R-loops in hepatocellular carcinoma: Bridging genomic instability and therapeutic opportunity (Review)

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Abstract. R-loops, three-stranded nucleic acid structures composed of an RNA:DNA hybrid and displaced single-stranded DNA, have emerged as important regulators of gene expression and genome maintenance. Although physiological R-loops participate in normal cellular processes, their dysregulation can threaten genomic integrity by inducing DNA damage and replication stress. The present review explores the role of R-loops in hepatocellular carcinoma (HCC), a malignancy characterized by marked genomic instability. In the present review, the formation mechanisms of R-loops, their dual functions in transcriptional regulation and DNA damage, and their specific implications for HCC pathophysiology were discussed. HCC cells exhibit altered R-loop homeostasis with aberrant accumulation linked to hepatitis B virus infection, inflammatory signaling and oncogene activation. The present review highlighted how HCC cells exploit or manage R-loops to promote tumor progression, particularly through the epigenetic silencing of differentiation genes and modulation of replication stress responses. Furthermore, emerging therapeutic strategies targeting R-loop biology were examined, including small molecules that induce synthetic lethality, gene-based interventions and combination approaches that exploit R-loop vulnerabilities. Challenges in targeting R-loops and future directions, including multi-omics profiling and biomarker development, were also addressed. Understanding the complex interplay between R-loops and HCC offers promising avenues for novel diagnostic and therapeutic approaches for this malignancy.

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Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related protein; BDH1, β-hydroxybutyrate dehydrogenase 1; BRCA, breast cancer susceptibility gene; CHK1, checkpoint kinase 1; DDR, DNA damage response; DRIP, DNA-RNA immunoprecipitation; DSB, double-strand break; FA, Fanconi anemia; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HR, homologous recombination; lncRNA, long non-coding RNA; MTA2, metastasis-associated protein 2; PARP, poly(ADP-ribose) polymerase; RNASEH, ribonuclease H; SETX, Senataxin; THOC, THO complex; TOP1, topoisomerase I

Key words: hepatocellular carcinoma, R-loops, genomic instability, RNA:DNA hybrids, DNA damage response, synthetic lethality

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1. Introduction

R-loops are triple-stranded nucleic acid structures comprising an RNA:DNA hybrid and displaced single-stranded DNA, typically forming co-transcriptionally when nascent RNA reanneals to its template DNA strand (1). Once considered by-products of transcription, R-loops have been recognized as important regulators of gene expression, DNA replication and genome maintenance (1). Under physiological conditions, controlled R-loop formation plays a role in normal transcriptional termination, RNA processing, epigenetic regulation and mitochondrial DNA replication (1,2). However, unscheduled or persistent R-loops can become pathological and pose a threat to genomic integrity (1). Excess R-loops can stall replication forks, induce DNA breaks and promote mutagenesis, thereby contributing to genomic instability, which is a well-known

hallmark of cancer (1-3). Indeed, aberrant accumulation of R-loops has been observed in breast cancer, colorectal cancer and leukemia and has been linked to dysregulated proliferation and oncogene activation (2,4,5).

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is a disease characterized by pronounced genomic instability (1,6). HCC typically arises in cases of chronic liver injury, including hepatitis B virus (HBV) or HCV infection, alcohol abuse or fatty liver disease, and is characterized by ongoing DNA damage and chromosomal aberrations (1,6). Such chronic stress may predispose the liver cells to aberrant R-loop accumulation. Recent evidence suggests that R-loops play a role in HCC pathophysiology; improper regulation of R-loop homeostasis can drive DNA breakage and replication stress, fueling the genomic instability that propels hepatocarcinogenesis (1,6).

