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MINI-REVIEW

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Genetic alterations affecting GTPases and T-cell receptor signaling in peripheral T-cell lymphomas

Rebecca L. Boddicker[a], Gina L. Razidlo [b,c], and Andrew L. Feldman [a]

[a]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA; [b]Center for Basic Research in Digestive Diseases, Division of Gastroenterology & Hepatology, Mayo Clinic, Rochester, MN, USA; [c]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA

ABSTRACT

Peripheral T-cell lymphomas (PTCLs) are rare, heterogeneous tumors with poor response to standard therapy and few targeted treatments available. The identification of mutations in the T-cell receptor (TCR) signaling pathway that either directly or indirectly affect Ras- and Rho-family GTPases is an emerging theme across PTCL subtypes. This review summarizes the role of GTPases in TCR signaling and highlights the constellation of mutations in this pathway among PTCLs. In particular, focus is given to the functional impact of the mutations and opportunities for targeted therapy. These mutations include activating mutations and gene fusions involving the guanine nucleotide exchange factor, *VAV1*, as well as activating and dominant negative mutations in the GTPases *KRAS* and *RHOA*, respectively. In addition to mutations directly affecting the GTPase pathway, TCR signaling mutations indirectly affecting Ras- and Rho-family GTPases involving genes such as *CD28*, *FYN*, *LCK*, and *PLCG1* are also reviewed.

Introduction

Peripheral T-cell lymphomas (PTCLs) are malignancies of mature T cells encompassing a heterogeneous group of over 20 lymphoma subtypes.[1] The frequency of individual subtypes varies widely by geographic region.[2,3] In Western countries, the most common subtype is PTCL, not otherwise specified (PTCL, NOS), a highly diverse group of tumors lacking sufficient criteria to be classified into one of the other, more specific entities. Angioimmunoblastic T-cell lymphoma (AITL) and anaplastic large cell lymphoma (ALCL) are other common subtypes, each with unique pathological, molecular, and clinical features. In contrast, adult T-cell leukemia/lymphoma (ATL) and NK/T-cell lymphoma subtypes are more common in Asian countries. Overall, the response to standard chemotherapy is quite poor, and few targeted therapeutic options have been identified for PTCL.

T-cell receptor (TCR) signaling plays a critical physiologic role in T-cell function and its dysregulation has been proposed to be a major factor in PTCL pathogenesis.[4,5] Of note, expression of TCR-associated proteins varies among PTCL subtypes.[6,7] Recent sequencing of PTCLs has revealed somatic mutations and genomic rearrangements in the TCR signaling pathway, bearing direct or indirect effects on GTPase signaling. Herein, we will summarize the role of GTPases in TCR signaling in normal T cells and detail the functional implications reported to date for genetic alterations occurring in this pathway.

