$); (b) selected genes must be normally expressed in primary CD4+ T cells; and (c) there must be strong correlation between promoter methylation and gene expression among the samples ($R < -0.4$). Except for one case (indicated with *), all cases with RRBS data have corresponding gene expression data. Mutational statuses of $IDH2^{R172}$, $TET2$, and $DNMT3A$ are listed at the top.

Figure 6. Poised genes tend to be downregulated and hypermethylated in $IDH2^{R172}$ mutant AITL

(A) GSEA analysis of genes differentially expressed between $TET2$-single mutant and $IDH2/TET2$-double mutant groups shows the enrichment of H3K27me3-associated genes. (B) Poised genes expressed in CD4+ T cells are downregulated in AITL with $IDH2^{R172}$ mutations. (C) Comparison of the DNA methylation patterns between poised genes and non-poised genes in AITL. The poised genes are significantly more likely to be hypermethylated than non-poised genes ($P = 1.3 \times 10^{-17}$, Fisher’s exact test).

Figure 7. $IDH2^{R172}$ mutant alters histone lysine trimethylation in Jurkat T cells and AITL

(A) Western blots showing ectopic expression of wild-type IDH2 and $IDH2^{R172}$ in whole cell lysates of transduced Jurkat cells. LC-MS/MS showing alteration of intracellular 2-HG in corresponding groups ($n = 4$ each, data are shown as mean ± SEM, *** $P < 0.001$ by two-tailed Student’s t test). (B) Western blots showing changes in trimethylation of various histone lysine residues in transduced Jurkat cells. Histone proteins were extracted and assessed for levels of histone lysine methylation. Total H3 was utilized as loading control. Blots shown are representative of at least three replicates. (C) Representative immunohistochemistry of AITL tissue microarrays with antibody against H3K27me3 ($n = 3$ in each group). Original magnification, x 40. (D) Image quantification using Image Scope (details in Methods section). Error bar indicates SEM of three independent samples in each group.
Figure 2
Figure 4
Figure 5
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Figure 7
IDH2R172 mutations define a unique subgroup of patients with angioimmunoblastic T-cell lymphoma

Chao Wang, Timothy W. McKeithan, Qiang Gong, Weiwei Zhang, Alyssa Bouska, Andreas Rosenwald, Randy D. Gascoyne, Xiwei Wu, Jinhui Wang, Zahid Muhammad, Bei Jiang, Joseph Rohr, Andrew Cannon, Christian Steidl, Kai Fu, Yuping Li, Stacy Hung, Dennis D. Weisenburger, Timothy C. Greiner, Lynette Smith, German Ott, Eleanor G. Rogan, Louis M. Staudt, Julie Vose, Javeed Iqbal and Wing C. Chan

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