Furthermore, HCC cells may co-opt mechanisms to tolerate or exploit R-loops. HCCs generally have defence mechanisms to minimize R-loop-induced damage (6) and aggressive tumors often upregulate R-loop-resolving factors to maintain survival despite high transcriptional output (2). HCC tumors frequently exhibit widespread transcriptional dysregulation due to inflammation and oncogene activation, which can alter signaling pathways, epigenetic marks and transcription factor activity, leading to aberrant gene expression, replication stress and increased R-loop formation. In addition, HBV infection, a major etiological factor of HCC, is associated with changes in the expression of R-loop regulatory genes (7). An integrative single-cell study found that HCC cells from HBV-infected livers tend to have higher 'R-loop scores' linking viral oncogenesis with R-loop dysregulation (1).

In summary, R-loops represent a notable interface between transcriptional regulation and genomic stability in cancer cells. In HCC, a cancer characterized by extensive genomic instability and complex molecular drivers, understanding R-loop biology is particularly relevant. The present review provided an overview of R-loop formation and functions and delves into their mechanistic impact on transcriptional regulation and genome stability in HCC. The key findings on the role of the R-loop in HCC are summarized in Table I (1,2,6,8,9). Selected R-loop-associated genes and their functions in HCC are presented in Table II (1,2,10-12). The R-loop-related signaling pathways in HCC are shown in Table III (1,8,9,13). Therapeutic strategies targeting R-loops in HCC are presented in Table IV (1,11,12,14). The present review also discussed how HCC cells manage or mismanage R-loops and how this knowledge has contributed to novel therapeutic strategies.

2. Mechanisms of R-loop formation and function in HCC

R-loop formation and homeostasis

Formation. R-loops commonly form during transcription when nascent RNA threads back and hybridizes with the template DNA strand, displacing the non-template strand (1). Certain DNA sequences and contexts promote R-loop formation; for example, GC-rich regions, especially those capable of forming G-quadruplexes, and repetitive sequences increase the stability of RNA:DNA hybrids. Negative supercoiling behind a moving RNA polymerase can also facilitate re-annealing of RNA strands to DNA (12). By contrast, positive supercoiling before the polymerase can impede hybrid formation. This topological balance is managed by topoisomerases (TOPs) such as TOP1, TOP2 and TOP3B, which relax the supercoils and prevent excessive R-loop formation (12).

Regulation and resolution. The cell maintains R-loop homeostasis through dedicated enzymes that remove R-loops once formed. Most of these are ribonuclease H (RNASEH)1 and 2, which specifically recognize RNA:DNA hybrids and cleave RNA strands (2). RNASEH1 is active throughout the cell cycle and serves as the first line of defence against R-loops, degrading RNA components and dismantling the hybrid (15). RNASEH2 operates especially during the S/G_2 phases, not only to resolve R-loops but also to excise ribonucleotides embedded in DNA (16).

Along with RNASEH, several DNA and RNA helicases resolve R-loops by unwinding the RNA:DNA hybrid. Senataxin (SETX) is a key helicase that resolves R-loops during transcription termination, preventing persistent hybrids at gene 3' ends (17). The Asp-Glu-Ala-Asp box (DEAD-box) RNA helicases DEAD-box helicase (DDX)19 and DDX21 are also important; DDX21 unwinds R-loops at ribosomal RNA (rRNA) genes and collaborates with NAD-dependent protein deacetylase sirtuin-7 to suppress R-loops during replication stress (18,19).

Other factors involved in R-loop suppression include the transcription-export (THO) complex/3' repair exonuclease complex, which couples transcription to mRNA export and various RNA-processing factors, such as splicing factors. Comprehensive mRNA splicing and 3' end processing are important to prevent R-loops; if transcription outpaces processing or if splicing is defective, the unprocessed nascent RNA is more prone to hybridize with DNA. This increase in hybridization due to insufficient mRNA processing explains why mutations in spliceosome genes, such as *splicing factor 3b subunit 1* and *U2 small nuclear RNA auxiliary factor 1*, can induce R-loop accumulation and activate DNA damage responses (DDRs) (12,14).

