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Role of Receptor Interacting Protein (RIP) kinases in cancer

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Abstract The Receptor Interacting Protein (RIP) kinase family consists of seven Serine/Threonine kinases, which plays a key signaling role in cell survival and cell death. Each RIP family member contains a conserved kinase domain and other domains that determine the specific kinase function through protein–protein interactions. RIP1 and RIP3 are best known for their critical roles in necroptosis, programmed necrosis and a non-apoptotic inflammatory cell death process. Dysregulation of RIP kinases contributes to a variety of pathogenic conditions such as inflammatory diseases, neurological diseases, and cancer. In cancer cells, alterations of RIP kinases at genetic, epigenetic and expression levels are frequently found, and suggested to promote tumor progression and metastasis, escape of antitumor immune response, and therapeutic resistance. However, RIP kinases can be either pro-tumor or anti-tumor depending on specific tumor types and cellular contexts. Therapeutic agents for targeting RIP kinases have been tested in clinical trials mainly for inflammatory diseases. Deregulated expression of these kinases in different types of cancer suggests that they represent attractive therapeutic targets. The focus of this review is to outline the role of RIP kinases in cancer, highlighting potential opportunities to manipulate these proteins in cancer treatment.

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Abbreviations

AAs	amino acids
AML	acute myeloid leukemia
5-aza-dC	5-aza-2′-deoxycytidine
CARD	caspase-recruiting domain
cIAP	cellular Inhibitor of Apoptosis
CARDIAK	CARD-containing ICE associated kinase
CD95L	CD95 ligand
CLL	chronic lymphocytic leukemia
COR	C-terminus of Roc
COX-2	cyclooxygenase 2
CRC	colorectal cancer
CSCC	cervical squamous cell carcinoma
CYLD	cylindromatosis
DAI	DNA-dependent activator of IFN regulatory factors
DAMP	damage associated molecular pattern
DFS	disease-free survival
DNMT1	DNA methyltransferase 1
DIK	PKC delta-interacting protein kinase
DR	death receptor
DSS	dextran sodium sulfate
EGFR	epidermal growth factor receptor
EMT	epithelial—mesenchymal transition
ESCC	esophageal squamous cell carcinoma
FADD	Fas-associated death domain
5-FU	5-fluorouracil
GEO	gene expression omnibus
GLUL	glutamate-ammonia ligase
HCC	hepatocellular carcinoma
HMGB1	high mobility group box 1
HNSCC	head and neck squamous cell carcinoma
HSP90	heat shock protein 90
ICD	immunogenic cell death
ICE	IL-1β converting enzyme
IFN	interferon
IL	interleukin
KD	knockdown
KIRC	kidney renal clear cell carcinoma
KO	knock-out
LRR	leucine-rich repeat
LRRK	leucine-rich repeat kinase
MDSCs	myeloid derived suppressor cells
MLKL	mixed lineage kinase domain like pseudokinase
NK	natural killer cell
NKT	natural killer T cell
NLR	nod-like receptor
NOD	nucleotide-binding oligomerization domain
NPC	nasopharyngeal carcinoma
OS	overall survival
OSCC	oral squamous cell carcinoma
PARP	poly-ADP ribose polymerase
PDA	pancreatic adenocarcinoma
PFS	progression free survival
PGAM5	phosphoglycerate mutase 5
PGE_2	prostaglandin E_2
PKC	protein kinase C
PKK	PKC-associated kinase
PRR	pattern recognition receptor
RICK	receptor-interacting serine/threonine kinase
RIP	receptor interacting protein
RIPK	receptor interacting protein kinase
RHIM	respective homotypic interaction motif
ROS	reactive oxygen species
Roc	Ros of complex proteins
SgK288	Sugen kinase 288
SNP	single nucleotide polymorphism
Sp1	specific-protein-1
STS	staurosporine
TCGA	the cancer genome atlas
TAM	tumor associated macrophage
TLR	Toll-like receptor
TME	tumor microenvironment
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis inducing ligand receptor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adaptor inducing IFN-β
TSCC	tongue squamous cell carcinoma
Ub	ubiquitin
UHRF1	ubiquitin-like, containing PHD and RING finger domains 1
WT	wild type
Zbp1	Z-DNA-binding protein 1

Introduction

Receptor Interacting Protein (RIP) kinases are a family of Serine/Threonine kinases that play a wide variety of functional roles in cellular signaling during pathogen infection, inflammation, DNA damage, and response to extracellular stimuli. RIP1 is the founding member initially described in 1995.[1] It was found to interact with the cell-surface receptor Fas/APO-1 (CD95) and therefore, named Receptor Interacting Protein (RIP).[1] Since then, six additional RIP kinases have been identified, each varying in function and being classified as a RIP family member based on their homologous kinase domains (Fig. 1). Among RIP kinases, RIP1 and RIP3 are best studied as critical players in necroptosis. RIP1 was initially shown to play a role in necroptosis in the early 2000s, while RIP3's role in necroptosis was not described until 2009.[2—5] Distinguished from other RIP kinases, RIP1 and RIP3 both contain a Respective Homotypic Interaction Motif (RHIM) domain that is involved in necroptosis through homo- or heterotypic interactions. Dysregulation of RIP kinases interferes with various signaling pathways, including cell survival and cell death pathways, which can promote oncogenic function. Because of this, it is not surprising

that RIP kinases are frequently upregulated or downregulated in certain types of cancer. These alterations are not only kinase-specific, but also cancer-specific. The goal of this review is to summarize the alterations of RIP kinases in cancer and discuss how these alterations impact cancer cell survival and death, metastatic potential, therapeutic response, and antitumor immune response. We will also briefly discuss various therapeutic agents known to induce necroptosis via RIP1 and/or RIP3 in cancer cells.

