SUPPLEMENTARY APPENDIX

# Early detection of T-cell lymphoma with T follicular helper phenotype by *RHOA* mutation analysis

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**SUPPLEMENTARY TABLES**

**Table S1:** Sequences of PCR primers and probes used for *RHOA* mutation detection.

| | **Sequence (5'-3')** | **Manufacturer** |
|---|---|---|
| Forward primer | CCTATGACTTCTTGTGCATTGC | Sigma-Aldrich |
| Reverse primer | TACACCTCTGGGAACTGGTC | Sigma-Aldrich |
| Total probe (Cy5) | [Cyanine5]GCTGCCATCCGGAAGAAACTG[BHQ3] | Sigma-Aldrich |
| PNA wild-type clamp | AGCCTGTGGAAAGACA | Panagene (Cambridge Research Biochemicals) |
| LNA mutant probe (Fam) | [6FAM]AGCC[+T]GT[+G][+T][+A]AAGA[+C]A[BHQ1] | Sigma-Aldrich |

**Table S2:** Mutational information for clonal hematopoiesis of indeterminate potential (CHIP) samples.

| Sample | *RHOA* qPCR | Gene | Nucleotide change | Amino acid change | VAF (%) |
|---|---|---|---|---|---|
| CHIP 1 | Negative | SRSF2 | c.C284A | p.P95H | 7.4 |
| CHIP 2 | Negative | JAK2 | c.G1849T | p.V617F | 14.7 |
| | | TET2 | c.A3797G | p.N1266S | 2.9 |
| CHIP 3 | Negative | TET2 | c.C2020T | p.Q674X | 17.5 |
| | | | c.G3893A | p.C1298Y | 15.8 |
| | | | c.G4249T | p.V1417F | 3.1 |
| | | TP53 | c.C523G | p.R175G | 3.5 |
| CHIP 4 | Negative | DNMT3A | c.1154delC | p.P385fs | 9.1 |
| | | SF3B1 | c.A2098G | p.K700E | 10.4 |
| | | | c.G1874T | p.R625L | 6.5 |
| CHIP 5 | Negative | CBL | c.2434+1G>A | Essential splicing site | 1.1 |
| | | DNMT3A | c.2173+1G>A | Essential splicing site | 26.1 |
| | | PPM1D | c.C1570T | p.Q524X | 4.8 |
| CHIP 6 | Negative | CBL | c.G570A | p.W190X | 2.3 |
| | | SF3B1 | c.A2098G | p.K700E | 0.9 |
| | | SF3B1 | c.G1998C | p.K666N | 34.2 |
| CHIP 7 | Negative | DNMT3A | c.2086delC | p.Q696fs | 0.9 |
| | | TP53 | c.G818A | p.R273H | 1.4 |
| | | U2AF1 | c.A470C | p.Q157P | 11.8 |
| CHIP 8 | Negative | DNMT3A | c.2567delA | p.E856fs | 5.4 |
| | | | c.1122+1G>- | Essential splicing site | 1.3 |
| | | TET2 | c.C863G | p.P288R | 1.9 |
| CHIP 9 | Negative | DNMT3A | c.G1904A | p.R635Q | 4.0 |
| | | TP53 | c.G1006T | p.E336X | 1.9 |
| CHIP 10 | Negative | SF3B1 | c.G1998T | p.K666N | 10.6 |
| | | U2AF1 | c.A470G | p.Q157R | 10.8 |
| CHIP 11 | Negative | DNMT3A | c.G2645A | p.R882H | 32.1 |
| CHIP 12 | Negative | GNB1 | c.A169G | p.K57E | 4.2 |
| | | SF3B1 | c.G1998T | p.K666N | 16.2 |
| | | TET2 | c.944delC | p.S315fs | 3.2 |
| | | TET2 | c.1305delC | p.H435fs | 1.2 |
| | | TET2 | c.A3821G | p.Q1274R | 3.3 |

