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REVIEW ARTICLE

Ribonucleotide reductase M2 (RRM2): Regulation, function and targeting strategy in human cancer

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Abstract Ribonucleotide reductase M2 (RRM2) is a small subunit in ribonucleotide reductases, which participate in nucleotide metabolism and catalyze the conversion of nucleotides to deoxynucleotides, maintaining the dNTP pools for DNA biosynthesis, repair, and replication. RRM2 performs a critical role in the malignant biological behaviors of cancers. The structure, regulation, and function of RRM2 and its inhibitors were discussed. RRM2 gene can produce two transcripts encoding the same ORF. RRM2 expression is regulated at multiple levels during the processes from transcription to translation. Moreover, this gene is associated with resistance, regulated cell death, and tumor immunity. In order to develop and design inhibitors of RRM2,

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appropriate strategies can be adopted based on different mechanisms. Thus, a greater appreciation of the characteristics of RRM2 is a benefit for understanding tumorigenesis, resistance in cancer, and tumor microenvironment. Moreover, RRM2-targeted therapy will be more attention in future therapeutic approaches for enhancement of treatment effects and amelioration of the dismal prognosis.

Introduction

Ribonucleotide reductases (RNR or RR) participate in nucleotide metabolism, by catalyzing the conversion of nucleotides to deoxynucleotides,[1] which is the rate-limiting step in the production of the precursors for DNA synthesis.[2] This is required for the maintenance of the relative ratios of dNTP pools, and DNA biosynthesis, repair, and replication.[3] RR catalyzes the reaction that the hydroxyl at C-2′ of ribonucleotide is replaced with a hydrogen using an organic radical. Four classes of RR are known according to the protein radical. Class I RR isolated from mammalian, some prokaryotes, and herpes simplex virus contains a tyrosyl radical at Tyr122.[4] Class II enzyme identified in many prokaryotes has an adenosyl cobalamin as the radical.[5] Class III enzyme has an *S*-adenosylmethionine.[6] The RR from *Brevibacterium ammoniagenes* is believed to be included in Class IV.[7] The mammalian RR contains two non-identical homodimeric subunits, a large protein M1 (RRM1) and a small protein M2 (RRM2) (Fig. 1). Throughout the cell cycle, the RRM1 level is excess and remains constant, while the level of RRM2 is fluctuating.[10] Thus, RRM2 expression is exquisitely controlled in the cell cycle.

The coding region of RRM2 comprises 1,170 nucleotides, which codes a protein of 389 amino acids with a calculated molecular mass of 44,883 Da.[11] The amino-terminal portion of RRM2 from various species has a high degree of sequence divergence. For example, there is only 69.2% homology in the first 68 amino acids between the human and mouse RRM2 (Fig. 1C).[12] It indicates that the human RRM2 sequence from aa 1 to aa 68 is not critical for its enzymatic activity.[11] While the carboxy-terminal amino acid sequence from aa 69 to aa 389 has considerable homology, suggesting that this portion is essential for producing functions. RRM2 subunit consists of 13 helices and two β-sheet strands (Fig. 1D).[13] These helices include the eight long helices named αA−αH and four shorter helices named α1−α4. After folding, the helices form into three layers, helices C, B, and D in layer one, helices F, E, and G in layer two, and helix H in layer three. The first layer contacts the other RRM2 subunit. RRM2 contains a non-heme iron center as well.[14] RRM2 contains a dinuclear iron center enclosed by six hydrophilic residues from four helices and two essential tyrosine residues from C-helix.[15] Hydrogen-bonding network connects the tyrosine and the iron cluster by the glutamine, which is a distinct way to regulate the enzymatic activities of RR. The tyrosine residue Tyr162 is essential for radical generation and stabilization.

RRM2 expression is regulated at multiple levels

In drug-resistant tumor cells, the RRM2 gene and its promoter region have significant amplification, leading to a high transcription level.[16] Unlike RRM1, of which levels are constant during the cell cycle, RRM2 expression levels are fluctuating.[10] The half-life of RRM2 ranges from 3 to 6 h.[17] RRM2 levels are strictly regulated throughout the cell cycle. RRM2 is undetectable in the G_1 phase, is stable and accumulates constantly to reach its maximum during the S phase, and is degraded after entry into mitosis. Chabes et al found that both promoter-activating and repressive elements regulate S phase-specific transcription of RRM2 gene.[18] Obviously, RRM2 expression is regulated at every stage all the time (Fig. 2).

Core elements in the promoter influence RRM2 gene transcription

Two RRM2 transcripts are expressed from the two separate promoters. For the first transcript, the fragment from −800 to +1 bp contributes the highest promoter activity, the fragment from −610 to +1 bp shows 80% of maximum activity, and the fragment from −125 to +1 bp has only more than 50% of maximum activity.[9] For the second transcript, the fragments from −610 to −125 bp and −800 to −125 bp contribute the highest activities. Promoter fragment from −125 bp to +1 bp contains three CCAAT sequences and a non-canonical TATA-box. The CCAAT sequences instead of the atypical TATA-box are responsible for the substantial transcription and S-phase-specific expression of RRM2. Each CCAAT sequence loses, and the promoter activity is decreased by about 20%−40%. The transcriptional efficiency of atypical TATA-box only has 25% of the consensus TATA-box sequence.[19] The TATA-box in mouse RRM2 promoter is dispensable for basal and S-phase-specific expression, but this element is required for full transcription.[20]

Transcription factor II D (TFIID) is a multi-subunit complex consisting of the TATA-binding protein (TBP) and TBP-associated factors (TAFs) and binds the core promoter to assemble the pol II preinitiation complex.[21] The TATA box is a core element for promoter function.[22] Besides, other core promoter elements have been identified.[23] A region located at about 30 bp downstream of the conserved atypical TATA-box contains a highly conserved palindrome sequence, GTGCACC, interacting with TFIID subunits.[20]