Structures and functions of RIP kinases

Structures of RIP kinases

RIP family kinases share 20–30% homology within a kinase domain, which is ~260–270 amino acids in length and most commonly located at the N-terminus (Fig. 1, 2). RIP1, RIP2, and RIP3 are the most similar RIP kinases based on their structures (Fig. 1, 2). RIP1 and RIP3 both contain an N-terminal kinase domain and a RHIM domain, allowing them

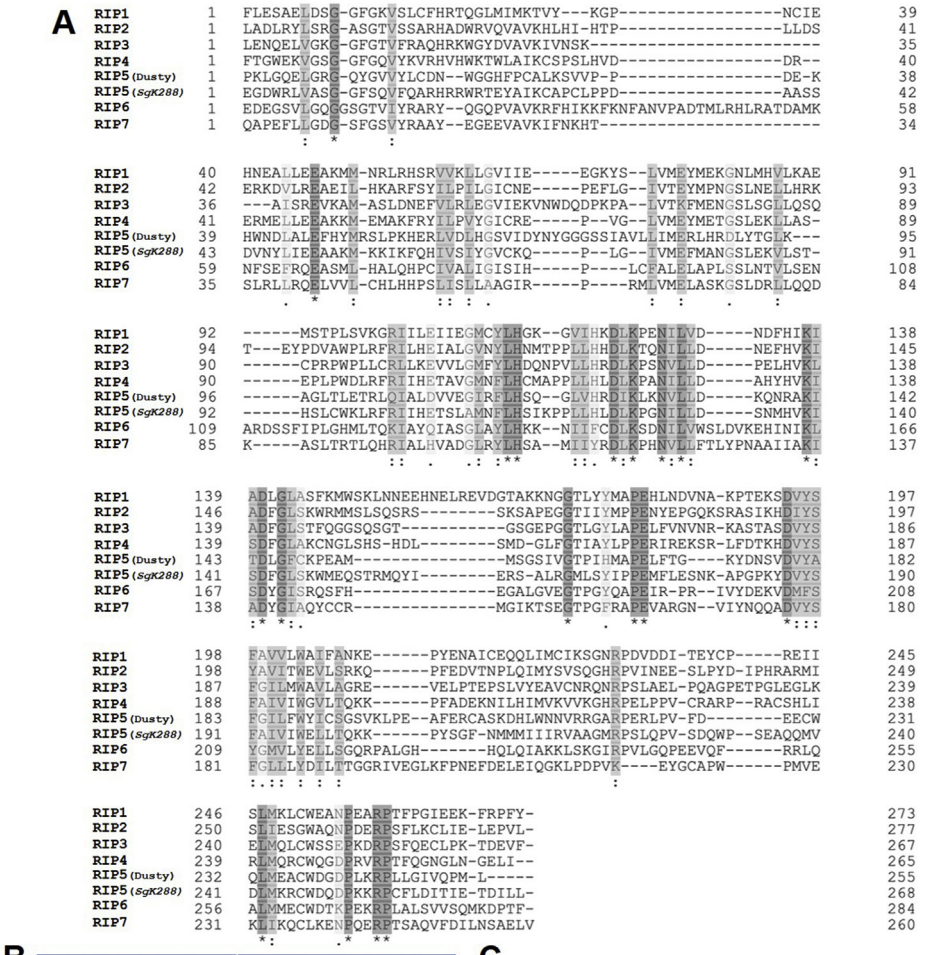

Figure 1 Sequence homology of the kinase domains of RIP family members. **(A)** Sequence alignment of RIP kinase domains generated through UniProtKB. Dark grey (*) indicates fully conserved residues, medium grey (:) indicates conserved residues with strong similarity, and light grey (.) indicates conserved residues with weak similarity. Two different RIP5s have been described, including Dusty Protein Kinase (Dusty) and Sugen kinase 288 (SgK288). **(B)** Percent similarity relative to RIP1 calculated through UniProtKB. Each kinase domain has approximately 20–30% similarity to RIP1. **(C)** Phylogenetic tree of RIP kinases generated through UniProtKB.

to interact with each other and other RHIM domain-containing proteins to trigger necroptosis. RIP1 also contains a death domain, which enables it to interact with other death-domain-containing proteins. RIP2 contains a caspase-recruiting domain (CARD) that can interact with and activate caspase-1, an inflammatory caspase.[6] RIP4 and RIP5 (Sugen kinase 288 [SgK288]/ANKK1) are structurally similar and both contain ankyrin repeats.[7] Two RIP5s have been reported, with one referring to a protein related to the Dusty protein kinase and the other referring to SgK288, also known as ANKK1.[8–10] RIP6 and RIP7 are most different from the other RIP family members, with each containing leucine-rich repeats (LRRs) and a Ros of complex proteins (Roc)/C-terminus of Roc (COR) domain.[7,8] RIP6, similar to RIP4 and RIP5, contains ankyrin repeats; but these are located at the N-terminus, rather than the C-terminus.[7] The presence of these non-kinase domains suggests protein–protein interactions are a key feature of RIP function in regulating cell death and immunity.

Functions of RIP kinases

The functions of each RIP kinase are summarized in Table 1, followed by a more detailed description below.

RIP1 and RIP3

RIP1 was initially reported to interact with Fas and Tumor Necrosis Factor (TNF) Receptor (TNFR) through its C-terminal death domain in yeast, which promoted cell death.[1]

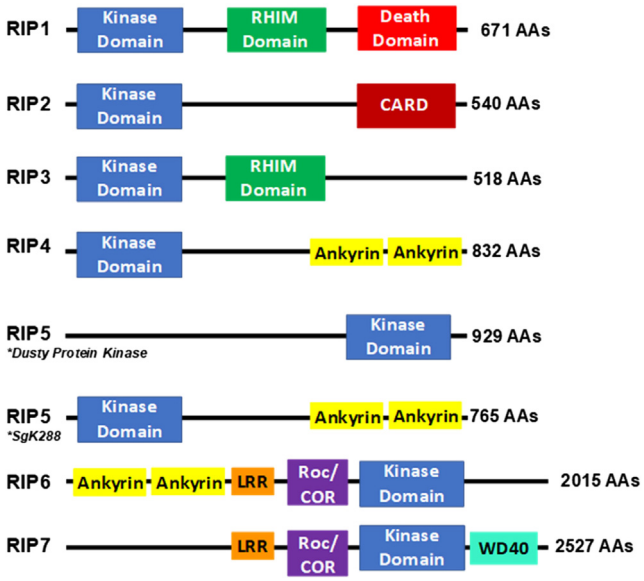

Figure 2 Structure of RIP Kinases. RIP1-RIP5 (SgK288) contain N-terminal kinase domains. RIP1 contains a C-terminal death domain, while RIP2 contains a C-terminal caspase-recruiting domain (CARD). Both RIP1 and RIP3 are RHIM-containing proteins, which is located within the intermediate domain. RIP4, RIP5 (SgK288), and RIP6 contain ankyrin repeats. RIP6 and RIP7 both contain leucine-rich repeats (LRRs) and a Ros of complex proteins (Roc)/C-terminus of Roc (COR) domain. RIP7 also contains WD40 repeats. The number of amino acids (AAs) in each protein is indicated on the right.