GTPases in T-cell receptor signaling

Signal transduction through GTP-binding proteins is coordinated to regulate multiple aspects of lymphocyte development and action. In particular, the small GTPases, such as those of the Ras and Rho families, play prominent roles in T-cell development, selection, activation, and migration (as reviewed in references 8–10). Small GTPases are 21 kDa "molecular switches" that bind and hydrolyze guanosine triphosphate (GTP). Multiple stimuli promote the loading of GTP onto this family of proteins (Fig. 1). When in the GTP-bound form, GTPases are in the active state and are capable of binding downstream effector proteins. These GTPases have weak intrinsic hydrolase activity, and thus rely on associated GTPase activating proteins (GAPs) to catalyze the hydrolysis of GTP to guanosine diphosphate (GDP). This conversion changes the conformation, thereby
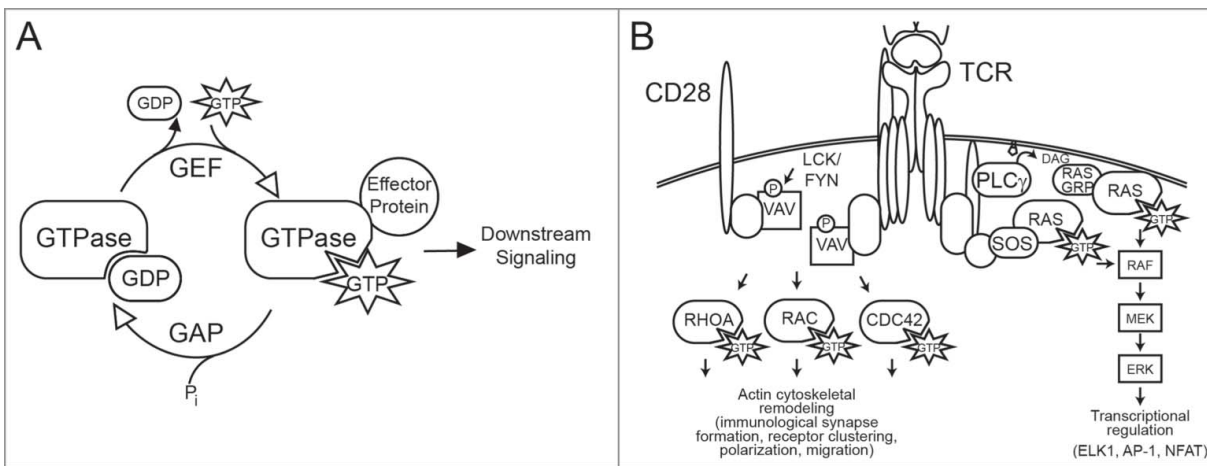
Figure 1. (A) Activation cycle of small GTPases. Ras and Rho family GTPases are activated at the cell membrane by GEFs, which catalyze the release of GDP and promote the loading of GTP, thereby inducing binding to effector proteins. GTPase activating proteins (GAPs) facilitate the hydrolysis of GTP to GDP, thereby inactivating the GTPase. Membrane targeting of GTPases is also regulated by guanine nucleotide dissociation inhibitors (GDIs, not shown). (B) Role of GTPases in T-cell receptor (TCR) signaling. Activation of the TCR stimulates activation of Ras through multiple mechanisms, including PLCγ-mediated generation of DAG, which recruits the Ras guanosine nucleotide exchange factor (GEF) GRP and through recruitment of another Ras GEF, SOS. Rho family GTPases including RAC are activated by multiple GEFs, including VAV1, which is activated by tyrosine phosphorylation (as reviewed in references, 8, 10, 52 and others).

inactivating the GTPase and preventing binding to effectors. Conversely, GTPases are activated by guanosine nucleotide exchange factors (GEFs), which facilitate the release of GDP from the GTPase and allow the binding of GTP. This action converts the protein to the GTP-loaded, active conformation, primed to bind effectors and propagate the signal to downstream factors.

Ras GTPases

The Ras family includes some of the best characterized GTPases, and the corresponding genes are among the most frequently mutated genes in multiple human tumor types. RAS is activated at the cell membrane by diverse stimuli, including activation of both the pre-TCR and mature TCR in T cells. Ligation of the TCR induces the formation of a proximal signaling complex, including phospholipase Cγ (PLCγ; encoded by PLCG1) and the adaptor growth factor receptor-bound protein 2 (GRB2), which are both required for activation of RAS. PLCγ hydrolyzes the lipid species phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] to the second messengers diacylglycerol (DAG) and inositol 3-phosphate (IP$_3$). In T cells, the GEF RASGRP1 binds to DAG, while the GEF Son of Sevenless (SOS) binds GRB2, thereby coupling RAS activation to TCR signaling. RAS activates multiple potent downstream signaling cascades, including the phosphatidylinositol-3-kinase (PI3K) pathway and mitogen-activated protein (MAP) kinase signaling through RAF, MEK, and ERK MAP; these signaling pathways are critical regulators of cell survival and proliferation.[11] The DAG produced by PLCγ also activates protein kinase C resulting in