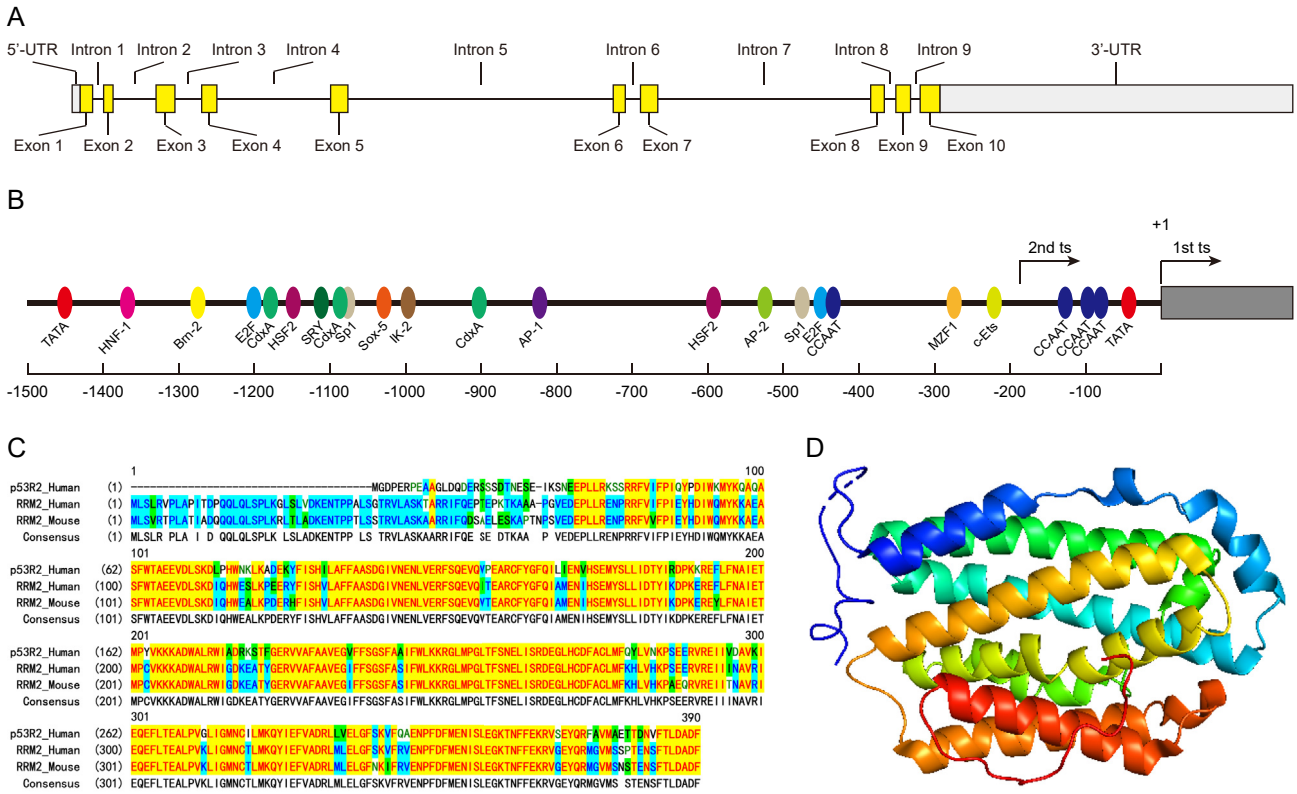

Figure 1 Structures of RRM2. **(A)** The RRM2 gene is transcribed to produce two transcripts, 1.65 kb, the first transcript, and 3.4 kb, the second transcript.[8] The transcription initiation site (ts) of the first transcript is designated as +1 bp, and the second ts is located at −187 bp. The difference in the transcript is the length of 5′-UTR and 3′-UTR. A TTTAAA sequence instead of a TATAA sequence, a canonical TATA-box, is found in the proximal promoter, and only regulates the first transcript.[9] **(B)** Potential DNA binding sites for transcription factors. ts, transcription initiation site. **(C)** Alignment of amino acid sequences of RRM2 from humans and mice and p53R2 from humans. **(D)** The tertiary structure of RRM2 protein (PDB code: 2UW2).

Methylation up-regulates RRM2 expression

In tumorigenesis, progression, and metastasis of breast cancer, aberrant gene expression results from many events including epigenetic modification such as DNA methylation.[24] More than 100 genes have an epigenetic modification in breast cancer.[25] Qi et al found that the up-regulated RRM2 is also hypomethylated.[26]

In eukaryotes, DNA wraps around a histone octamer, which leads to forming the smallest subunit of chromatin, nucleosome.[27] Different modifications, such as methylation, on core histones, have been well studied and reported.[28] Histone methylation participates in multiple cellular processes and diseases through the regulation of gene expression.[29] It has been known that histone 3 lysine 4 trimethylation (H3K4me3), H3K79me3 and H3K36me3 active gene transcription, and H3K27me3 and H3K9me3 suppress transcription.[30] Xiao et al proposed that H3K36me3 is a promising target for epigenetic therapeutics in cancer.[31] H3K36me3 provides a positive effect on the regulation of RRM2 transcription as well. SET-domain 2 protein (SETD2) is responsible for the methylation of H3K36 including its mono-, di-, and tri-methylation, and is believed to be a druggable target for precision cancer therapy as well.[32] In the human osteosarcoma U2OS cells, RRM2 mRNA and protein levels are reduced by either SETD2 knockdown or CRISPR SETD2 knockout.[33] Reducing H3K36me3 also suppresses RRM2 levels. H3K36me3 actives gene expression through multiple mechanisms such as alteration of genomic H3K36me3 landscape, transcriptional elongation, and collaborative regulation with other histone marks.[31] Based on the finding that TAFs are enriched at H3K36me3,[34] Pfister et al found that H3K36me3 recruits TAFs to facilitate transcription initiation.[33]

RRM2 expression is regulated by multiple signaling pathways

Insulin-like growth factor 1 (IGF1) belongs to the insulin-like growth factor family, which binds specifically to the IGF1 receptor (IGF1R) and activates intracellular signaling pathways.[35] Subsequently, IGF1R activates the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and the Ras/Raf/extracellular signal-regulated kinase (ERK) signaling pathways.[36]

In MDA-MB-231 cells, Zhuang et al found that RRM2 inhibition decreases Akt phosphorylation level, suggesting that RRM2 can regulate PI3K/Akt pathway.[37] The RRM2/PI3K/Akt signal pathway also exists in liposarcoma cells.[38] Intriguingly, they also showed that, in RRM2-silenced MDA-