| | | | | | |
|---|---|---|---|---|---|
| | | TET2 | c.T3965A | p.L1322Q | 3.4 |
| | | TET2 | c.C4624T | p.Q1542X | 1.3 |
| | | TET2 | c.C5473T | p.Q1825X | 40.9 |
| CHIP 13 | Negative | SRSF2 | c.C284T | p.P95L | 11.7 |
| CHIP 14 | Negative | DNMT3A | c.G989A | p.W330X | 6.5 |
| | | SRSF2 | c.C284A | p.P95H | 4.0 |
| | | TET2 | c.C3116A | p.S1039X | 2.9 |
| | | | c.4228delC | p.P1410fs | 2.3 |
| | | | c.4931_4935del | p.P1644fs | 1.6 |
| CHIP 15 | Negative | SF3B1 | c.2098A>G | p.K700E | 40.4 |
| (High-risk) CHIP 16 | Negative | TET2 | c.2031_2032delTG | Cys677fs | 36.4 |

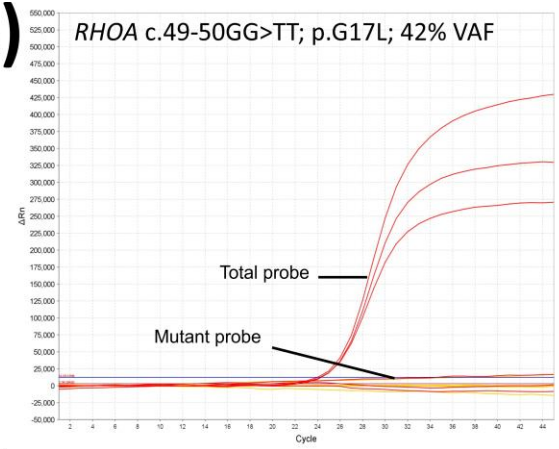
**Table S3:** Summary of laboratory data of non-diagnostic biopsies in AITL with *RHOA* p.Gly17Val mutation.