CARD11 phosphorylation and subsequent activation of the CARD11-BCL10-MALT1 complex, a key mediator of NF-κB activation. In addition, RAS activates the GTPase RAC[12] and cooperates with IP$_3$-regulated calcium signaling to regulate NFAT (nuclear factor of activated T cells) transcription factors.[13,14] RAS is also required in T-cell development for positive and negative selection.[15]

Rho GTPases

The Rho family of GTPases is comprised of 22 members. This review will focus specifically on RAC1, CDC42, and RHOA, which are best characterized as regulators of actin cytoskeletal remodeling. TCR ligation or engagement of the costimulatory protein CD28 is followed by a burst of actin reorganization to induce TCR clustering and formation of the immunological synapse, thereby stabilizing the interaction between T cells and antigen-presenting cells. Active, GTP-bound Rho GTPases bind to specific effector proteins. As many as 70 effector proteins for RHOA, RAC1, or CDC42 have been described, including various proteins involved in actin cytoskeletal organization such as p21-activated kinase (PAK) and WASp/WAVE family proteins.[9] These GTPases also regulate cell migration, adhesion, trafficking, and transcription, thereby regulating T-cell differentiation, proliferation, and activation.

A primary activator of RAC in T cells is the haematopoietic -specific GEF VAV1. VAV1 integrates signals from tyrosine kinases to activate RAC, and potently regulates lymphocyte development and activation. Genetic knockout of VAV1 results in dramatic defects in TCR

signaling and clustering, reduced calcium flux, and impaired NFAT transcriptional activity.[16-19] VAV1 is phosphorylated by Src family kinases, including LCK, which is activated following TCR ligation.[20,21] Phosphorylation of critical tyrosine residues near the N-terminus of VAV1 induces a conformational change that relieves autoinhibition and exposes the Dbl homology (DH) domain, which is responsible for GEF function, primarily toward RAC.[22-24] In addition, VAV1 also demonstrates GEF-independent activities, likely due to the adaptor function of its Src homology- (SH) 2 and SH3 domains.[25,26] Interestingly, VAV1 has been found to be aberrantly expressed in multiple human tumor types, and deletion of key regulatory domains converts VAV1 to an oncogene.[27-29] Thus, while critical for lymphocyte function, VAV1 is also a proto-oncogene implicated in human cancers.

Somatic alterations in GTPase-related genes in in peripheral T-cell lymphomas

VAV1

Through an integrated mate-pair DNA sequencing and RNA sequencing approach for fusion detection, we recently discovered recurrent VAV1 fusion genes in PTCL.[30] Fluorescence in situ hybridization (FISH) studies demonstrated that chromosomal rearrangements of the VAV1 locus were present with a frequency of 11% in PTCL, NOS and ALCL, but absent in other subtypes (Fig. 2). Both of the VAV1 fusions studied by next generation-sequencing showed identical fusion breakpoints at residue 777, just proximal to the C-terminal SH3 domain and resulting in complete loss of this domain in the fusion protein. The C-terminal SH3 domain is responsible for autoinhibition of VAV1 activation, and deletion of this domain results in constitutive activation.[31] Consistent with loss of this autoinhibitory function, we demonstrated in vitro that the VAV1 fusion promoted cell growth and migration. We further showed that RAC1 was the major GTPase activated by the VAV1 fusion protein, and that the resultant cell growth was RAC1-dependent. Importantly, we also showed that the VAV1 fusion was targetable with the clinically available RAC1 inhibitor azathioprine.