Subsequent studies showed that RIP1 can bind to other death receptors, including TNF-Related Apoptosis Inducing Ligand Receptor 1 (TRAILR1 or Death Receptor 4 [DR4]) and TRAILR2 (DR5), as well as various adaptor proteins including TNF Receptor-Associated Factor 1 (TRAF1), TRAF2, TRAF3, TNF Receptor-Associated Death Domain (TRADD), and Fas-Associated Death Domain (FADD).[7,11] In addition to mediating cell death, RIP1 has also been shown to promote cell survival, specifically through the NF-κB and MAPK pathways.[12–14] Whether RIP1 promotes cell survival or cell death is dependent on the specific cellular context and likely, its binding partners. RIP1's kinase activity, which can be blocked by Necrostatin-1, has been shown to be critical for its role in cell death, but dispensable for RIP1-mediated cell survival.[15,16] Necrostatin-1 interferes with RIP1's conformation, and therefore, can potentially affect its non-kinase functions.[17] The RHIM domain of RIP1 allows it to interact with other RHIM-containing proteins, such as RIP3, DNA-dependent Activator of IFN regulatory factors (DAI)/Z-DNA-binding protein 1 (Zbp1), and TIR-domain-containing adaptor Inducing Interferon (IFN) β (TRIF), which is involved in the Toll-Like Receptor (TLR) pathways.[18,19] Knock-out (KO) of *RIP1* in mice leads to perinatal lethality with increased systemic inflammation and cell death.[15,16,20]

Table 1 Functions of RIP kinases.

RIP kinases (alternative names)	Functions	References
RIP1 (RIPK1)	• NF-κB and MAPK signaling • TLR signaling • Apoptosis and necroptosis	1,8,12–14,19,38
RIP2 (RIPK2; RICK; CARDIAK)	• NOD signaling	7,8,14,50,51
RIP3 (RIPK3)	• IFN signaling • Metabolism • Necroptosis	3–5,33,34,36
RIP4 (RIPK4; DIK[human]; PKK [mouse])	• NF-κB and JNK signaling • Wnt/β-catenin signaling • Epidermal differentiation • Cutaneous inflammation	7,8,14,57–60
RIP5 (RIPK5; Dusty Protein Kinase; SgK288; ANKK1)	• Dusty Protein Kinase: cell death • SgK288/ANKK1: neurodevelopment	7–9,61,62
RIP6 (RIPK6; LRRK1)	• Endocytosis	64,65
RIP7 (RIPK7; LRRK2)	• Vesicle trafficking	65–67

Abbreviations: CARDIAK: CARD-containing ICE associated kinase; DIK: PKC delta-interacting protein kinase; IFN: Interferon; LRRK: Leucine-rich repeat kinase; NOD: Nucleotide-binding oligomerization domain; PKK: PKC-associated kinase; RICK: Receptor-interacting serine/threonine kinase; RIP: Receptor interacting protein; RIPK: Receptor interacting protein kinase; SgK288: Sugen kinase 288; TLR: Toll-like receptor.

RIP3 was initially described in 1999 as an inducer of apoptosis independent of its kinase activity.[21–23] There are conflicting reports on whether RIP3 can also activate the NF-κB pathway.[21–24] Unlike RIP1 and RIP2, RIP3 does not contain a death domain or CARD domain (Fig. 2) and is not suspected to directly interact with death domain-containing proteins. In 2009, RIP3 was reported to be a crucial mediator of TNF-α-dependent necroptosis, which requires its kinase activity.[3–5,25] Several studies showed that RIP3, when overexpressed or under certain conditions such as kinase inhibition, is able to promote apoptosis, in addition to necroptosis.[26–29] A recent study revealed that in certain cancer cells, whether cells die by necroptosis or apoptosis is determined by the levels of heat shock protein 90 (HSP90) and CDC37.[30] In some cell lines, RIP3 expression alone was sufficient to cause apoptosis via its autophosphorylation at S164/T165.[30] Treatment with small-molecule RIP3 kinase inhibitors at a high concentration also results in apoptosis with blocked necroptosis.[26,31] Similar to RIP1, RIP3 can interact with DAI/Zbp1 and TRIF through its RHIM domain, which are involved in necroptosis and TLR pathways, respectively.[31,32] These interactions of RIP3 are also involved in IFN production.[33,34] In some cases, such as during TLR stimulation, necroptosis can occur independent of RIP1 by direct activation of RIP3 through another RHIM domain-containing protein.[31,35] In addition, RIP3 also regulates metabolism by interacting with various metabolic enzymes, such as Glutamate-Ammonia Ligase (GLUL), which leads to increased production of reactive oxygen species (ROS).[5,36] Furthermore, *RIP3* KO mice are viable and can rescue the embryonic lethality of *caspase-8* and *FADD* KOs.[37]

RIP1 and RIP3 functions have been elucidated largely through studies of the key steps in TNF-α-induced-cell death (Fig. 3). Upon TNF-α binding to the TNFR, complex I forms, which typically consists of TRADD, TRAF2, RIP1, and E3 ubiquitin ligases cellular Inhibitor of Apoptosis 1 (cIAP1) and cIAP2.[38,39] From this point, either cell survival or cell death can occur. The cIAPs can ubiquitinate RIP1, which leads to activation of NF-κB and MAPK pathways to promote cell survival.[39,40] Upon cIAP degradation, RIP1 can be de-ubiquitinated by deubiquitinase cylindromatosis (CYLD).[41] Upon deubiquitination, Complex IIa forms (Fig. 3), which typically includes RIP1, caspase-8, and FADD.[38,42] Formation of this complex can trigger cell death by apoptosis. However, when caspase activity is inhibited, RIP1 can interact with RIP3 through their RHIM domains, forming a functional amyloid signaling complex termed "necrosome" or Complex IIb, which triggers necroptosis.[38,43,44] Upon interaction, RIP3 becomes auto-phosphorylated, which leads to recruitment and phosphorylation of necroptotic executor Mixed Lineage Kinase domain Like pseudo-kinase (MLKL).[3–5,45] Phosphorylated MLKL then translocates and oligomerizes at the plasma membrane to induce rupture, which causes release of Damage Associated Molecular Patterns (DAMPs), such as High Mobility Group Box 1 (HMGB1), stimulating an immune response.[45,46] It is important to note that RIP1 and RIP3 can promote cell death independently during drug treatment or infection.[38]