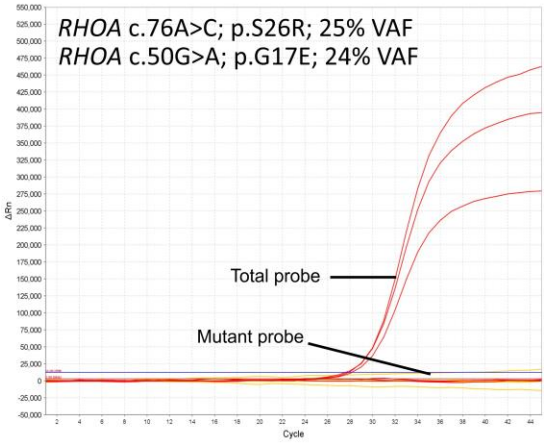
| Case | Age/ Sex | *RHOA* mutation | Biopsy site | Core or excision | Preceding or follow up biopsy | Diagnostic category | Histological & immunohistochemical findings | T-cell clonality | B-cell clonality | Final histological opinion |
|---|---|---|---|---|---|---|---|---|---|---|
| C2 | 64/F | Positive | Bone marrow | Trephine biopsy | Follow up | C | There is a nodular and interstitial infiltrate of small lymphoid cells, mainly T-cells, predominantly $CD4^+$, no evidence of loss of pan T-cell markers | Weak clonal in polyclonal background | n/a | No definite aberrant T-cell phenotype, lack of molecular evidence, thus not diagnostic |
| C3-1 | 66/F | Positive | Bone marrow | Trephine biopsy | Preceding | C | Non-specific changes. A focal cluster of multiple perivascular lymphoid aggregates, dominantly $CD4^+$ T cells, but very few expressing PD1 | Polyclonal | n/a | Suspicious of bone marrow involvement by AITL, but low number of assumed neoplastic cells and no evidence of clonal TCR rearrangement, thus not diagnostic |
| C3-2 | 66/F | Positive | Liver | Core biopsy | (Preceding) Same date as diagnostic biopsy | C | Focal inflammatory cells including lymphocytes and histiocytes in parenchyma and portal tracts | Weak polyclonal | | No histological evidence of involvement by a T-cell lymphoma |
| C5 | 72/ M | Positive | Skin | Punch biopsy | Follow up | C | There is a nodular dermal inflammatory infiltrate, involving all levels of the dermis and also the subcutis. The infiltrate is perivascular and periadnexal, comprising sheets of histiocytes, in areas forming granulomas with occasional giant cells, and inflammatory cells and lymphocytes. Immunohistochemistry shows mixed $CD4^+$ and $CD8^+$ T cells with no evidence of loss of pan T-cell markers | Clonal, identical pattern to that of previous biopsy showing AITL | n/a | No histological evidence of involvement by a T-cell lymphoma, although shown by clonality analysis |
| C7-1 | 82/ M | Positive | Skin | Punch biopsy | Preceding | C | Mild perivascular chronic inflammatory cell infiltration, plus a predominantly lobular panniculitis associated with focal fat necrosis. The infiltrate is predominantly small T cells, admixed with B cells and histiocytes | n/a | n/a | Panniculitis (please also refer to Table 1) |
| C7-4 | See Table 1 | | | | | | | | | |
| C7-5 | | | | | | | | | | |
| C9 | 82/ M | Positive | Bone marrow | Trephine biopsy | Preceding | C | There are numerous lymphoid aggregates, with a paratrabecular location, which are composed of small mixed B and T cells. The majority of T cells are $CD4^+$, no loss of pan T-cell markers. EBER-ISH negative | Polyclonal | n/a | Suspicious lymphoma involvement, but not diagnostic as no supporting evidence by clonality and flow cytometry analyses |
| C11 | 77/ M | Positive | Right inguinal lymph node | Core biopsy | Preceding | C | Small, fragmented and poorly fixed tissue cores showing polymorphous infiltrates, particularly histiocytes and T cells, and FDC expansion. T cells are predominantly $CD4^+$, expressing pan T-cell markers, with a small subset PD1 positive. EBER-ISH negative | Weak clonal | Polyclonal | Suspicious for a T-cell lymphoma, but lacking convincing histological and molecular evidence, advised for re-biopsy |
| C13 | 81/ M | Positive | Bone marrow | Trephine biopsy | Follow up | C | There are multiple intraparenchymal & occasionally paratrabecular aggregates of histiocytes, numerous small to slightly enlarged lymphoid cells which are mainly $CD4^+$ T cells, showing possible loss of CD7, occasionally PD1 positive, but negative for CD10 and BCL6. Occasional $EBER^+$ immunoblasts | Weak polyclonal | n/a | Low lesional cell content and no evidence of clonal TCR gene rearrangement, thus suspicious but not diagnostic of involvement by a T-cell lymphoma |