In further support of an oncogenic role for VAV1 in PTCLs, somatic mutations in the VAV1 gene have also been identified in 18% of ATLs and in occasional cases of AITL and other PTCLs derived from T follicular helper (T_{FH}) cells.[5,32] Similar to the role of the breakpoint in VAV1 fusions, most VAV1 mutations in ATL and AITL/T_{FH}-like PTCLs affected the C-terminal SH3 domain, while additional mutation clusters in acidic, pleckstrin homology (PH), and zinc-finger protein domains were also identified in ATL.[32] One of the residues in the SH3 domain found to be mutated in both studies, D797, was previously demonstrated to have a role in oncogenic transformation.[29] In addition, a mutation occurring in the acidic domain Y174C was predicted to disrupt autoinhibitory interaction with the Dbl homology (DH) domain, and this residue has been shown to negatively regulate VAV1 activity.[33] Although functional characterization of these specific mutations in the context of PTCL has not been reported, the clustering of mutations in specific regions of VAV1, in particular the C-terminal SH3 domain, suggest a role for VAV1 activation in T-cell malignancies. To this end, RAC inhibition should be
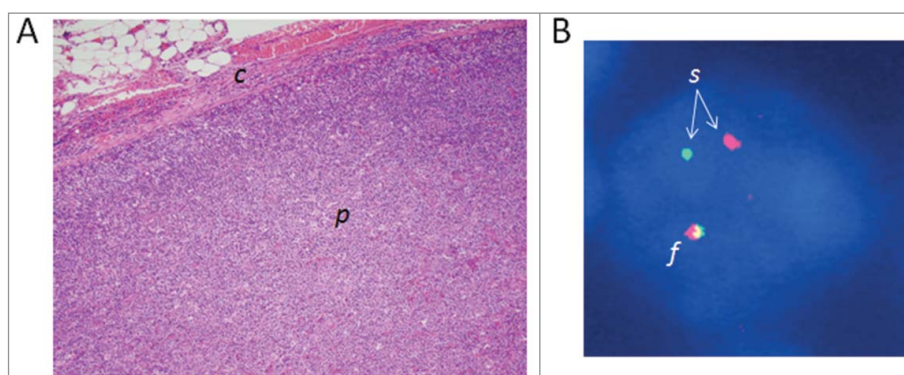

Figure 2. Three-centimeter lymph node from a 55 year-old male with peripheral T-cell lymphoma, not otherwise specified, bearing a chromosomal rearrangement of the GEF gene *VAV1. VAV1* rearrangements are seen recurrently in PTCL (see text).[30] (A) Photomicrograph of the lymph node biopsy (hematoxylin and eosin stain; original magnification, 10×). The architecture of the lymph node parenchyma (*p*) has been effaced by the infiltrating lymphoma cells. *c*, lymph node capsule. (B) Fluorescence *in situ* hybridization (FISH) image of a single lymphoma cell nucleus (stained blue). The DNA has been hybridized with red and green fluorescent probes flanking the *VAV1* locus. One *VAV1* allele shows a normal red-green fusion signal (*f*), whereas the red and green signals of the other allele are split (*s*), indicating a *VAV1* rearrangement.

evaluated as a potential targeted therapy approach for PTCLs with activating mutations or structural alterations of VAV1. In addition, the potential effect of these genetic alterations on the non-GEF activity of VAV1, as well as the role of mutations or altered function of other VAV family members,[5,34] merits further study.