HCC context. Homeostatic mechanisms may be altered in HCC. Tumor cells often have markedly elevated transcriptional activity, which can favor R-loop formation beyond the capacity of normal regulatory mechanisms. There is evidence that HCC cells upregulate certain R-loop-resolving factors, presumably to cope with the increased R-loop load (1,2). In HCC, dysregulated R-loops contribute to genomic instability by inducing replication stress and DNA double-stranded breaks, thereby activating the ataxia telangiectasia and Rad3-related protein (ATR)-checkpoint kinase (CHK)1 and ataxia telangiectasia mutated (ATM)-CHK2 DNA damage response pathways (2,6). Oncogenic drivers such as metastasis-associated protein 2 (MTA2) promote R-loop formation at the promoters of differentiation genes such as *β-hydroxybutyrate dehydrogenase 1* (*BDH1*), leading to epigenetic silencing and enhanced stemness in HCC cells (9). Furthermore, unresolved R-loops can initiate inflammatory responses through cytosolic DNA-sensing pathways, such as cyclic GMP-AMP synthase and stimulator of interferon genes (cGAS-STING), contributing to immune modulation within the tumor microenvironment (TME) (1). For instance, RNASEH1 expression is elevated in breast cancer, colorectal cancer, leukemia, and HCC and associated with poor prognosis (2). One study noted

Table I. Key findings on the roles of R-loops in HCC.

Key finding	Description and significance	(Refs.)
R-loops contribute to genomic instability in HCC	Persistent R-loops induce DNA breaks and replication stress, fueling HCC genomic instability	(1,6)
HCC cells modulate R-loop levels for survival	Cancer cells upregulate defense mechanisms to limit R-loop-associated damage	(2,6)
High R-loops linked to HBV-related HCC	HBV-infected HCCs show elevated R-loop regulator-gene expression and higher R-loop scores	(1)
R-loops impact HCC tumor microenvironment	R-loops activate innate immune pathways, influencing tumor-immune interactions	(1)
R-loop dysregulation alters metabolism in HCC	Aberrant R-loops are linked to metabolic reprogramming affecting tumor progression	(1)
R-loop-associated factors affect chemosensitivity	THO complex 1 knockdown increases R-loops and DNA damage, sensitizing cells to cisplatin	(8)
Oncogenic drivers exploit R-loops	Metastasis-associated protein 2 enhances R-loop formation to silence differentiation genes such as β-hydroxybutyrate dehydrogenase 1	(9)

HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

Table II. Selected R-loop-associated genes and proteins and their roles in HCC.

Gene/factor	Role in R-loop regulation	Relevance in HCC	(Refs.)
RNASEH1	Degrades RNA in hybrids	RNASEH1-antisense 1 long non-coding RNA is upregulated in advanced HCC	(2,10)
RNASEH2 (A/B/C)	Removes RNA from hybrids	RNASEH2 deficiency creates synthetic lethality when combined with ATR inhibition	(11)
Senataxin	Unwinds R-loops during transcription	Important for resolving R-loops at highly transcribed HCC genes	(12)
Fanconi anemia group G protein (Fanconi anemia complex)	Binds and resolves R-loops at forks	Part of the 8-gene prognostic signature; affects HCC outcomes	(1,2)
Breast cancer susceptibility gene 1/2	Recruits factors to suppress R-loops	Suppresses telomeric R-loops that cause DNA damage	(1,2)
TOP1	Prevents excessive R-loop formation	TOP1 inhibitors induce DNA damage partly via R-loop accumulation	(12)
ATR/checkpoint kinase 1 pathway	Responds to R-loop-stalled forks	High R-loop HCCs show ATR dependency; this shows potential for ATR inhibition in therapeutics	(2,12)
Metastasis-associated protein 2/histone deacetylase 2 (nucleosome remodeling and deacetylase)	Promotes R-loops at target genes	Enhances stemness via R-loop-mediated silencing of differentiation genes	(1)

RNASEH, ribonuclease H; HCC, hepatocellular carcinoma; TOP, topoisomerase; ATR, Ataxia telangiectasia and Rad3-related protein.

that a long non-coding RNA (lncRNA) antisense to RNASEH1 (RNASEH1-AS1) is frequently upregulated in HCC and associated with worse survival prognosis (10). Additionally, certain oncogenic pathways in HCC actively induce R-loops to alter gene expression, such as the chromatin modifier MTA2, which increases R-loops at specific gene promoters (9). Thus, the balance between R-loop formation and resolution is maintained in HCC cells.