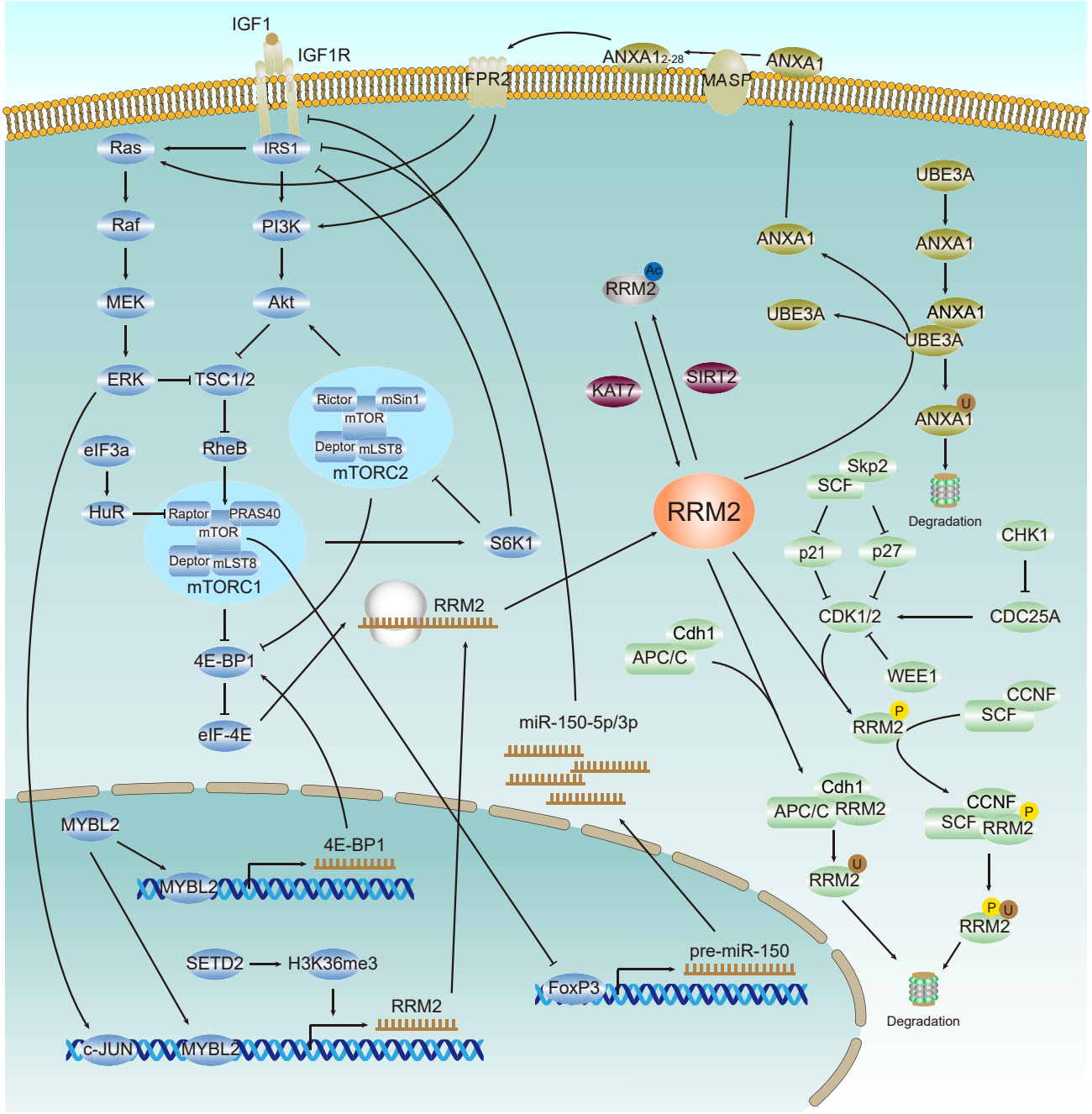

Figure 2 Mechanisms of regulation of RRM2. RRM2 expression is regulated at transcriptional, post-transcriptional, transcriptional, and post-transcriptional levels. Ac, acetylation; P, phosphorylation; U, ubiquitylation.

MB-231 cells, PI3K/Akt pathway agonist IGF1 restores the Akt phosphorylation level. It seems that PI3K/Akt pathway can be regulated both by IGF1/IGF1R and RRM2.

In the breast cancer cell line MCF-7, Li et al found that the phosphorylation levels of mitogen-activated protein kinase kinase (MEK), ERK, and p38 are decreased in the si-RRM2 cells, suggesting that the mitogen-activated protein kinase (MAPK) signaling pathway is inhibited by RRM2 down-regulation.[39] On the other hand, also in MCF-7 cells, Rieunier et al showed that MEK inhibitor trametinib inhibits RRM2 up-regulation induced by IGF1.[40] Moreover, c-JUN is a transcriptional effector of ERK,[41] and c-JUN is a mediator of IGF effects on RRM2,[40] suggesting that RRM2 expression is regulated by MEK/ERK/JUN.

IGF1R influences gene transcription by recruitment to promoters,[42] but in MCF-7 cells, Rieunier et al did not detect IGF1R at the RRM2 promoter by chromatin immunoprecipitation sequencing (ChIP-seq).[40] Akt inhibitor AZD5363 inhibits RRM2 up-regulation induced by IGF1. Annexin A1 (ANXA1) is an anti-inflammatory protein and shows pro-invasive and pro-tumoral properties in cancers.[43,44] In cervical carcinoma cells and renal cancer cells, ANXA1 interacts with ubiquitin-protein ligase E3A (UBE3A), thereby leading to degradation.[45] Furthermore, Xiong et al

found that in renal cancer cells RRM2 competes to seize ANXA1 from ANXA1-UBE3A which causes ANXA1 degradation, and stabilizes ANXA1.[45] ANXA1 will be externalized and is linked to the cell surface in a calcium-dependent manner.[46] After translocated to the outer cell surface, ANXA1 is cleaved between Ser28 and Lys29 by a membrane-associated serine protease, releasing a bioactive N-terminal acetyl Ala2-Ser28 ($ANXA1_{2-28}$) peptide. Then, the $ANXA1_{2-28}$ interacts with the formyl peptide receptor 2 (FPR2) belonging to the G protein-coupled receptor family,[47] leading to the activation of PI3K/Akt pathway in breast cancer[48] and glioma cells,[49] and also the activation of extracellular signal-regulated kinase (ERK) MAPK pathway through, probably, Ras/MEK[50] in melanoma cells[46] and breast cancer cells.[51]

Transcription factors positively or negatively regulate RRM2 expression

Besides the motif for FOS/JUN dimer AP-1, the RRM2 promoter also contains a motif binding with the transcription factor adenoviral early region 2 binding factor (E2F) (Fig. 3). The cyclin-dependent kinase (CDK)-retinoblastoma tumor suppressor (Rb)-E2F axis forms the core regulatory machinery to drive cell cycle progression. The regulation of E2F transcription factors is the most critical event during the transition from G_1 to S.[52] ERK can directly phosphorylate and activate cyclin D (CycD).[53] The activated CycD binds with CDK4/6 to form an active holoenzyme CDK4/6-CycD, which cooperates with or without CDK2-CycE in Rb phosphorylation.[54] The phosphorylated Rb release and activate E2F. The E2F family comprises eight distinct genes encoding transcriptional activators such as E2F1 and repressors such as E2F4.[52] E2F1 induces RRM2 expression and E2F4 represses the activity of RRM2 promotor.[18,40] E2F4 has a nuclear export signal and is predominantly cytoplasmic.[55] Associated with retinoblastoma protein family members, also called pocket proteins such as p107 and p130, E2F4 is localized in nuclear during G_0/G_1.[56] When the pocket protein is phosphorylated, the complex dissociates and E2F4 is free, resulting in the progressive increase of cytoplasmic E2F4.[56] E2F1 has a nuclear localization signal and is constitutively nuclear.[57] When being activated, E2F1 induces expression of cell cycle-related and S phase-promoting genes, and then the cells enter the S phase.[58]

E2F1 has opposing functional roles in proliferation and apoptosis, called the "yin and yang" of E2F1.[59] The biological activity of E2F1 is regulated by arginine methylation.[60] E2F1 methylated by asymmetric dimethylating protein arginine methyltransferase (PRMT) 1 augments