RHOA

A highly recurrent mutation in the GTP/GDP binding domain of RHOA, G17V, was identified by several groups to be present in both AITL (50% to 71% of cases) and PTCL, NOS (8–18% of cases, particularly those with AITL-like features).[35-37] In vitro experiments revealed a dominant negative role for the G17V mutation whereby mutant RHOA prevents binding of GTP by both mutant and wild type RHOA, presumably by sequestering RHOA GEFs.[35-37] This dominant negative function was further supported by data demonstrating inhibition of serum response factor-responsive element (SRF-RE) reporter activity and decreased actin stress fiber formation.[35,36] In a T-cell model, overexpression of wild type but not mutant RHOA resulted in growth suppression, although mutant RHOA itself did not promote growth over mock-transduced control cells.[35] Interestingly, gene set enrichment analysis of PTCLs with the G17V mutation compared to those with wild type RHOA revealed enrichment of the RAC1 pathway as well as T-cell cytokine signaling, NOTCH, and NF-κB pathways.[38] On the basis of the reciprocal relationship between RAC1 and RHOA,[39] it would be of interest to evaluate RAC1 activation in the RHOA G17V model for potential therapeutic targeting strategies via RAC1 inhibition. In addition, understanding mechanisms for cooperativity between RHOA G17V and other mutations with which it is associated, including those involving IDH2, DNMT3A, and particularly TET2, may elucidate additional potential therapeutic strategies.[40]

Other RHOA mutations, albeit far less frequent than G17V, have been reported in PTCL, affecting amino acids 16–19 (C16R, G17E, T19I, and K18N) of the GTP/GDP binding domain.[5,36] Unlike G17V, the K18N mutation appears to activate rather than inhibit RHOA; hence, its specific role in PTCL tumorigenesis is unclear.

PLCG1/KRAS

In addition to mutations directly or indirectly affecting Rho family GTPases, genetic aberrations have also been reported in genes involved in Ras family GTPase signaling, including PLCG1 and KRAS. Of these, PLCG1 is the most commonly involved and is recurrently mutated in ATL, cutaneous T-cell lymphoma (CTCL), AITL, and PTCL, NOS.[5,32,41,42] Mutations in PLCG1 affect regions that encode several protein domains and have been shown to promote MALT1 cleavage and NFAT activity to varying degrees.[5,41]

The GTPase KRAS is highly mutated across human cancers, most commonly in carcinomas of the pancreas, lung, and colon. Recently, KRAS mutations also were reported in PTCLs.[5,43] Most of the KRAS mutations identified in PTCL overlap with previously characterized mutation hotspots, including amino acids 12 and 13 as well as the less frequently mutated amino acids 18 and 146. Although the function of these mutations has not been reported in PTCL, these mutations are well characterized in other tumor types. Primarily, mutations in Ras family genes lead to Ras activation, subsequent activation of targets such as ERK, and promotion of cell growth, invasion, and survival, and represent a promising candidate for targeted therapies.[44,45]

CD28, LCK, and FYN

Mutations upstream in the TCR pathway may also dramatically impact GTPase activity. The Src-family kinases LCK and FYN play a critical role in the early steps of TCR signaling including phosphorylation of the TCRζ chain, leading to recruitment and activation of ZAP-70 and subsequent phosphorylation of adaptor molecules such as LAT, which in turn facilitate downstream kinase signaling and T-cell activation.[46] LCK and FYN also are required for CD28 engagement and subsequent VAV1 phosphorylation (Fig. 1).[47] Recurrent somatic mutations in CD28 have been reported in AITL (9–11%), ATL, and, less frequently, in PTCL, NOS.[5,32,48,49] In vitro study of 2 commonly mutated residues, D124 and T195, revealed activation of TCR signaling.[48,49] The D124V mutation, located near the ligand binding site of CD28, conferred greater affinity for its CD86 ligand.[48] Likewise, the T195P mutation, situated between the SH2 and SH3 domains, exhibited higher affinity to the adaptor proteins GRB2 and GRAP2.[48,49] NF-κB activity also was enhanced by these CD28 mutants, suggesting increased GTPase activity, although GTPases were not measured directly.[48,49] CD28 gene fusions, namely CTLA4-CD28 and ICOS-CD28, have been reported across numerous PTCL subtypes and at greater frequency than CD28 mutations.[32,48,50,51] Focal amplifications of CD28 also have been reported in PTCLs.[32,51] However, further study is needed to clearly define the functional role of these fusions and their possible effect on GTPase signaling.