R-loops in transcriptional regulation and epigenetics. In addition to their impact on DNA stability, R-loops can influence gene expression and the chromatin state. Physiological

Table III. R-loop-related signaling pathways in HCC.

Pathway	Trigger/context	Function in HCC	(Refs.)
DNA damage response (ATR/ATM-checkpoint kinase 1)	Transcription-replication conflicts cause R-loop accumulation, stalling replication forks and causing DNA double-strand breaks. For example, the loss of the RNA export factor THO complex 1 in HCC cells increases R-loops and activates DNA damage signaling	Activates ATR/ATM and p53 pathways, leading to genomic instability or growth arrest. In HCC models, R-loop-induced DNA damage reduces tumor cell proliferation and sensitizes cells to chemotherapy	(8)
Innate immune (cGAS-STING) pathway	Unresolved nuclear R-loops can be processed into cytosolic RNA:DNA hybrids, such as in breast cancer susceptibility gene 1 or Senataxin-deficient contexts. These hybrids and R-loop-derived DNA fragments are sensed by cGAS and other pattern recognition receptors	Triggers STING-interferon regulatory factor 3 signaling and inflammatory cytokine production. In HCC, excessive R-loops aberrantly activate innate immunity, which can induce tumor cell apoptosis and reshape the tumor microenvironment	(13)
Metabolic reprogramming (lipid metabolism)	Upregulation of certain R-loop regulator genes, such as CLTC, in HCC promotes persistent R-loop formation at metabolic gene loci, for example at fatty acid synthase regulators	Enhances lipid biosynthesis and storage to fuel tumor growth. High R-loop levels in HCC cells are linked to increased fatty acid metabolism and a more aggressive tumor phenotype. For instance, CLTC-driven R-loops upregulate lipogenic pathways, supporting rapid proliferation	(1)
Epigenetic regulation (histone modification)	R-loops can be exploited by chromatin-modifying complexes to silence genes. In HCC, the chromatin remodeler metastasis-associated protein 2, acting with histone deacetylase 2/chromodomain-helicase-DNA-binding protein 4, induces R-loop formation at the *BDH1* locus, which is a metabolic gene	Transcriptional repression of *BDH1* leads to altered β-hydroxybutyrate levels and increased histone β-hydroxybutyrylation, an epigenetic mark that promotes cancer stem cell traits. This R-loop-dependent epigenetic silencing enhances HCC stemness and tumor propagation	(9)

HCC, hepatocellular carcinoma; BDH1, β-hydroxybutyrate dehydrogenase 1; CLTC, clathrin heavy chain; ATR, ataxia telangiectasia and Rad3-related protein; ATM, ataxia telangiectasia mutated; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes.

R-loops often occur in the promoter or terminator regions of genes and have regulatory functions (2). For example, R-loops formed over 5'-cytosine-phosphate-guanine-3' island promoters maintain an open chromatin configuration by displacing nucleosomes, thereby facilitating transcription initiation of these genes (19). R-loops at the gene termini have been implicated in pausing RNA polymerase II and promoting transcription termination (20,21).

These co-transcriptional R-loops can recruit or occlude specific factors. One study showed that promoter-proximal R-loops can attract DNA demethylases, leading to local DNA hypomethylation and robust gene expression (2). Conversely, stable R-loops on gene bodies or regulatory elements may interfere with transcriptional elongation, effectively repressing these loci. Furthermore, R-loops interact with chromatin-modifying complexes and alter histone marks, modulate chromatin accessibility and either promote or repress gene transcription (5). For example, R-loop structures are recognized by the polycomb repressive complex and DNA methyltransferases, linking hybrid formation to heritable epigenetic changes (2).