| C17 | 76/ M | Positive | Cervical lymph node | Core biopsy | Preceding | C | Small and poorly preserved tissue fragments. A mixed population of small lymphoid cells, CD30 positive immunoblasts, and plasma cells. There are no recognizable follicular structures on morphology and immunohistochemistry. No evidence of loss of pan T-cell markers, high number of $EBER^+$ immunoblasts | Weak clonal | Clonal | Little tissue for evaluation and dominant B-cell component render difficulty in diagnosis |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| C19 | 67/M | Positive | Lymph node | Core biopsy | Preceding | C | A mixture of B and T cells in a vaguely nodular intermixed distribution. T cells are predominantly $CD8^+$ and show partial loss of CD7. The smaller $CD4^+$ population shows CD10, PD1 and possibly BCL6 expression. There is FDC expansion and a high number of diffuse $EBER^+$ B cells across a range of B-cell differentiation | Clonal | Clonal | The B cell component looks like immunodeficiency associated LPD and there is a relatively small TFH component. Advised for re-biopsy if lymphadenopathy persists or progresses |
| C30 | 79/M | Positive | Cervical lymph node | Excision | Preceding | C | Partial effacement of the lymph node architecture and expansion of the interfollicular area by a polymorphous population of lymphoid cells. B cells express pan B-cell markers (CD20, CD79a, CD19), and MUM1, but negative for CD10 and BCL6. A high proportion of the B cells were $EBER^+$ and showed IG kappa light chain restriction. The lymphoid follicles appeared to be reactive and showed no apparent expansion of TFH cells, with only a few $CD10^+$ cells spilling out of the germinal centers | Weak multiple clonal re-arrangements | Clonal | Clonal EBV positive polymorphous lymphoproliferation, advised for follow up with a low threshold for re-biopsy. On review with *RHOA* mutation positivity, pattern-I AITL is considered |
| C37-1 | 81/M | Positive | Lymph node | Core biopsy | Preceding | C | There are multiple, variably preserved germinal centers with slightly expanded FDC-meshworks and a population of $CD4^+/PD1^+$ follicular helper T cells in a predominantly peri-germinal centre localization, which express pan T-cell markers and CD10. Germinal center cells are negative for CD10 and BCL6 and these large B cells spill beyond the follicles and show CD30 expression. EBER-ISH highlights scattered positive cells | Weak clonal | Weak clonal | Suspicious of a T-cell lymphoma, particularly AITL, but not diagnostic due to small tissue and lack of strong molecular evidence |
| C37-3 | | Positive | Skin | Punch biopsy | Follow up | C | Multiple perivascular infiltrates comprising a mixed population of small to medium atypical lymphoid cells admixed with histiocytes and plasma cells. The lymphoid cells are mixed $CD4^+$ and $CD8^+$ T cells. Many T cells express PD1 and show no loss of expression of pan T-cell markers. There are also numerous B cells and scattered $EBER^+$ cells, which are also $CD30^+$. The plasma cells display lambda light chain restriction | Weak polyclonal | Weak clonal | A clonal B-cell process with plasmacytoid differentiation, and not entirely excluding a T-cell lymphoma, but not diagnostic |
| C38-1 | 80/M | Positive | Lymph node | Core biopsy | Preceding | C | The nodal architecture is effaced by a diffuse mixed infiltrate of small, intermediate and large lymphoid cells. There is mixed CD4 and $CD8^+$ T cells, with CD4 expression in some large cells. There is loss of CD7 expression in T cells | Weak clonal | Clonal | Suspicious of a T-cell lymphoma, not diagnostic |
| C38-2 | 80/M | Positive | Cervical lymph node | Excision | Preceding | C | This is a mostly necrotic sample. In viable areas, a marked proliferation of variably sized blood vessels is noted. The T cells are small to medium sized with preserved pan T-cell marker expression. They are predominantly $CD8^+$ with a smaller $CD4^+$ population. There is no significant CD30, PD1, CD10, BCL6 expression in the T cell population. There is expansion of FDC-meshworks. EBER-ISH negative | Weak multiple clonal re-arrangements | Clonal | Not diagnostic, not excluding B-cell lymphoma |
| C43 | 64/M | Positive | Cervical lymph node | Core biopsy | Preceding | C | Lymph node architecture appears essentially preserved. There is a striking paracortical hyperplasia by small lymphocytes and histiocytes. T cells are dominantly $CD4^+$, show no loss of pan T-cell markers, and appear negative for CD10 and BCL6. PD1 staining is largely restricted to follicular T cells. Some FDC-meshworks appeared slightly ragged. EBER-ISH negative | Polyclonal | n/a | Reactive T-cell hyperplasia |