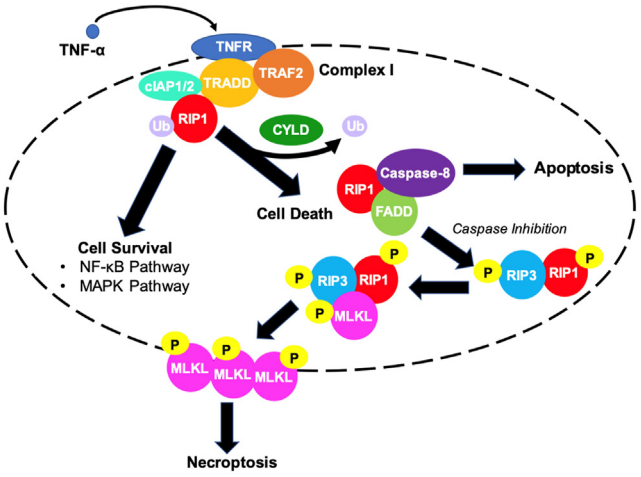

Figure 3 RIP1 and RIP3 are critical mediators of necroptosis. TNF-α binds to the TNFR, promoting formation of Complex I, consisting of TRADD, TRAF2, cIAP1/2, and RIP1. cIAP1/2 ubiquitinates (Ub) RIP1, which can promote cell survival through the NF-κB and MAPK pathways. Upon de-ubiquitination by CYLD, RIP1 can interact with caspase-8 and FADD, promoting apoptosis. When caspase is inhibited, RIP1 can promote necroptosis by interacting with RIP3, leading to its autophosphorylation. Phosphorylated RIP3 recruits and phosphorylates MLKL, which translocates and oligomerizes at the plasma membrane, inducing rupture.

Other RIP kinases

RIP2. RIP2, also known as Receptor-interacting serine/threonine kinase (RICK) or CARD-containing interleukin (IL)-1β converting enzyme (ICE) associated kinase (CARDIAK), was described in 1998.[6,47,48] RIP2 was shown to activate the NF-κB and JNK pathways independent of its kinase activity.[6,48] RIP2 can bind to and activate inflammatory caspase-1 through their respective CARD domains.[6] Caspase-1 is involved in the activation of proinflammatory cytokines, such as pro-IL-1β and pro-IL-18.[49] RIP2 was also shown to interact with various adaptor proteins, such as TRAF1 and TRAF2, and with E3 ubiquitin ligases cIAP1 and cIAP2.[6] Overexpression of RIP2 in some cells resulted in apoptosis.[48] Furthermore, RIP2 plays a role in the innate immune response through interaction with Nucleotide-binding Oligomerization Domain 1 (NOD1) and NOD2, which are a type of pattern recognition receptor (PRR) that recognizes bacterial infection.[50,51] Interaction between NOD1/2 and RIP2 can also result in activation of the NF-κB pathway.[52–54]

RIP4. RIP4, also known as Protein Kinase C (PKC) Delta-Interacting protein Kinase (DIK) in humans and Protein Kinase C-associated Kinase (PKK) in mice, was first described in the early 2000s.[55,56] RIP4 was found to activate NF-κB and JNK pathways, which require its kinase activity.[8,14] The kinase domain of RIP4 alone was sufficient to stimulate these pathways.[57] RIP4 interacts with various TRAF proteins and can be cleaved in a caspase-dependent manner during apoptosis, a common

characteristic of different RIP proteins.[7] RIP4 can also phosphorylate Dishevelled proteins to stimulate Wnt/β-catenin signaling and is involved in epidermal differentiation and cutaneous inflammation.[58–60] *RIP4* KO in mice results in perinatally lethality and defects in the epidermis.[59]

RIP5. In 2004, Zha et al first reported RIP5, a protein related to Dusty protein kinase.[9] Overexpression of RIP5 resulted in cell death with characteristics of apoptosis, such as nuclear fragmentation.[9] However, inhibition of caspases did not affect RIP5-induced death, suggesting non-apoptotic cell death.[9] Due to the difference in structure of RIP5 compared to other RIP kinases (Fig. 2), Meylan et al proposed to abandon the term RIP5 for this protein kinase.[8] Instead, they identified another protein commonly known as SgK288 or ANKK1 that was fairly similar to RIP4 with approximately 35% homology, containing both an N-terminal kinase domain and C-terminal ankyrin repeats (Fig. 2).[8,10] They decided to refer to this kinase as RIP5.[8] Studies suggest RIP5 plays a role in the central nervous system, specifically in neurodevelopment, and variations in this protein may be involved with psychiatric disorders, such as addiction.[61,62] However, the exact physiological and biochemical functions of RIP5 remain unclear.

RIP6 and RIP7. RIP6 and RIP7 are also known as leucine-rich repeat kinase 1 (LRRK1) and LRRK2, respectively, due to their LRR domains (Fig. 2).[7,8] LRR domains are found in a variety of immune-related proteins, such as Nod-Like Receptors (NLRs), and mutations in LRR-containing proteins are associated with a variety of human inflammatory diseases.[63] RIP6 plays a role in endocytosis and endosome trafficking, specifically with the epidermal growth factor receptor (EGFR).[64,65] RIP7 has been shown to be involved in vesicle trafficking.[65–67] These kinases are also known to play a role in neurodegenerative diseases, with most information reported being in the context of these diseases.[68,69] For example, mutations in RIP6 and RIP7 are often associated with Parkinson's disease.[70,71]

Roles of RIP kinases in cancer development

Depending on specific cancer types, RIP kinases can be either oncogenic or tumor suppressive. The dual roles most likely reflect the functions of these kinases in cell survival, cell death, and inflammation.

Alterations of RIP kinase expression and impacts in cancer

RIP kinases are frequently altered in cancer mainly at their expression levels. The types and impacts of the alterations of RIP kinases in different types of cancer are outlined in Table 2 and further discussed below. RIP5 and RIP7 are not included in the discussion due to lack of their information in cancer.

RIP1

RIP1 is commonly downregulated in breast cancer, colorectal cancer (CRC), and head and neck squamous cell carcinoma (HNSCC).[38,72,73] Low RIP1 expression in triple negative breast cancer (TNBC) was associated with worse clinical outcomes.[74] It was specifically shown that RIP1 negatively regulates Aquaporin-1, a known oncogenic transport protein, and when RIP1 was overexpressed, both invasiveness and tumor burden were decreased.[74] RIP1 was also associated with increased drug sensitivity in esophageal squamous cell carcinoma (ESCC) and chemotherapy-induced necrosis in CRC cells when apoptosis was inhibited.[75,76] RIP1 is upregulated in glioblastoma, melanoma, lung cancer, pancreatic adenocarcinoma (PDA), gastric cancer, and gallbladder cancer.[38,72,77–81] One study revealed approximately 30% of grade IV glioblastomas have upregulated RIP1 expression, with high RIP1 expression being associated with decreased overall survival (OS) and progression free survival (PFS).[77] Furthermore, high levels of RIP1 resulted in activation of the NF-κB pathway, promoting cell survival and decreased p53 induction.[77] RIP1 was also shown to promote the PI3K–Akt pathway in glioma cells, further suggesting an oncogenic role of RIP1 in glioblastomas.[82] Similarly, RIP1 expression was found to be upregulated in melanocytic tumors and melanoma cell lines, which led to enhanced NF-κB activation and cell proliferation, suggesting that RIP1 promotes cell survival in melanomas.[79] RIP1 was also found to be increased in human and mouse lung tumors compared to normal tissue.[78]

Table 2 Alterations of RIP kinase expression in cancer.