These R-loops may be co-opted or dysregulated in cancer cells, including HCC cells. A notable HCC-related example is the MTA2 pathway; MTA2, a component of the nucleosome remodeling and deacetylase chromatin-remodeling complex, increases R-loop formation at the promoter of *BDH1*, which is a gene that influences both differentiation and metabolism (9,22). The R-loop recruits histone deacetylase (HDAC)2/chromodomain-helicase-DNA-binding protein 4 (CHD4) specifically to the promoter region of BDH1, leading to the epigenetic silencing of *BDH1* and stemness in HCC cells (9,23). Thus, an oncogenic factor exploits R-loop

Table IV. Therapeutic strategies impacting R-loops in HCC.

Therapeutic agent/class	Mechanism (R-loop context)	Potential application in HCC	(Refs.)
Topoisomerase I inhibitors	Increase R-loop formation and associated DNA breaks	Target HCCs with high transcriptional output; combine with DNA damage response inhibitors	(12)
ATR inhibitors	Block responses to R-loop-induced fork collapses	Target HCCs with R-loop resolution defects or high stress	(11,14)
Poly(ADP-ribose) polymerase inhibitors	Block repair of R-loop-induced DNA breaks	Effective in HCCs with homologous recombination deficiencies or R-loop-mediated breast cancer susceptibility gene sequestration	(12)
Splicing modulators	Induce R-loop overload via unspliced transcripts	Create synthetic lethality when combined with ATR inhibitors	(14)
Guanine-quadruplex stabilizers	Prevent R-loop resolution by locking Guanine-quadruplex structures	Target R-loop-prone regions in HCC, such as recombinant DNA and telomeres	(12)
Histone deacetylase inhibitors	Alter chromatin states affecting R-loop dynamics	Reverses metastasis-associated protein 2-induced R-loop silencing of differentiation genes	(1,12)

HCC, hepatocellular carcinoma; ATR, ataxia telangiectasia and Rad3-related protein.

formation as part of a transcriptional repression mechanism that favors tumor cell dedifferentiation and self-renewal.

Another example involves the THO complex (THOC). THOC1 knockdown impairs the THO complex's role in co-transcriptional RNA processing and export, causing RNA-DNA hybrids to accumulate, which increases R-loop formation and consequent DNA damage, which paradoxically leads to reduced tumor cell proliferation, indicating that THOC1 aids HCC cells by preventing lethal levels of R-loop accumulation (8). This suggests that HCC cells require proper mRNA processing to avoid transcription-associated damage. Therefore, R-loops serve as both outputs and regulators of transcription. With widespread transcriptional reprogramming, HCC cells are likely to exhibit an altered landscape of R-loops across the genome. The influence of R-loops on oncogenic gene expression is bidirectional: Certain R-loops promote tumor progression by activating oncogenes or enhancing stemness, while others suppress tumor progression by inducing transcriptional repression or DNA damage-mediated checkpoints (1,6). The interplay between R-loops and epigenetic machinery is a frontier area; for instance, how R-loop formation might interface with the frequent epigenetic alterations seen in HCC.

R-loops and genome instability. One of the most important aspects of R-loops is their ability to induce DNA damage, if not properly mitigated. During the S phase, the coexistence of transcription and replication machinery on the DNA template can lead to transcription-replication conflicts. R-loops are a prominent source of such conflicts. An R-loop can impede the replication fork, especially when it collides with the transcription complex (2). Conflicts are particularly deleterious in a head-on orientation, when transcription and replication move toward each other on opposite strands, as the replication fork encounters the R-loop and stalled RNA polymerase headlong (23). Head-on transcription-replication conflicts markedly increase the likelihood of fork stalling or collapse, resulting in DNA double-strand breaks (DSBs) and chromosome rearrangements (2,24).