**SUPPLEMENTARY FIGURES**

**A)**

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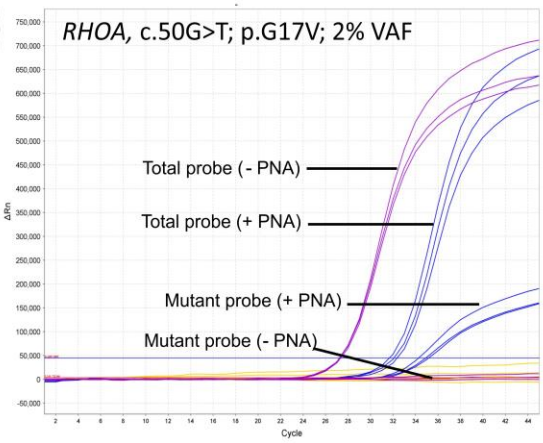
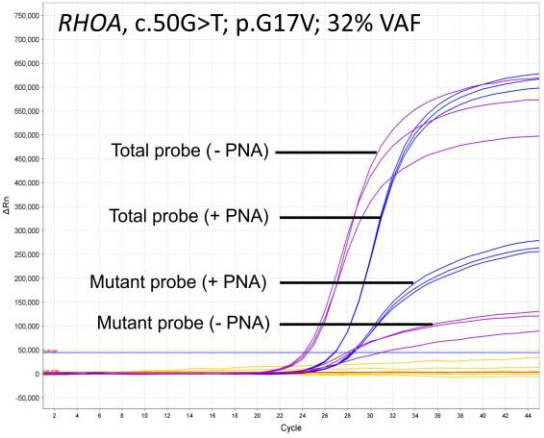
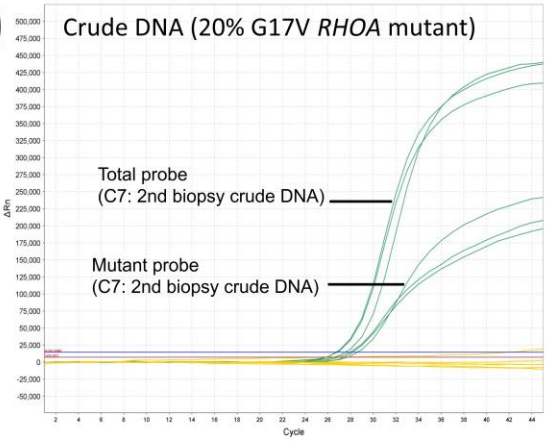
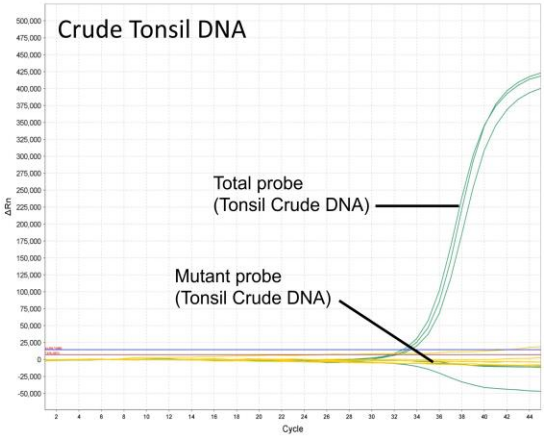