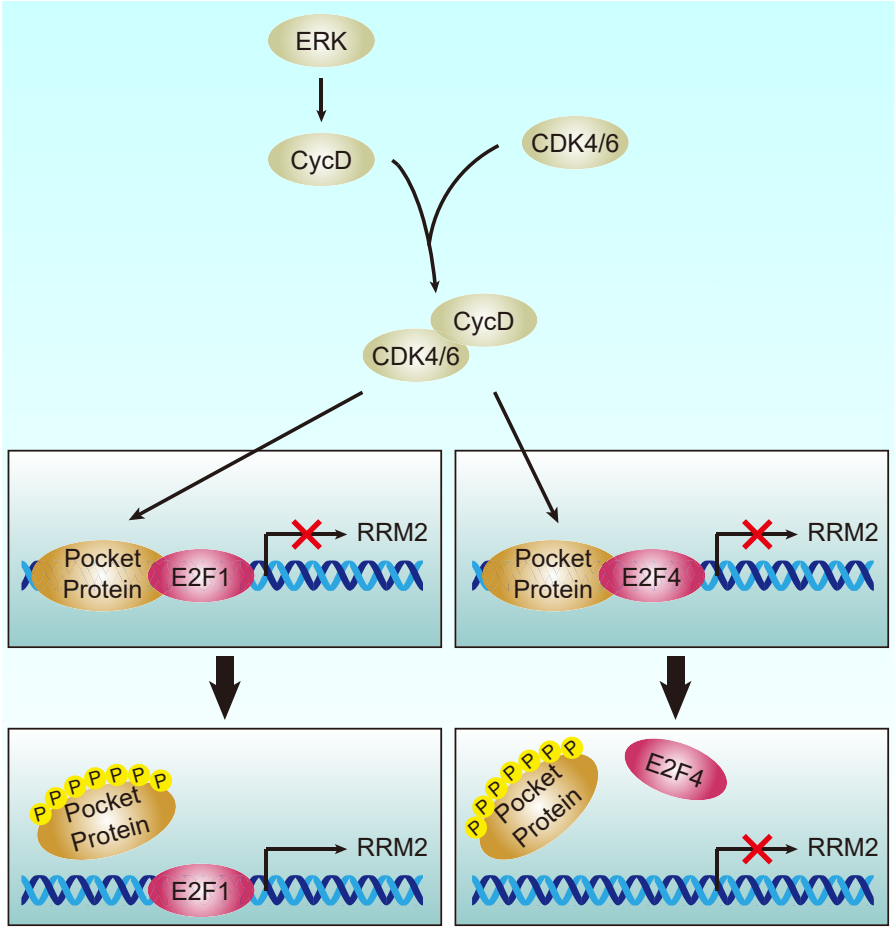

Figure 3 Regulation of RRM2 transcription by E2F1 and E2F4. Pocket proteins including Rb, p107, and p130 regulate the activation of E2Fs.

apoptosis, and symmetric dimethylating PRMT5-dependent methylation promotes E2F1-dependent proliferation.[61] PRMT1 and PRMT5 competitively methylate E2F1, but cyclin A binding to E2F1 hinders PRMT1 methylation, thereby facilitating PRMT5 methylation. PRMT5 belongs to type II PRMT. Another type, type I PRMT, contains PRMT2, which directly binds and interacts with Rb through its *S*-adenosyl methionine binding motif, forming a ternary complex with E2F1, to negatively regulate E2F1 activity.[62]

Breast cancer gene 1 (BRCA1) is a tumor suppressor. In breast cancer cells, BRCA1 suppresses epithelial-to-mesenchymal transition (EMT) through binding to the TWIST promoter and suppressing its activity,[63] while BRCA1 depletion promotes EMT activation through activation of transforming growth factor beta receptor 2 (TGFβR2) signaling pathway.[64] Interestingly, BRCA1 is found to be also a transcriptional co-activator of RRM2 in glioblastoma cells, but not in non-glioblastoma cells.[65]

MYBL2 (B-Myb, MYB proto-oncogene like 2) belongs to the Myb transcription factor family and plays a critical role in the cell cycle and tumorigenesis.[66] MYBL2 is overexpressed and is believed to be a prognostic and predictive biomarker in several types of cancers including breast cancer.[67] Liu et al found that two MYBL2 binding motifs exist in the RRM2 promoter at −36 to −51 bp and −1857 to 1872 bp.[68] MYBL2 directly binds to the RRM2 promoter and promoted its transcription in colorectal cancer cells.

Non-coding RNAs (ncRNAs) engage in RRM2 gene expression

Long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) are different kinds of ncRNAs, and both participate in cancer initiation and development.[69,70] lncRNAs can be a miRNA "sponge". These sponges interact with miRNAs, resulting in the release of miRNA target genes. Using bioinformatical and experimental methods, some lncRNAs and miRNAs regulating the RRM2 gene have been identified, and the ones verified by experiments in breast cancer are listed in Table 1.

Table 1 lncRNAs and miRNAs regulating RRM2 gene in breast cancer cells.

lncRNA	miRNA	Target	Cell lines	Reference
DSCAM-AS1 ↑	miR-204-5p ↓	RRM2 ↑	HCC1937	71
SNHG16 ↑	miR-30a ↓	RRM2 ↑	MDA-MB-231	72
			MCF-7	
TTN-AS1 ↑	miR-524-5p ↓	RRM2 ↑	T47D	73
			BT549	
/	miR-4500 ↓	RRM2 ↑	MCF7	39
			BT474	
			MDA-MB-468	
			MDA-MB-231	

The protein synthesizing machine regulates RRM2 gene translation

Protein synthesis is limited by mRNA translation. In eukaryotes, protein synthesis is regulated by eukaryotic translation initiation factors (eIFs) and translation repressors. The mTOR forms two structurally and functionally distinct complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), which participates in protein synthesis through mTORC1-mediated phosphorylation of 70-kDa ribosomal protein S6 kinase 1/2 (S6K1/2) and eIF4E-binding proteins 1/2/3 (4EBP1/2/3), and mTORC2-mediated phosphorylation of Akt (Akt1/2/3).[74–76]

The eIFs act as complexes called eIF1, eIF2, eIF3, eIF4, eIF5, and eIF6. The eIF3 comprises 13 subunits,[77] of which eIF3a, the largest subunit, is believed to be a potential oncogene, and is also a potential drug target.[78] The eIF3a binds to the mRNA 5'-UTR of regulatory associated protein of mTOR (Raptor), a subunit of mTORC1, through interacting with the RNA-binding protein human antigen R (HuR), and inhibits Raptor protein synthesis, resulting in negative regulation of mTORC1 activity.[79] The eIF4E-binding protein 1 (4E-BP1) belongs to a family of translation repressor proteins and a family of eIF4E-binding proteins. 4E-BP1 is regulated and phosphorylated by mTORC1/2.[80] The phosphorylated 4E-BP1 dissociates from eIF4E, thereby initiating cap-dependent translation. In rhabdomyosarcoma cells, mTORC1/eIF-4E cap-dependent protein translation participates in the regulation of RRM2 expression.[81] Similar results are reported in sarcoma cell lines, in which 4E-BP1 is activated by inhibition of mTORC1/2, thereby inhibiting RRM2 translation.[82] Moreover, 4E-BP1 transcription can be induced by MYBL2.[83] Thus, MYBL2 regulates RRM2 expression at transcriptional and translational levels. Simultaneously. Zhang et al found that RRM2 knockdown may suppress the activity of the Akt/mTOR/4E-BP1 pathway in retroperitoneal liposarcoma cells.[84] Perhaps, this mechanism is ANXA1 mediated.