RIP kinases	Alterations and cancer types	References
RIP1	• Downregulation: breast cancer, CRC, HNSCC • Upregulation: gallbladder cancer, gastric cancer glioblastoma, lung cancer, melanoma, PDA	38,72,73,77–81,97
RIP2	• Downregulation: OSCC • Upregulation: breast cancer, CRC, gastric cancer, KIRC	85–91
RIP3	• Downregulation: AML, breast cancer, CLL, CRC, lung cancer, malignant mesothelioma, melanoma, prostate cancer • Upregulation: PDA	38,81,94,95,98–100,103
RIP4	• Downregulation: HCC, lung cancer, TSCC • Upregulation: bladder cancer, CSCC, CRC, osteosarcoma, ovarian cancer, pancreatic cancer	58,108–110,113–119
RIP6	• Downregulation: HCC	120

Abbreviations: AML: acute myeloid leukemia; CLL: chronic lymphocytic leukemia; CRC: colorectal cancer; HCC: hepatocellular carcinoma; HNSCC: head and neck squamous cell carcinoma; KIRC: kidney renal clear cell carcinoma; OSCC: oral squamous cell carcinoma; PDA: pancreatic adenocarcinoma; RIP: receptor interacting protein; TSCC: tongue squamous cell carcinoma.

Exposure to carcinogens led to elevated RIP1 levels in normal mouse lung tissue and human bronchial epithelial cells, and, knockdown (KD) of *RIP1* resulted in increased cell death and decreased transformation upon carcinogen exposure, most likely through abrogating its function in cell survival pathways.[78] Another study reported that expression of kinase-inactive RIP1 increased survival of mice with pancreatic tumors, suggesting the kinase activity of RIP1 is oncogenic in these tumors.[83] Additionally, RIP1 was found to be upregulated in gastric cancers, which was correlated with worse clinical outcomes, also suggesting a pro-tumor role.[72] Interestingly, in ovarian cancer, RIP1 appears to play dual roles, where depletion of the kinase resulted in decreased proliferation but also reduced sensitivity to cisplatin.[84] This dual role makes sense given RIP1's established involvement in both cell survival and cell death. It would be interesting to determine in some tumors whether RIP1 is initially upregulated to aid in cell proliferation but eventually downregulated as the cancer progresses to suppress its ability to induce cell death.

RIP2

RIP2 was reported to be downregulated during the progression of oral squamous cell carcinoma (OSCC), suggesting RIP2 plays a tumor suppressive role in this type of cancer.[85] RIP2 is commonly overexpressed in cancer, with reports of high expression levels in breast cancer, kidney renal clear cell carcinoma (KIRC), CRC, and gastric cancer.[86–91] RIP2 overexpression in TNBC was associated with worse PFS and, at the cellular level, resulted in activation of the NF-κB and JNK pathways, which can promote growth and survival.[87] Pharmacological inhibition of RIP2 resulted in decreased activation of these pathways, along with decreased migratory abilities of the TNBC cells.[87] High levels of RIP2 were also associated with worse clinical outcomes in KIRC, with high expression being associated with increased tumor grade.[89] Higher levels of RIP2 in CRC were also associated with worse clinical outcomes.[88] Despite overexpression of RIP2 in human CRC, RIP2 was shown to suppress colorectal tumor development in mice due to its role in regulating inflammation via NOD2.[92] KO of *NOD2* or *RIP2* in mice resulted in an increased number of tumors and enhanced activation of various inflammatory genes.[92] Furthermore, co-housing wild-type (WT) mice with *RIP2* KO mice treated with dextran sodium sulfate (DSS) promoted tumorigenesis and resulted in increased tumor burden in the WT mice, compared to mice that were separated.[93] In this study, WT mice co-housed with *NOD2* KO or *RIP2* KO mice had an increased incidence of colitis, which is a known risk factor for CRC, suggesting enhanced transmission of microorganisms and a role of the microbiome.[93]

RIP3

RIP3 was reported to be downregulated in CRC, malignant mesothelioma, breast cancer, melanoma, lung cancer, acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and prostate cancer.[38,94–100] Low RIP3 expression was associated with worse clinical outcomes in metastatic CRC, malignant mesothelioma, and breast cancer patients.[99,101,102] RIP3 expression was also shown to be decreased during melanoma development.[103] Reconstituting RIP3 expression in *RIP3*-silenced melanoma cells restored the ability of these cells to undergo necroptosis, suggesting a role of RIP3-mediated necroptosis in suppressing melanoma development.[103] Furthermore, stable expression of *RIP3* in lung xenograft tumors resulted in decreased tumor burden and increased immune cell infiltration.[104] Contrarily, in some cancer cells, RIP3 appears to play an oncogenic role. Increased RIP1 and RIP3 levels were found in PDA compared to normal tissue.[81] Treating PDA cells with chemotherapeutic agents further increased the expression of RIP3, which was associated with induction of chemokine CXCL1, increased levels of myeloid derived suppressor cells (MDSCs), and an immunosuppressive tumor microenvironment (TME).[81] In addition to altered expression, single nucleotide polymorphisms (SNPs) of *RIP3* have been reported in non-Hodgkin's lymphoma, which again, can restrict its ability to induce cell death to inhibit oncogenesis.[105] Furthermore, nasopharyngeal carcinoma (NPC) tumors often have loss of heterozygosity 14q11.2, where the *RIP3* genomic locus resides, which could promote tumorigenesis through compromising RIP3's ability to induce cell death.[24,106,107]

RIP4

RIP4 was reported to be downregulated in lung cancer.[108] High RIP4 expression levels in lung cancer are associated with tumor differentiation and better clinical outcomes in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets, suggesting a tumor suppressive role.[108] This is further supported by the finding that *RIP4* KD in lung cancer cells leads to enhanced STAT3 signaling and de-differentiation.[108] Similarly, decreased RIP4 expression was correlated with poor differentiation of tongue squamous cell carcinoma (TSCC).[109] RIP4 expression was also found to be reduced in primary human hepatocellular carcinoma (HCC) samples compared to normal tissue.[110] Furthermore, there are conflicting reports on RIP4 expression in NPC. While one study reported RIP4 downregulation in a small number of NPC patient samples compared to normal tissue, another study described upregulation of RIP4 in NPC and an inhibitory effect of *RIP4* KD on NPC cell growth.[111,112] On the other hand, RIP4 expression was found to be upregulated in cervical squamous cell carcinoma (CSCC), bladder cancer, pancreatic cancer, ovarian cancer, CRC, and osteosarcoma.[58,113–116] High RIP4 expression was associated with a shorter 5-year OS and disease-free survival (DFS) in CSCC,[113,117] and also, shorter OS in bladder cancer, pancreatic cancer, ovarian cancer and osteosarcoma.[115,116,118,119] Therefore, like the other RIP kinases, RIP4 has dual roles in cancer depending on the specific cancer type.