ATR and ATM activation. ATR kinase is a central responder to replication stress caused by R-loops. Stalled replication forks with stretches of single-stranded DNA coated with replication protein A activate ATR, which, in turn, phosphorylates CHK1 and coordinates fork stabilization (25). ATR signaling is associated with head-on collisions. Notably, ATR not only halts the cell cycle to provide time for repair, but also actively promotes R-loop resolution (3), as ATR signaling recruits SETX to transcription-replication clash sites to help unwind R-loops (2).

Similar to ATR, the ATM kinase is activated when R-loop-associated DSBs are formed. ATM can promote end resection and homologous recombination (HR) or facilitate non-homologous end joining, depending on the cell cycle stage and local chromatin environment (2). ATM-CHK2 and ATR-CHK1 are both engaged in response to R-loops; however, R-loops that result in DSBs preferentially trigger ATM, whereas those that cause stalled forks without immediate breakage primarily trigger ATR (26).

Notably, the ATR and ATM signaling pathways suppress R-loop-driven instability. R-loop accumulation is notably toxic to cells lacking these kinases or their downstream factors. For example, deficiencies in the Fanconi anemia (FA) pathway lead to hypersensitivity to R-loop-induced DNA damage (27). FA proteins such as FA group D2 protein bind R-loops and stalled forks, stabilizing them and recruiting RNASEH2 to remove RNA:DNA hybrids. Breast cancer susceptibility gene (BRCA)1, apart from its role in HR repair, directly

contributes to resolving R-loops by recruiting SETX to stalled transcription sites (28). Similarly, BRCA2 knockdown leads to abnormal R-loop accumulation, DNA breaks and chromosomal aberrations (29).

Genomic instability in HCC. The relevance of these mechanisms in HCC is supported by several observations. HCC tumors often harbor mutations or dysregulation of DNA repair genes; for example, *tumor protein P53* is commonly mutated, whilst *BRCA2* or *ATM* mutations are less frequent but occur in subsets. Even without specific DDR mutations, chronic replication stress in HCC likely indicates that HCC cells rely heavily on ATR-CHK1 signaling to survive (30,31).

If R-loops are prevalent in HCC cells, ATR would be expected to remain continuously engaged in managing replication-transcription conflicts. Recent research has shown that HCC cells with high R-loop scores exhibit an activated defined set of genes involved in the DDR pathway and are more reliant on stress response pathways (1). When R-loop levels become excessive, cells may enter senescence or apoptosis because of irreparable DNA damage (1,6). This could act as a barrier to tumor proliferation, which HCC cells must overcome by enhancing R-loop tolerance. The aforementioned THOC1 example illustrates that reducing the ability of HCC cells to resolve R-loops leads to notable DNA damage and slow proliferation in HCC cells (8).

Furthermore, etiological factors such as HBV can exacerbate genomic instability in HCC via R-loops. HBV genome integration and HBV X protein (HBx) expression interfere with host DNA repair. Although not yet supported by evidence, it is conceivable that HBV infection facilitates R-loop accumulation, for instance, by causing replication stress or by HBx-mediated downregulation of the RNASEH or ATR pathways (32).

In summary, R-loop-induced DNA damage is likely associated with myriad sources of genomic instability in HCC. The ability of HCC cells to survive and proliferate may depend on how effectively they activate ATR/ATM and associated repair factors to manage R-loop conflicts.