Dong et al showed that eIF3a positively regulates RRM2 expression in HeLa and NIH3T3 cells.[85] He et al also found that depletion of S6K1, another target of mTORC1, leads to up-regulation of RRM2.[81] Raptor binds to the tor signaling motif within S6K1 for promoting interaction with mTOR and mediating phosphorylation.[86] S6K1 has two downstream effectors, mTORC2 and insulin receptor substrate 1 (IRS1).[86,87] S6K1 is implicated in the phosphorylation and negative regulation of Rictor (sirolimus-insensitive companion of mTOR) within mTORC2, which phosphorylates and activates Akt.[75] In addition to its role in the inhibition of mTORC2, S6K1 inhibits IRS1 through phosphorylation of serine of IRS1[88,89]; while the activated IRS1 with tyrosine phosphorylation activates PI3K/Akt/mTOR pathway.[90] Forkhead box protein 3 (FoxP3), first found in regulatory T (Treg) cells, is a transcription factor involved in the development and function of Treg cells,[91] and serves vital roles in cancers including breast cancer.[92] The mTOR has the ability to block Foxp3 induction.[93] Zhang et al also showed that FoxP3 positively regulates miR-150-5p/3p by binding to the mir-150 promoter, and miR-150-5p/3p directly targets IRS1 and IGF1R.[90] This forms a feedback loop of FoxP3/miR-150/IGF1R-IRS1/PI3K/AKT/mTOR, like Akt/mTOC1/S6K1/mTOC2 and PI3K/Akt/mTOC1/S6K1/IRS1.

The post-translational modification will activate RRM2

Protein post-translational modifications (PTMs) are key steps in structural changes of proteins and participate in multiple biological processes.[94] Lysine acetylation is a versatile PTM and is involved in many important cellular processes.[95] Histone acetyltransferase KAT7 (MYST2 or HBO1), a member of the MYST KAT family, is essential for histone H3 lysine 14 acetylation and is involved in replication-associated processes.[96] Sirutin 2 (SIRT2), a member of the Sirtuin family, is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase, and participates in various biological processes by deacetylation.[97] Chen et al found that acetylation/deacetylation of the RRM2 is a molecular switch of RR activity.[98] RRM2 is directly acetylated by KAT7. The acetylation of RRM2 at K95 disrupts RRM2 homodimerization, resulting in RR inactivation. While SIRT2 directly deacetylates RRM2, thereby leading to RR activation. They also found that SIRT2 regulation of RRM2 deacetylation is cell-cycle-dependent, and ionizing radiation or camptothecin significantly decreases RRM2 acetylation by promoting an interaction between SIRT2 and RRM2 instead of KAT7 and SIRT2 protein levels. Moreover, the RRM2 deacetylation is regulated by its phosphorylation induced by the serine/threonine-protein kinase ATR (ataxia telangiectasia and Rad-3 related protein), solving genome maintenance challenges and integrating the information stored in the DNA,[99] at the S150 site, the only one SQ motif in human RRM2.[100]

Multiple pathways are responsible for RRM2 degradation

Two E3 ubiquitin protein ligases, the anaphase-promoting complex/cyclosome (APC/C) and the Skp1/cullin/F-box (SCF) complex, highly regulate the cell cycle and play an important role in tumorigenesis.[101] RRM2 is regulated by the ubiquitination-proteasome system (UPS). The APC/C is an evolutionarily conserved multi-subunit E3 ubiquitin ligase. Cdh1 is an activator of APC/C.[102] The APC/C^{Cdh1} can recognize a conserved KEN box located at the N-terminus of RRM2.[103] Then the RRM2 binds to APC/C^{Cdh1} and is polyubiquitinated.

The F-box protein is a component of SCF E3 ubiquitin ligase and is responsible for the recognition of target proteins for ubiquitylation and degradation.[104] Cyclin F (CCNF) is a member of the F-box protein family.[105] D'Angiolella et al identified that RRM2 is an interactor of the F-box protein CCNF, and found that RRM2 is degraded *via* SCF^{CCNF} after CDK-mediated phosphorylation of Thr33 during G_2, maintaining a balance of dNTP pools and stability of the genome.[106] They also showed that CCNF down-regulation in an ATR-dependent manner allows RRM2 accumulation in response to DNA damage, which is required for efficient DNA repair. Similar results are observed in breast cancer cell lines MCF-7 and MDA-MB-231. Chang et al showed that CCNF overexpression reduces the RRM2 protein level.[107]

S-phase kinase-associated protein 2 (Skp2) is also a part of the SCF complex, an E3 ubiquitin ligase, and is a substrate-recruiting F-box protein.[108] During the G_1/S transition, SCF^{Skp2} degrades the CDK1 inhibitors p21 and p27, thereby controlling cell cycle and cancer progression.[109,110] Unexpectedly, Xiao et al found that Skp2 and RRM2 are synchronously overexpressed in the hydroxyurea (HU)-resistant cell line KB.[111] Furthermore, they demonstrated that Skp2 interacts with RRM2 directly, and the interaction enhances the RR activity.

WEE1 is a tyrosine kinase and is a master regulator of the G_2/M transition during the cell cycle.[112] CDK1 is a target of WEE1 and its phosphorylation at Tyr15 can be inhibited by WEE1.[113] WEE1 inhibition can be applied in cancer therapy.[114] Pfister et al showed that WEE1 inhibition activates CDK1/2, which increases RRM2 phosphorylation at Thr33, promoting RRM2 degradation.[33] The same results are confirmed in sarcoma cells by Gordon team, where inhibition of CHK1-WEE1 signaling promotes the CDK-mediated degradation of RRM2.[82,115]

RRM2 has a dual function

RR has an important function in DNA synthesis, cell proliferation, and cancer development. RRM2, as the small subunit, has RR-related enzymatic functions and non-enzymatic functions.

Enzymatic function

DNA replication is a hallmark of cancer.[116] Some chemotherapy drugs used in cancer treatment, such as Cisplatin,[117] exert an anti-tumor function by interfering with DNA replication. Contrarily, sustained DNA synthesis and replication contribute to drug resistance. For example, RRM1 activation is an important hallmark of cancer cells acquiring drug resistance.[118] Thus, RRM2 overexpression has been shown to be a potential factor causing drug resistance.