RIP6

RIP6 was found to be downregulated at both mRNA and protein levels in primary human HCC samples and in liver cancer cell lines compared to normal tissue.[120] Low RIP6 expression was associated with larger tumors, suggesting an anti-tumor function of RIP6 in HCC.[120] Stable expression of

RIP6 in liver cancer cell lines that have reduced *RIP6* expression led to decreased proliferative abilities and enhanced apoptosis with an unclear mechanism. RIP6 expression *in vivo* also led to reduced tumor growth, suggesting RIP6 has antitumor activity against liver cancer.[120]

Genetic, epigenetic and other alterations of RIP kinases in cancer

Few studies have described genetic alternations of the RIP family members in cancer. Analysis of COSMIC database revealed amino acid sequence changes of RIP1 and RIP3 in some cancers, resulting in missense or RHIM domain mutations that may impact their expression and functions.[43,97] We analyzed cancer genomics datasets at cBioPortal (https://www.cbioportal.org/), and surprisingly, found many genetic alterations of RIP kinases in cancer, predominantly genomic amplification and mutations. Table 3 summarizes the top four tumor types with the most frequent genetic alterations of each RIP family member. The highest alteration frequencies range from 15.69% to 3.60%, corresponding to *RIP7* in uterine corpus endometrial carcinoma and *RIP3* in skin cutaneous melanoma, respectively. *RIP2* is the most frequently amplified RIP family member in cancer, with 8.77% amplification in uterine carcinosarcoma and 8.58% amplification in breast invasive carcinoma. *RIP7* is the most frequently mutated RIP family member, with 14.93% mutations in uterine corpus endometrial carcinoma and 13.86% mutations in stomach adenocarcinoma. Other genetic alterations, including structural variants, deep deletions, and multiple alterations, are rare and not included in Table 3. The functions of these genetic alterations are not well characterized. The amplification of RIP kinases likely leads to upregulation of their expression in cancer. The mutations, including silent, missense, and nonsense mutations, may result in silencing, downregulation, upregulation, or altered functions of RIP kinases in cancer.

A major cause of downregulation of RIP kinases in cancer is promoter hypermethylation.[96,121] Hypermethylation often occurs at CpG islands, which are genomic regions of 200—2000 nucleotides with at least 50% of CpG dinucleotides existing in approximately 60%—70% of promoters.[122] RIP3 downregulation in various cancer cell lines was found to be

Table 3 The most frequent genetic alterations of RIP kinases in human cancer.

RIP kinases	Tumor types	Number of cases	Frequency of genetic alterations		
			Total	Amplification	Mutations
RIP1	Uterine Corpus Endometrial Carcinoma	529	6.99%	5.48%	1.51%
	Ovarian Serous Cystadenocarcinoma	584	6.85%	5.99%	0.34%
	Skin Cutaneous Melanoma	444	6.08%	2.93%	2.70%
	Liver Hepatocellular Carcinoma	372	4.84%	3.76%	0.81%
RIP2	Breast Invasive Carcinoma	1084	9.04%	8.58%	0.28%
	Uterine Carcinosarcoma	57	8.77%	8.77%	0%
	Prostate Adenocarcinoma	494	7.89%	7.69%	0.20%
	Liver Hepatocellular Carcinoma	372	7.53%	6.99%	0.27%
RIP3	Skin Cutaneous Melanoma	444	3.60%	0.23%	3.38%
	Uterine Corpus Endometrial Carcinoma	529	3.59%	0.38%	3.21%
	Bladder Urothelial Carcinoma	411	3.16%	0.73%	0.73%
	Lung Adenocarcinoma	566	2.83%	1.77%	0.71%
RIP4	Skin Cutaneous Melanoma	444	5.86%	0.23%	5.41%
	Stomach Adenocarcinoma	440	5.45%	0.23%	3.18%
	Bladder Urothelial Carcinoma	411	4.62%	0.24%	3.89%
	Uterine Corpus Endometrial Carcinoma	529	4.35%	0.76%	3.59%
RIP5 (Dusty Protein Kinase)	Breast Invasive Carcinoma	1084	9.13%	8.49%	0.46%
	Skin Cutaneous Melanoma	444	8.11%	3.15%	4.95%
	Uterine Corpus Endometrial Carcinoma	529	7.56%	2.27%	5.29%
	Liver Hepatocellular Carcinoma	372	6.72%	6.45%	0.27%
RIP5 (SgK288;ANKK1)	Skin Cutaneous Melanoma	444	7.88%	0.23%	4.95%
	Uterine Corpus Endometrial Carcinoma	529	6.05%	0%	5.48%
	Uveal Melanoma	80	3.75%	0%	1.25%
	Uterine Carcinosarcoma	57	3.51%	1.75%	0%
RIP6 (LRRK1)	Sarcoma	255	10.20%	7.45%	2.35%
	Uterine Corpus Endometrial Carcinoma	529	10.02%	0.76%	8.70%
	Skin Cutaneous Melanoma	444	9.91%	1.13%	8.56%
	Stomach Adenocarcinoma	440	9.09%	3.64%	5.23%
RIP7 (LRRK2)	Uterine Corpus Endometrial Carcinoma	529	15.69%	0.76%	14.93%
	Stomach Adenocarcinoma	440	14.55%	0.45%	13.86%
	Lung Squamous Cell Carcinoma	487	13.96%	1.03%	12.73%
	Skin Cutaneous Melanoma	444	13.29%	0.45%	12.61%

Data from https://www.cbioportal.org/.