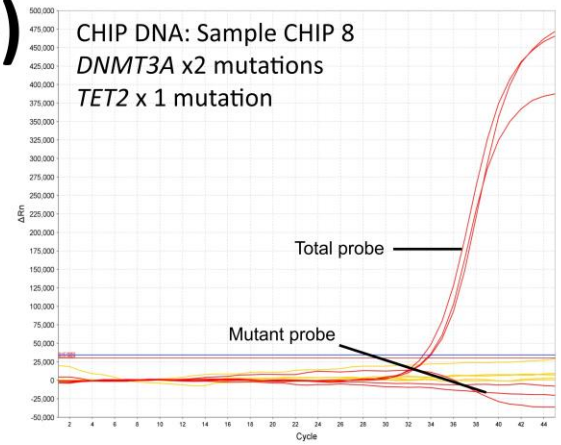




**C)**




**D)**



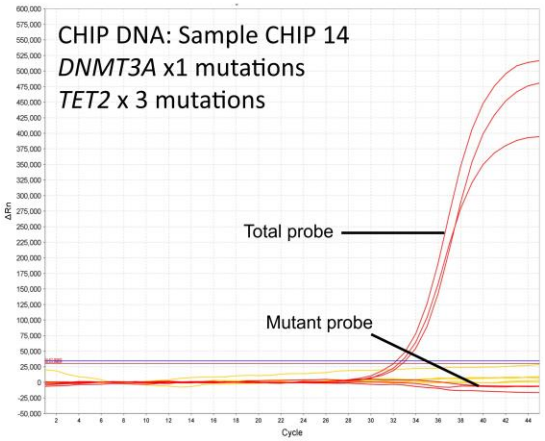



**Figure S1.** Representative LNA-PNA qPCR plots for detection of *RHOA* p.Gly17Val.

A) The qPCR is highly specific for *RHOA* (c.50G>T; p.Gly17Val) mutation, not detecting any other *RHOA* changes.

B) Examples of qPCR for detection of *RHOA* (c.50G>T; p.Gly17Val) mutation in the presence or absence of PNA probe which represses the amplification of the wild-type *RHOA* sequence, thus boosting the detection of the *RHOA* mutation. As demonstrated by the total probe amplification curve shift in presence of PNA clamp, while the mutant probe amplification curve is enhanced.

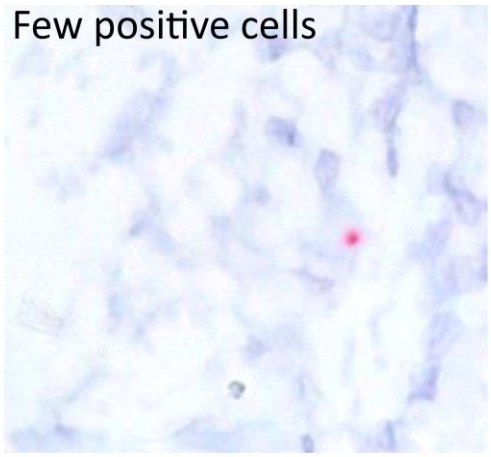
C) Examples of qPCR for detection of *RHOA* (c.50G>T; p.Gly17Val) mutation using crude DNA preparations.

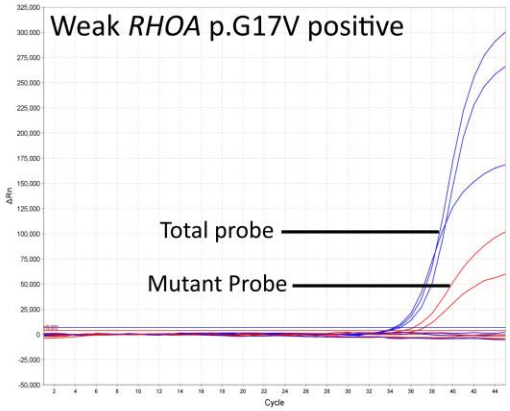
D) Examples of qPCR for detection of *RHOA* (c.50G>T; p.Gly17Val) mutation for clonal hematopoiesis of indeterminate potential (CHIP) cases with both *TET2* and *DNMT3A* mutations.

**BaseScope-ISH**

**qPCR for *RHOA* p.G17V**

First Biopsy (12/08/2015)