A HU-resistant human KB cell line was developed and exhibited a 15-fold resistance to HU, with a gene amplification of RRM2, increased levels of RRM2 mRNA and protein, and enhanced activity of RR.[119,120] Inhibition of RRM2 can overcome the resistance and increase chemosensitivity in fibrosarcoma,[121] pancreatic cancer,[122] *etc*. Several studies also identified that MMR2 is a marker for resistance of HU,[123] tamoxifen,[124–126] and Adriamycin.[127] ICBP90, also known as Uhrf1 (ubiquitin-like with PHD and ring finger domains 1) and Np95, is a Rb-associating transcription factor,[128] and has an essential role in the maintenance of DNA methylation by histone H3 ubiquitylation.[129] In human head and neck cancer cells, dNTP depletion caused by HU treatment induces ICBP90, thereby transactivating RRM2.[123] Gemcitabine can also increase RRM2 expression in a dose-dependent manner.[130] Nuclear factor Y (NF-Y) interacts with proximal CCAAT-boxed in the RRM2 promoter and is essential for its high-level expression of RRM2.[131] Liu et al believed that this plays a pivotal role in human oropharyngeal epidermal carcinoma cell line KB with gemcitabine resistance (KB^{Gem}).[132] Another transcription factor E2F1 can also be increased by gemcitabine and then induces RRM2 expression.[133] In KB^{Gem}, RRM2 overexpression increases the dCTP pool, and the available dCTP is able to compete with gemcitabine.[16,134]

In breast cancer, several regulatory mechanisms of RRM2 overexpression have also been proposed, and involve Akt, NF-κB, HIF1α, and MAPK/c-Jun N-terminal kinase (JNK) pathways.[124,125]

Non-enzymatic function

Crosstalk of RRM2 and regulated cell death

In 2012, Brent R. Stockwell team reports a new cell death modality, an iron-dependent form of regulated cell death, called ferroptosis, which is triggered by unrestricted lipid peroxidation and subsequent plasma membrane rupture.[135,136] There are some characterizations, such as iron overload, glutathione (GSH) depletion, inactivation of glutathione peroxidase 4 (GPX4), and lipid peroxidation. GSH is a tripeptide synthesized by glutathione synthetase (GS) from glycine, glutamate, and cysteine.[137] RRM2 participates in nucleotide synthesis in a glutathione-dependent manner,[138] and is believed to be a ferroptosis-related gene.[139] Zhang et al showed that RRM2 is involved in ferroptosis in liver cancer.[140] RRM2 was also believed to be a ferroptosis-related metabolic gene in prostate cancer[139] and lung adenocarcinoma.[141] It has been reported that RRM2 is an endogenous ferroptosis suppressor in liver cancer cells and lung adenocarcinoma, and RRM2 knockdown can induce ferroptosis.[141] In liver cancer cells, the phosphorylation of RRM2 at T33 facilitates the expression of GS protein, which is critical for GSH synthesis. The dephosphorylation of RRM2 enhances the interaction of RRM2 and GS interaction and, at the same time, triggers proteasome degradation, thereby leading to their degradation.

Chen et al revealed a relationship between nucleotide pools and autophagy in cancer cells.[142] RRM2 plays an important role in this process. Reduction of intracellular dNTP pools by knocking down RRM2 expression induced autophagy and conversely, an increase in intracellular dNTP pool concentrations by RRM2 overexpression attenuates autophagy induced by rapamycin.

RRM2 facilitates immune escape

Cancer immunotherapy is a novel strategy and a breakthrough approach for cancer treatment through the activation of the immune system.[143] Clinically, it has been proved that immunotherapy has achieved promising outcomes.[144] Based on experimental and bioinformatic studies, it has been confirmed that there is an association between RRM2 and tumor immunity, and RRM2 contributes to immune escape.[145]

Immune checkpoint inhibition is an effective means of therapy for improving the prognosis of patients, such as programmed cell death 1/programmed cell death ligand 1 (PD-1/PD-L1) blockade.[146] PD-1, also known as CD279 and belonging to the CD28 family, is an immunoinhibitory receptor that is expressed on activated T cells, B cells, and macrophages.[147] PD-1 has a role in the inhibition of T-cell intracellular signaling, proliferation, and cytokine production.[148] PD-L1, PD-1 ligand, also known as CD274 and belonging to the B7 family, is expressed by lymphoid and nonlymphoid tissues, as well as various tumors.[149,150] PD-L1 overexpression contributes to evading immune responses.[151] Binding of PD-1 to PD-L1 and PD-L2 triggers a cascade of intracellular signaling that results in T cell inhibition and exhaustion,[152] thereby leading to tumor immune escape.[153] PD1/PD-L1 blockade therapy is considered a promising strategy in cancer treatment.[154] Based on RNA-seq data, Xiong et al found that RRM2 modulates the PD-1 pathway in renal cancer 786-O cells.[45] The GEPIA web tool also shows a significant association between RRM2 and PD-L1 levels in renal cancer, bladder cancer, breast cancer, and prostate cancer tissues. Then, experimentally, they confirmed that RRM2 silencing decreases and its overexpression increases the mRNA and protein levels of PD-L1 in 786-O and A498 cells. Hypoxia is a prominent feature of most tumors and leads to the stabilization of hypoxia inducible factor 1 subunit alpha (HIF-1α) in cancer cells.[155] HIF-1α inhibition promotes anti-tumor immunity in non-small cell lung cancer.[156] Patients with high HIF-1α expression exhibit an immunosuppressive phenotype. Inhibition of HIF-1α alleviates tumor immunosuppression through HIF-1α/LOXL2 signaling pathway. Moreover, targeting HIF-1α can also suppress PD-L1 expression in tumor cells.[157] In breast cancer, Shah et al showed that RRM2 overexpression specifically up-regulates HIF-1α.[125] Furthermore, this may explain the reason that knockdown of RRM2 enhances the anti-tumor efficiency of PD-1 blockade in renal cancer.[45]

Macrophages play significant roles in immunity and are generally categorized into the pro-inflammatory M1 and the anti-inflammatory M2 phenotypes.[158] In the tumor microenvironment (TME), M1 macrophages exhibit anti-tumor immunity, whereas M2 macrophages, also called "tumor-associated macrophages" (TAMs), have pro-tumor features (promoting tumor growth and metastasis).[159] Xiong et al found that RRM2 levels are positively correlated with the number of M2 macrophages in the TCGA-KIRC dataset.[45] Tang et al also showed that RRM2 promotes macrophage infiltration, and its inhibition suppresses macrophage infiltration in lung adenocarcinoma.[141] RRM2 regulates macrophage polarization *in vitro* and *in vivo* as well. Inhibition of RRM2 effectively promotes M1 polarization and suppresses M2 polarization of macrophages.

Strategies for designing inhibitors targeting RRM2

RRM2 is the small subunit, two of which combine with two large subunits RRM1 to form a protein tetramer RR, catalyzing the reaction of nucleotides to deoxynucleotides. Stubbe and van der Donk proposed three approaches to targeting RR.[1] They involve nucleotide analogs, reduction of the essential tyrosyl radical, and inhibition of the interaction of RRM1 and RRM2. RR, as a holoenzyme, is directly involved in tumor growth and drug resistance. RRM2 expression is tightly regulated, but RRM1 overexpression does not affect RR activity.[160] As compared with RRM1, RRM2 is a promising and important target for the design of anti-cancer agents (Fig. S1). There are also several strategies for developing inhibitors targeting RRM2.