caused by promoter hypermethylation, which could be restored by treatment with hypomethylating agents, such as the DNA methyltransferase 1 (DNMT1) inhibitor 5-aza-2′-deoxycytidine (5-aza-dC).[96,99,100] Another study reported that RIP1, but not RIP2, was downregulated by promoter hypermethylation in HNSCC, which could also be reversed by 5-aza-dC treatment.[73] Many previous studies have shown that combining 5-aza-dC, or its analogue 5-azacitidine, with other anticancer agents can enhance therapeutic sensitivity, which may involve restoration of RIP3 expression in *RIP3*-silenced cancer cells. However, a contradicting report showed that 5-aza-dC treatment did not restore the expression of RIP1 or RIP3 in various cancer cell lines.[97] Methylation of the *RIP3* promoter has been shown to be maintained by Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) by recruiting DNMT1 in CRC cell lines.[123] KD of *UHRF1* led to enhanced RIP3 expression, which is mediated by the zinc finger transcription factor specific-protein-1 (Sp1).[123] It was also suggested that hypoxia can trigger *RIP1* and *RIP3* silencing, indicated by decreased RIP1 and RIP3 expression in RIP1/3-expressing CRC cells cultured under hypoxic conditions.[97]

In addition, RIP kinase expression in cancer can be regulated by other mechanisms. One study suggests post-translational modifications and autocrine TNF-α impact upregulation, specifically for RIP1 in melanoma.[79] Increased expression of oncogenes may also play a role in upregulation of these kinases.

Impact of RIP kinase alterations on cancer metastasis

Alterations in RIP kinases can either promote or suppress the metastatic potential of cancer cells depending on the specific type of cancer and context.

Promoting metastasis

Several studies showed that loss or inhibition of RIP1 kinase activity reduced tumor metastasis *in vivo*.[124–127] RIP1 inhibition led to reduced vessel sprouting, a key event in metastasis.[124] Exposing non-small cell lung cancer cells to γ-ionizing radiation enhanced metastatic potential by increasing RIP1 expression and NF-κB activation.[128] KD of *RIP1* in gallbladder and gastric cancer cells resulted in reduced invasiveness *in vitro* and decreased tumor size *in vivo*.[72,80] RIP1 was shown to promote lymph node metastasis of gallbladder cancer in an orthotopic model in nude mice.[129] This activity of RIP1 can be explained by its role in regulating TNF-α-mediated lymphangiogenesis and lymphatic metastasis via the NF-κB–VEGF-C pathway in gallbladder cancer, further suggesting that the pro-tumor function of RIP1 is dominant in this type of cancer.[130]

RIP2 was shown to enhance metastatic properties in TNBC, where depletion of RIP2 reduced migration and invasion of these cells *in vitro* and decreased tumor size *in vivo*.[87] Similar *in vivo* results were observed in *RIP1* or *RIP3* KO TNBC cells.[127,131] These observations are most likely due to activation of cell survival pathways by these RIP kinases. Depletion of RIP2 in KIRC also led to decreased proliferative and migratory abilities, along with decreased tumor burden, which was shown to be due to down-regulation of the NF-κB and JNK pathways.[89]

Oncogenic roles of RIP4 have been largely attributed to NF-κB, JNK, and Wnt signaling, where KD of this kinase in various cancers reduced invasiveness both *in vitro* and *in vivo*.[115–118] Furthermore, depletion of RIP4 in osteosarcoma cells reduced not only invasiveness, but also the epithelial–mesenchymal transition (EMT), indicated by increased E-cadherin and decreased N-cadherin expression.[119] This effect of RIP4 is mediated by stimulation of the Wnt/β-catenin pathway, which is known to play a role in EMT.

Suppressing metastasis

It was shown that RIP1 is downregulated in metastatic-tumor-derived HNSCC cells, which increased invasiveness assessed by wound healing and trans-well migration assays.[73] Similarly, depletion of RIP3 in AML and HCC models resulted in enhanced tumorigenesis.[127,132,133] In CRC cells, RIP3 overexpression caused decreased metastatic potential.[123] The effects of RIP1 and RIP3 described in these studies are most likely due to their functions in cell death, in particular necroptosis. As described above, when necroptosis is triggered, DAMPs are released, which can stimulate a local immune response and, in certain cases, can lead to activation of the adaptive immune response through immunogenic cell death (ICD).[134] Furthermore, the effects of RIP kinases can also be mediated through the TME.[127] For example, RIP3 expression was found to be decreased in MDSCs in the TME of CRC, which promoted tumor development via increased cyclooxygenase 2 (COX-2) and prostaglandin E_2 (PGE_2) levels, along with enhanced NF-κB activity.[135]

RIP4 was also found to play a tumor suppressive role in lung cancer and TSCC. RIP4 overexpression in lung cancer cells reduced invasiveness *in vitro* and decreased tumor size *in vivo*, in part due to its inhibition of oncogenic STAT3 signaling.[108] Similarly, depletion of RIP4 led to enhanced migration and invasion of TSCC cells, along with decreased caspase-8 activation during cisplatin treatment.[109]

Roles of RIP kinases in anticancer therapies

Role in determining therapeutic response

Various anticancer agents have been shown to induce cell death mediated by RIP1 and/or RIP3. There has been a strong interest to restore cell death in cancers with *RIP1* or *RIP3* silencing. Variations in the expression of RIP3 was shown to impact therapeutic sensitivity in different cancer cell lines, with *RIP3*-silenced cancer cells having reduced sensitivity to necroptosis-inducing therapeutic agents, which could be restored through ectopic expression of RIP3.[96,123] Reconstituting RIP3 expression in *RIP3*-silenced cancer cells, either ectopically or through 5-aza-dC treatment, resulted in enhanced response to various chemotherapeutic drugs, such as taxol, doxorubicin, etoposide, camptothecin, cisplatin, and 5-fluorouracil (5-FU), as shown by decreased cell viability *in vitro* and reduced

tumor burden *in vivo*.[96,97,99,100,136] Similarly, *RIP3* KO lung cancer and thymoma cells were also less responsive to mitoxantrone and oxaliplatin.[137] Furthermore, caspase inhibition in some CRC cells can enhance the sensitivity to 5-FU, which is abrogated by RIP1 and/or RIP3 depletion.[138] These studies suggest RIP kinase expression can enhance therapeutic sensitivity in certain cancer types, while loss of their expression may be a mechanism of therapeutic resistance.