Signaling pathway and function of R-loop in HCC. Aberrant R-loop accumulation can provoke DDRs, contributing to genomic instability in liver cancer (6). THOC1 knockdown triggers R-loop accumulation and DNA damage and curbs tumor proliferation (8). Excess R-loops can also initiate innate immune signaling; cytosolic RNA:DNA hybrids excised from nuclear R-loops activate the cGAS-STING pathway and related receptors, leading to interferon regulatory factor 3-driven inflammatory cascades and tumor cell apoptosis (13). Furthermore, the dynamics of R-loops intersect with cancer metabolism. High R-loop levels are associated with metabolic reprogramming, for instance, the HCC R-loop score correlates with elevated lipid synthesis and an immunosuppressive microenvironment (1). Elevated lipid synthesis provides energy and membrane components to support rapid HCC cell proliferation, while an immunosuppressive microenvironment inhibits anti-tumor immune responses, together promoting tumor growth and progression. Upregulation or increased activity of specific R-loop regulators such as clathrin heavy chain 1 (CLTC) enhances fatty acid metabolism, providing energy and biosynthetic substrates that support tumor growth and survival (1). Finally, R-loops interact with epigenetic mechanisms and result in both tumor promotion and suppression; the chromatin modifier MTA2 creates R-loops to repress *BDH1*. The increase in histone β-hydroxybutyrylation is a direct consequence of BDH1 repression. BDH1 normally metabolizes β-hydroxybutyrate (BHB); when BDH1 is repressed, BHB accumulates, serving as a substrate for histone β-hydroxybutyrylation, which promotes pro-tumorigenic gene expression and stemness (9).

Similarly, RNA-binding proteins such as G patch domain-containing protein 4 (GPATCH4) guard ribosomal DNA loci by unwinding nucleolar R-loops. This activity of GPATCH4 primarily drives HCC progression. By unwinding nucleolar R-loops, it maintains rRNA transcription and protein synthesis, supporting rapid proliferation and tumor growth. However, GPATCH4 could also represent a therapeutic vulnerability, as its inhibition may increase R-loop accumulation, impair ribosome biogenesis, and selectively stress HCC cells (33). Collectively, these findings indicate that R-loops engage multiple signaling pathways, including DNA damage repair, immune surveillance, metabolic adaptation and epigenetic regulation, all of which can drive HCC progression or present therapeutic vulnerabilities (34).

R-loop dysregulation and its dual role in HCC. Dysregulation of R-loops in HCC is associated with context-dependent pro- and antitumor effects (6). Abnormal R-loop accumulation causes genomic instability and DNA breaks that, beyond repair thresholds, trigger replicative senescence or cell death, thereby suppressing cancer proliferation (6). For example, unresolved R-loops arising from THOC1 depletion or GPATCH4 loss in HCC cells can lead to DNA damage, impaired proliferation and heightened chemosensitivity (8). Thus, cancer cells often upregulate R-loop-resolving factors such as THOC1, GPATCH4 and tonicity-responsive enhancer-binding protein to avoid lethal R-loop stress.

By contrast, dysregulation of R-loop formation can drive HCC progression. R-loop formation can activate oncogenic pathways or silence tumor suppressors (1,6). As aforementioned, MTA2 induces R-loops at the *BDH1* locus, repressing differentiation-linked metabolism and sustaining cancer stemness (9). Similarly, elevated R-loop levels (via CLTC or other R-loop regulators) reprogram lipid metabolism and correlate with an immunosuppressive microenvironment, ultimately promoting HCC proliferation (1). Excessive R-loops activate cGAS-STING signaling, inducing type I interferons and cytokines that recruit cytotoxic immune cells, which directly kill tumor cells and enhance immune surveillance, thereby constraining HCC growth and progression (1).

R-loops and the TME. R-loop dysregulation contributes to the malignant behavior of HCC cells and is closely associated with changes in the TME. As aforementioned, MTA2 promote R-loop accumulation at the *BDH1* locus, silencing this metabolic gene via the recruitment of HDAC2/CHD4 and enhancing cancer stemness and invasiveness (9). Additionally, elevated R-loop levels in HCC have been shown to upregulate lipid metabolic pathways via regulators such as CLTC, promoting tumor cell proliferation and tumor aggressiveness (1). These metabolic

shifts reshape the TME by increasing immunosuppressive signaling and nutrient competition with paracancerous cells. Furthermore, unresolved R-loops generate cytosolic DNA fragments that activate cGAS-STING signaling, drive type I interferon responses and alter immune cell infiltration (6,35). Hypoxic or inflammatory TMEs may also exacerbate R-loop formation by impairing RNA processing or TOP activity, further aggravating replication stress (3). Thus, R-loops and HCC TMEs exist in a bidirectional relationship that fuels tumor progression.