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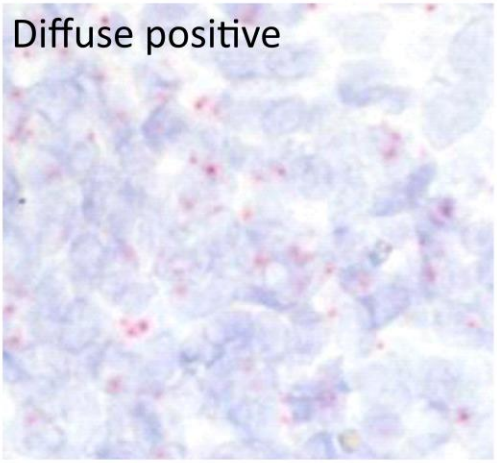
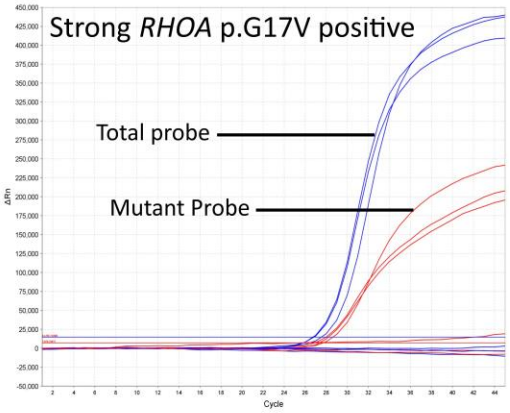
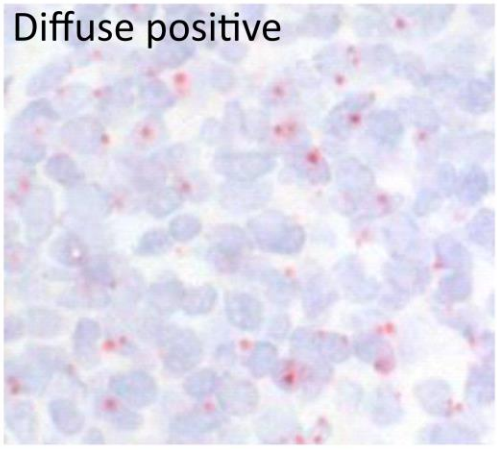
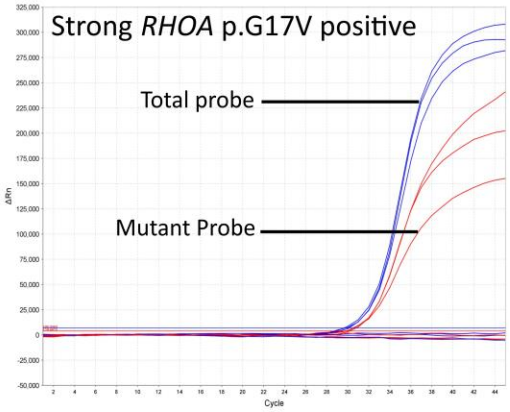



Third Biopsy (1/08/2016)

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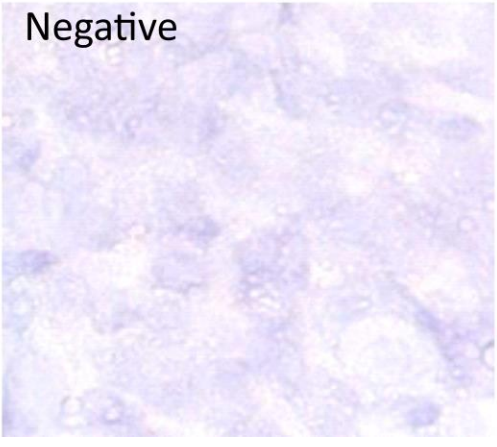


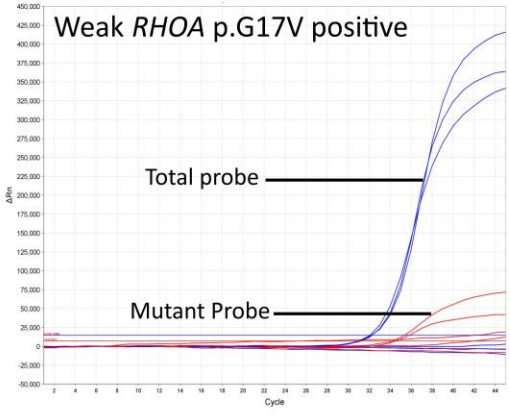

**Figure S2:** Detection of the AITL cells by BaseScope in situ hybridization (ISH) using a specific probe to the unique VDJ junction sequence of the lymphoma cells (TRB-V5 clone), and qPCR of *RHOA* Gly17Val in case 7.

Left panel: Representative images of BaseScope-ISH;

Right panel: *RHOA* Gly17Val qPCR plots for various biopsies of case 7.