Destroying free radical and iron center

HU is synthesized for the first time in 1869 and is approved by the Food and Drug Administration (FDA) for the

treatment of solid tumors in 1967.[161] HU inactivates RRM2 by destroying its tyrosyl-free radical and the iron center, accompanied by the release of iron from the protein.[162]

Didox (3,4-Dihydroxybenzohydroxamic acid) is a derivative of HU. As an iron chelator and a free-radical scavenger for targeting RRM2,[163] didox is one of the most potent pharmaceutical inhibitors of RR[163,164] and has been used as an anti-tumor agent for many years.[165,166]

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a natural antioxidant,[167] has anti-tumor effects.[168] But its effects are controversial. Recently, Chen et al found that, in MDA-MB-231 cells, low concentrations of resveratrol have a protumorigenic effect of promotion of proliferation and migration by activating the JAK3/STAT3 signaling pathway, while high concentrations of resveratrol exhibit anti-tumor performance of inhibition of cell growth and induction of autophagy and apoptosis through MAPK signaling pathway.[169] Resveratrol also has the ability to inhibition of RR by quenching the tyrosyl radical of RRM2.[170]

Capturing iron from RRM2

Due to being iron-dependent, depleting iron can inhibit RRM2.[122] Iron chelators such as DFX (desferrioxamine, deferoxamine mesylate), HBED (*N*,*N*′-bis(*o*-hydroxybenzyl) ethylenediamine-*N*,*N*′-diacetic acid) and BPYTA (2,2′-bipyridyl-6-carbothioamide) can be used as RRM2 inhibitors.[171,172] Moreover, an agent inhibiting cellular iron incorporation can also decrease the activity of RRM2, such as transferrin-gallium.[173]

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP), one of the heterocyclic carboxaldehyde thiosemicarbazones (HCTs), is a synthesized RR inhibitor with antineoplastic activity.[174] Although several models are proposed to explain the mechanisms by which 3-AP inhibits RRM2 through chelating iron or producing oxygen reactive species (ROS), Aye et al presented another model, in which Fe(II)-(3-AP) directly reduces Y· of the [(Fe_2^{III}-Y·)(Fe_2^{III})] cofactor of RRM2.[175] Due to minor or even no anti-tumor activity and side effects, some clinical trials are stopped.[176,177] Considering these problems, novel derivatives are discovered. Stefani et al designed a novel RBpT series, namely 2′-benzoylpyridine thiosemicarbazones bearing hydrophobic, electron-donating substituents at the *para* position of the phenyl group, especially *t*-BuBpT series.[178] Based on the formation of the Fe(II)$(3\text{-AP})_2$ complex, Plamthottam et al synthesized three classes of 3-AP analogs including 12 compounds.[179] Group I compounds with methylation on the secondary amine show no RNR-specific activity. Group II compounds have the most similar structures with that of 3-AP, and have less potent (non-aromatic substituents on the pyridine ring) or slightly more potent (substituents to the primary amine part of the thiosemicarbazone moiety) than 3-AP. Group III compounds with isoquinolines instead of pyridine ring exhibit much stronger activity than 3-AP, in which IQ-2 is four-fold more potent than 3-AP.

Decreasing RRM2 mRNA level

Flavopiridol (also called alvocidib) is a synthetic analog of a natural flavone derivative that is isolated from *Dysoxylum binectariferum*.[180] It is a CDK inhibitor, inhibits the growth of diverse human tumor cells, and treats acute myeloid leukemia and chronic lymphocytic leukemia.[181,182] PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine, is a Src tyrosine kinase inhibitor.[183] It is found that both of them suppress RRM2 at the transcriptional level through regulation of E2F1.[184,185]

Arginine is a non- or semi-essential amino acid for it can be synthesized from citrulline and aspartate using argininosuccinate synthase 1 (ASS1) and argininosuccinate lyase (ASL).[186] In most tumors, ASS1 transcription is suppressed and, thus, arginine deprivation is believed to be a novel antimetabolite strategy for treating arginine-dependent cancers.[187] Some enzymes such as arginase (ARG), arginine decarboxylase (ADC), and arginine deiminase (ADI) participate in arginine metabolism. Using these enzymes to target exogenous arginine is a promising therapy to treat cancers.[188] ADI-PEG 20 is a stabilized, pegylated arginine deiminase. It has been assessed against various cancers including breast cancer in clinical trials.[189] Prudner et al found that both acute and long-term treatment of ADI-PEG20 can significantly decrease RRM2 expression.[190] Daylami et al believed that this is PRMT2-mediated.[130]

RRM2 mRNA can be effectively repressed by RNA interference. GTI-2040, a 20-mer antisense nucleotide against RRM2, decreases its mRNA and protein levels in various cancers including breast cancer.[191] Interestingly, Xiao et al found that GTI-2040 can decrease both RRM2 and Skp2, suggesting that it interrupts their interaction by dissociation of the complex.[111] Moreover, in the less sensitive group, the RRM2 mRNA does not change. Duxbury et al used a novel engineered replication-deficient retrovirus to induce stable siRNA targeting RRM2.[192] P-element induced wimpy testis (PIWI)-interacting RNA (piRNA) guide PIWI proteins to bind and cleave RNAs.[193] Das et al used a piRNA, piR-39980, to directly target 3′-UTR of RRM2 for repression.[194]

5-Azacytidine (5-AZA) is a pyrimidine analog and is approved by FDA for the treatment of the myelodysplastic syndrome.[195] It also has anti-tumor effects on acute myeloid leukemia (AML).[196] Aimiuwu et al found a novel molecular target of 5-AZA in AML.[197] It can attenuate RRM2 mRNA stability and induce its expression inhibition.

Promoting RRM2 protein degradation

GW8510 is also a CDK inhibitor and reverses chemoresistance through the inhibition of RRM2 in cancers such as lung squamous cell carcinoma[133] and breast cancer.[126] In human colon cancer HCT116 cells, Hsieh et al found that GW8510 targets the RRM2 protein and promotes its degradation, thereby resulting in inhibition.[198]

Sorafenib (BAY 43-9006), an FDA-approved multikinase inhibitor, mainly targets the vascular endothelial growth factor receptor family and platelet-derived growth factor receptor family.[199] In human lung cancer cells, sorafenib reduces RRM2 gene expression.[200] Yang et al showed that sorafenib inhibits RRM2 expression by suppressing its transcription and promoting its protein degradation. But the mechanism is unclear and needs further investigation.[201]

Chen et al identified a synthetic analog of resveratrol, DHS (trans-4,4′-dihydroxystilbene), which can inhibit

pancreatic, ovarian, and colorectal cancer cells in mouse models of tumor xenografts.[202] Cellular and biochemical experiments confirmed that DHS binds directly to RRM2, and docking analysis showed that the binding involves residues Val146, Ser150, Gln151, Thr156, Arg159, Cys160, and Ile166. At the molecular level, DHS induces ubiquitination of RRM2 by cyclin F, and then the ubiquitinated RRM2 is degraded by the proteasome.