Several other classes of anticancer agents can induce necroptosis in cancer cells either alone or in combination with chemotherapy. For example, Smac mimetics can stimulate the formation of the ripoptosome complex, comprised of FADD, caspase-8, and RIP1, through depletion of cIAPs, which can then trigger either apoptosis or necroptosis in various cancer cell lines.[139] BH3 mimetics, such as obatoclax, can stimulate necroptosis in various cancer cell lines when used in combination with other anticancer agents.[38,140] CD95 ligand (CD95L) used along with gemcitabine can induce necroptosis in pancreatic cancer cells.[141] Additionally, poly-ADP Ribose Polymerase (PARP) inhibitor rucaparib has also been shown to induce necroptosis, specifically in ovarian cancer cells.[142] Furthermore, PI3K inhibitor GDC-0326 has been shown to upregulate expression of RIP1 and RIP3 in CRC cells, which led to increased sensitivity to 5-FU treatment.[143]

A number of natural compounds can also induce necroptosis, such as shikonin and its analogues, staurosporine (STS), neoalbaconol, trichothecin, and 2-methoxy-6-acetyl-7-methyljuglone.[144–150] Shikonin was shown to induce cell death through necroptosis in breast cancer cells, which was not blocked by overexpression of anti-apoptotic proteins.[145] Shikonin treatment led to increased RIP1 and RIP3 expression in pancreatic cancer cells and a synergistic effect when combined with gemcitabine.[151] STS could induce necroptosis in lymphoma cells under caspase inhibition.[144,152] Oncolytic viruses, such as vaccinia virus, have also been shown to induce necroptosis in cancer cells, such as ovarian cancer cells.[153] Together, these data suggest inducing necroptosis can be used to overcome therapeutic resistance and enhance treatment efficacy, especially when the apoptotic pathway is inactivated.

Therapeutic targeting of RIP kinases

Various RIP kinase-targeting agents have been developed but not yet approved for cancer treatment. Some agents have advanced more in the treatment of other diseases. For example, a RIP1 inhibitor is currently under review for treating inflammatory diseases, such as rheumatoid arthritis.[154] Other RIP1 inhibitors showed promising anticancer effects, such as reducing metastasis in mouse models and enhancing T cell activation in patient-derived tumor samples.[155,156] With promising results in preclinical studies, the RIP1 inhibitor GSK3145095 was tested in Phase 1 clinical trials for certain solid tumors.[155] FDA-approved Braf inhibitor Dabrafenib, which is currently used for treatment of Braf-mutated (V600E) melanoma, can also inhibit RIP3.[154,157] These inhibitors could potentially be used in cancers where these RIP kinases play an oncogenic role. Perhaps these inhibitors could be used alone or in combination with targeted or chemotherapy drugs to enhance therapeutic efficacy and improve clinical outcomes for these types of cancer. Furthermore, utilizing drugs to restore expression or activate RIP kinases could also be beneficial by enhancing therapeutic sensitivity in cancers where RIP kinases play a tumor suppressive role. As noted above, cell death mediated by RIP kinases, in particular necroptosis, can increase inflammation, which may or may not be beneficial for cancer treatment. There is a fine line between the beneficial and negative effects of increased inflammation. While the antitumor immune response stimulated by inflammatory cell death is beneficial, too much inflammation can promote tumorigenesis and metastasis. Therefore, we need to better understand cancer-specific functions of RIP kinases and develop corresponding therapeutic strategies to target or restore these proteins.

Impact of RIP kinase alterations on antitumor immune response

In addition to impacting tumor intrinsic therapeutic response, emerging evidence supports that RIP1- and RIP3-dependent necroptosis influences the antitumor immune response. RIP1-dependent cell death triggered by RIP3 oligomerization has been shown to promote $CD8^+$ T cell priming through activation of the NF-κB pathway and enhance both ICD and tumor infiltration of immune cells.[158] RIP1-mediated cell death enhanced activation of $CD8^+$ T cells and natural killer (NK) cells and potentiated immune checkpoint blockade against soft-tissue sarcomas.[159] RIP3 was also reported to be involved in natural killer T (NKT) cell function, as depletion of RIP3 led to decreased NKT cell activation, cytokine production, and inflammation upon treatment with an immunostimulant in mouse melanoma and liver inflammation models.[38,160] This activity of RIP3 is mediated by mitochondrial phosphoglycerate mutase 5 (PGAM5), which activates a transcription factor required for cytokine production in NKT cells.[160] Furthermore, a recent study showed that intratumoral delivery of constitutively active RIP3 enhanced T cell response, improved survival of tumor-bearing mice, and potentiated anti-PD-1 therapy.[161] Additionally, KO of *RIP3* in mouse syngeneic lung cancer or lymphoma models resulted in reduced ICD and decreased immune cell infiltration, which is most likely due to RIP3's critical role in necroptosis.[137] Together, these data suggest that RIP1 and RIP3 enhance the immune response against various types of cancers.

On the other hand, necroptosis can also promote tumorigenesis due to its inflammatory effects.[52] In a PDA model, RIP1 inhibition not only decreased tumor size, but also led to increased T cell activation and enhanced efficacy of anti-PD-1 therapy, suggesting oncogenic immunosuppressive activity of RIP1 in this model.[127,162] Inhibition of RIP3 in small intestine tumors reduced levels of various inflammatory cytokines and the amount of MDSCs, suggesting that RIP3 promotes tumorigenesis through its pro-inflammatory activity in this type of tumor.[127,163] Another study showed that in contrast to the *in vitro* growth inhibitory effects of RIP1 and RIP3 in PDA cells, KO of *RIP3* or inhibition of RIP1 suppressed PDA progression in mice.[81]

In this study, *RIP3* KO tumors showed decreased tumor associated macrophages (TAMs) and MDSCs, both of which promote an immunosuppressive TME.[81] These surprising results indicate a role of RIP1/3-mediated inflammation in PDA. Thus, alterations in RIP1 and RIP3 can both positively and negatively impact immune response depending on the cancer type due to their roles in promoting cell survival, cell death, and inflammation.

Conclusions

In conclusion, the RIP family kinases are involved in a wide variety of cellular functions and signaling pathways, with each kinase having its own unique functions. These kinases are frequently upregulated or downregulated in cancer depending on cancer types and cellular contexts, which contributes to tumor progression. A major cause of reduced expression of RIP1 and RIP3 in cancer is promoter hypermethylation, which results in gene silencing and decreased expression. Alterations in RIP kinases can lead to enhanced metastatic potential, reduced therapeutic sensitivity, and decreased antitumor immune response. Expression of RIP kinases can potentially be used as a predictive biomarker for certain therapies. However, further studies are needed to better delineate how alterations of RIP kinases influence the development and treatment response of different types of cancer. Such information is essential for the development of RIP-kinase-targeted therapies. The current knowledge suggests that RIP kinases represent attractive targets for developing improved treatments for certain types of cancer.

Author contributions

K.E., J.Y., and L.Z. contributed to the conception, design and writing of this review.

Conflict of interests

The authors declare no conflict of interests.

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