Several mutations have been identified in FYN, primarily affecting its SH2, SH3, and C-terminal domains in PTCL, NOS, AITL, and ATL, with frequencies of about 3–4%.[5,32,36] In a manner similar to that observed for VAV1 and RHOA mutations, mutations in FYN are predicted to interfere with an inhibitory interaction

Table 1. Summary of mutations and fusion genes involving GTPase and TCR–related genes in peripheral T-cell lymphomas.

Gene	Mutation	Affected domain/Functional consequence	References
VAV1	SNV	Activating	5, 32
	Fusion	Truncation of SH3 domain in *VAV1-GSS* and *VAV1-MYO1F* fusions; increased RAC activation	30
RHOA	SNV	G17V in GTP binding domain; dominant negative function	35–37
FYN	SNV	SH2 domain; activating through loss of inhibitory SH2 domain and Y531 interaction	5, 32, 36
CD28	SNV	D124, T195; activation of NF-κB and TCR signaling	32, 48, 49
	Fusion	*CTLA4-CD28* and *ICOS-CD28*	5, 32, 48, 50
LCK	SNV	Predicted activating	5
PLCG1	SNV	MALT1 and NFAT activation	5, 32, 41
KRAS	SNV	Activating	5, 43

SNV, single nucleotide variant; TCR, T-cell receptor.

between the FYN SH2 domain and the C-terminal Src kinase (CSK)-phosphorylated Y531 residue.[36] In vitro studies of FYN mutations identified by Palomero et al revealed that these mutations indeed activated FYN through loss of the interaction between the SH2 domain and phosphorylated Y531.[36] Furthermore, mutant FYN activation was targetable with the kinase inhibitor dasatinib. However, the functional implications of these FYN mutations and specifically their effect on GTPase activity, GTPase-dependent signaling, and downstream functions such as actin structure, migration, and cell growth have not been reported. LCK mutations in the tyrosine kinase domain (N446K, P447R) were recently reported in a single AITL patient, but although predicted to be activating, their recurrence and function have not yet been determined.[5]

Summary

With the recent progress in mutational profiling of PTCLs, somatic mutations and gene fusions have been reported in several GTPase-related genes, including VAV1, RHOA, KRAS, PLCG1, CD28, FYN, and LCK (Table 1). In addition to the TCR-related genes upstream of GTPases (VAV1, CD28, FYN, LCK, PLCG1) and GTPases themselves (RHOA and KRAS), activating mutations have been reported in downstream TCR signaling genes such as CARD11.[5,32] Among TCR-related genes, PLCG1 is the most frequently mutated in PTCL, and PLCG1 mutations often co-occur with one of the other TCR-related mutations.[5,32] In contrast, mutations in non-PLCG1 TCR-related genes do not often occur together in the same case.

With the exception of RHOA G17V, most of these genomic events either have been demonstrated to be activating in vitro or are predicted to be activating. Because

RHOA opposes RAC1 activity, the dominant negative RHOA G17V mutation may have some similarities to VAV1 rearrangements; in fact, ectopic expression of both VAV1 fusions and RHOA G17V cause cytoskeletal rearrangement. However, the effect of RHOA G17V on RAC1 has not been reported. Likewise, KRAS has been shown to promote RAC1 activation, although KRAS mutations remain to be studied in T cell models. Hence, GTPases, especially RAC1, may become increasingly recognized for a role in PTCL biology including cell growth, cytoskeletal remodeling, and cell migration. Targeted therapeutic strategies such as RAC inhibition (azathioprine) and tyrosine kinase inhibition (dasatinib) have been shown to target VAV1 fusions and FYN mutations, respectively. It will be important moving forward to evaluate the specific role of other GTPase-related mutations on GTPase signaling and to test these and related drugs for efficacy against these candidate therapeutic targets.

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ORCID

Gina L. Razidlo http://orcid.org/0000-0002-9042-2738
Andrew L. Feldman http://orcid.org/0000-0001-5009-4808

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