Developing small molecule inhibitors that target active sites of RRM2

Zhou et al identified five ligand binding pockets based on the human RRM2 structure and believed that Pocket 5 close to the RRM1-RRM2 interface is ideally suited for designing the inhibitor.[203] Based on these structural features of the ligand binding pocket, the most effective inhibitor COH29, *N*-(4-(3,4-dihydroxyphenyl)-5-phenylthiazol-2-yl)-3,4-dihydroxybenzamide, is synthesized after structure-based optimization. Residues Gly233, Asp271, Tyr323, Phe326, Val327, Arg330, Glu334 and Met350 are involved in COH29 binding, and the 41 C-terminal amino acids of RRM2 are important for COH29 binding. COH29 can also disrupt RRM1-RRM2 interaction at higher doses, but enhance it at low doses. Mechanistically, COH29 directly affects enzyme activity and does not regulate protein expression. It inhibits most of the cancer cells including ovarian, leukemia, and prostate cancer cells.[204] In KB epithelioid cells, COH29 has similar activity to gemcitabine, and is over 20-fold more active than HU; moreover, it overcomes resistance to both of them.[203] COH29 also enhances the chemosensitivity to doxorubicin in breast cancer MDA-MB-231 cells.[205]

When Liu et al performed computer-assisted virtual screening against the RRM2 structure, they identified a potential RRM2 inhibitor osalmid from the Comprehensive Medicinal Chemistry (CMC) database, with 10-fold more activity than HU.[206] They also found a novel derivative of osalmid, 4-cyclopropyl-2-fluoro-*N*-(4-hydroxyphenyl) benzamide (YZ51), which shows higher efficacy than osalmid. Docking studies show that residues Phe240, Phe244, Cys270, Asp271, Cys274, Tyr323, Arg330, Glu334, and Met350 are involved in the two compounds binding. Osalmid exhibits significant cytotoxicity in esophageal cancer[207] and hepatocellular carcinoma cells.[208] Wu et al studied the metabolic profile of osalmid.[208] During the processes of hydroxylation, glucuronidation, sulfonation, acetylation, and degradation, osalmid will produce ten metabolites, in which metabolites M7, M8, and M10 have higher binding affinities with the RRM2 active site than osalmid.

Conclusion and prospects

Searching and identifying key genes from next-generation sequencing data of bulk normal and tumor samples is an effective strategy for tumor research. Undoubtedly, RRM2 is believed to be a promising target for diagnosis, treatment, and prognosis prediction, such as in breast cancer.[209,210] RRM2, one of the subunits of RR which is essential for DNA replication and cancer cell proliferation, is tightly regulated. The molecular mechanisms of regulation are extremely complicated. For example, RRM2 can be regulated by one pathway, and can also regulate this pathway, forming feedback signaling. During the process from transcription to translation, regulation happens in almost every step all the time.

Although chemotherapy treatment has a significant role in the treatment of cancers and prevention of their recurrence and spreading,[211] the drug-resistance is the main problem. For example, gemcitabine is a famous RR inhibitor and is widely used in cancer therapies, but resistance limits its therapeutic efficacy.[212] In the treatment of breast cancer cells, RRM2 is up-regulated and is involved in the resistance to GTI-2040, tamoxifen, adriamycin, and cisplatin. The acquired resistance is readily overcome by RRM2 inhibition. Besides chemoresistance, RRM2 participates in radiation resistance. After exposure to ionizing radiation or UV, the RRM2 level is increased.[213,214] Chemotherapy or radical therapy combined with RRM2 inhibition significantly enhance the anti-tumor efficiency in cell models of fibrosarcoma cells[121] and pancreatic ductal adenocarcinoma cells,[212] and animal models of lung squamous cell carcinoma,[133] pancreatic cancer,[202] breast cancer,[126] and renal cell carcinoma.[45]

However, unfortunately, many RRM2 inhibitors themselves will promote chemoresistance, and are effectless or have side effects. Thus, continued efforts to develop and design effective and less toxic RRM2 inhibitors are needed. A better understanding of the structure and mechanism of action of RRM2 may improve the design of novel RRM2 inhibitors for cancer therapy. Two main functions which are enzymatic and non-enzymatic provide two different kinds of inhibitors to be developed. Natural products can also be a source of anti-tumor agents. They have obvious advantages, such as safety, few side effects, and low toxicity, and exert chemopreventive activities against cancers including breast cancer.[215] For example, Zhou et al showed that curcumin can rescue part of adriamycin resistance in breast cancer, which may be associated with RRM2.[127] In addition to traditional therapies using small molecular compounds or natural products, gene therapy, such as RNA-based treatments using ncRNA including lncRNA and miRNA, creates a new avenue for treating diseases, such as cancers.[216]

Overall, RRM2 has been uncovered to have a tremendous impact on the biological processes of cancer cells including breast cancer cells in proliferation, prognosis, and resistance. A deeper understanding of the regulatory mechanisms of RRM2 will advance our understanding of tumor development and resistance. Targeting RRM2 therapy is a promising approach in the treatment of breast cancer.

Author contributions

Zuo Zanwen: writing - original draft, writing - review & editing, visualization. Zhou Zerong: writing - original draft. Chang Yuzhou: writing - original draft. Liu Yan: writing - original draft, writing - review & editing. Shen Yuping: writing - original draft, writing - review & editing. Li Qizhang: conceptualization, writing - original draft, writing - review & editing, visualization, supervision, funding

acquisition. Zhang Lei: conceptualization, writing - review & editing. All authors read and approved the final version of the work to be published.

Conflict of interests

The authors report there are no competing interests to declare.

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Appendix A. Supplementary data

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Abbreviations

4E-BP1	eIF4E-binding protein 1
ANXA1	annexin A1
Akt	protein kinase B
APC/C	anaphase-promoting complex/cyclosome
CCNF	cyclin F
CDC25A	cell division cycle 25A
CycD	cyclin D
Cdh1	epithelial-cadherin
CDK	cyclin-dependent kinase
CHK1	checkpoint kinase 1
eIF	eukaryotic translation initiation factor
ERK	extracellular signal-regulated kinase
FPR2	formyl peptide receptor 2
H3K36me3	histone 3 lysine 36 trimethylation
HuR	human antigen R
IGF1	insulin-like growth factor 1
IGF1R	insulin like growth factor 1 receptor
IRS1	insulin receptor substrate 1
KAT7	lysine acetyltransferase 7
MASP	membrane-associated serine protease
MEK	mitogen activated protein kinase kinase
mLST8	mammalian lethal with sec13 protein 8
mSin1	mammalian stress-activated mitogen-activated protein kinase-interacting protein 1
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
PI3K	phosphatidylinositol 3-kinase
PRAS40	proline-rich Akt substrate of 40 kDa
Raptor	regulatory associated protein of mammalian target of rapamycin
RheB	Ras homolog enriched in the brain
Rictor	rapamycin-insensitive companion of mammalian target of rapamycin
S6K1	70-kDa ribosomal protein S6 kinase
SETD2	methyltransferase SET-domain-containing 2
SCF	Skp1/cullin/F-box
SIRT2	sirutin 2
Skp2	S-phase kinase-associated protein 2
TSC	tuberous sclerosis complex
UBE3A	ubiquitin protein ligase E3A

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