3. Therapeutic strategies targeting R-loops in HCC

Given their notable roles in genome stability and gene regulation, R-loops and their regulatory machinery are promising targets for any other cancer therapy methods (2). In HCC, where standard treatments often achieve a relatively low rate of curing HCC in advanced stages, exploiting R-loop vulnerabilities may offer novel approaches. Therapeutic strategies can broadly aim to either reduce pathological R-loops or increase the R-loop burden beyond a tolerable threshold. The latter approach leverages the concept of synthetic lethality, targeting the 'Achilles heel' of R-loop processing in cancer cells.

Small-molecule approaches

TOP inhibitors. TOPs prevent excessive R-loop formation; therefore, drugs that inhibit them can increase R-loop levels, leading to cytotoxic effects (12). TOP1 inhibitors such as camptothecin and its derivatives trap TOP1 on DNA, preventing it from relieving supercoils (36,37). This increases negative supercoiling behind transcription complexes and promotes R-loop formation (12). Trapped TOP1 also leads to transient DNA breaks that are converted into lethal collisions in the presence of R-loops (36). In HCC, TOP inhibitors have shown some therapeutic efficacy in preclinical experimental models, including cell lines and mouse xenograft models, by inducing DNA damage; their ability to exacerbate R-loop formation is a notable contributor to this damage (12).

ATR and CHK1 inhibitors. As described, ATR kinase is important for cancer cells to survive R-loop-driven replication stress. Therefore, inhibition of ATR can experimentally reveal the lethal consequences of R-loops on tumor cells. Preclinical studies have shown strong synergy between ATR inhibitors and R-loop accumulation (24,38). For example, cancer cells with RNASEH2 dysfunction accumulate R-loops and rely on ATR to mitigate the harmful effects of accumulated R-loops; treating these cells with an ATR inhibitor causes notable DNA breaks and apoptosis (1). The same study found that cells harboring spliceosome mutations accumulate R-loops and show selective sensitivity to ATR inhibitors (1). Translating this to HCC, ATR inhibitors may be effective therapeutic agents in tumors with a high R-loop load or impaired R-loop resolution. ATR inhibitors are currently in clinical trials for solid tumors and hold potential for use in combination with an agent that elevates R-loops for the treatment of HCC (28).

Poly(ADP-ribose) polymerase (PARP) inhibitors. PARP1 inhibitors are the preferred treatment option for tumors with HR deficiencies. There is an emerging connection between R-loop incidence and sensitivity to PARP inhibition (39). R-loops can cause replication fork collapse and one-ended DSBs, which normally require HR for repair (40,41). If HR is compromised, cells become markedly reliant on PARP-mediated repair of replication-associated breaks, making them vulnerable to PARP inhibition; in cancer and HCC, PARP inhibitors exacerbate DNA damage, promote R-loop-induced genomic instability and trigger apoptosis, selectively suppressing tumor proliferation (39). Furthermore, R-loops can sequester BRCA1 and prevent it from localizing to DNA damage sites, impairing homologous recombination repair (12). Although BRCA1 is wild-type, its functional unavailability creates an HR defect, increasing genomic instability (12). In Ewing sarcoma cells, transcription of the *EWS RNA binding protein 1-friend leukemia integration 1 transcription factor* fusion oncogene leads to R-loops that sequester BRCA1, rendering the cells sensitive to PARP inhibitors, similar to BRCA1 mutants (12). Similarly, conditions in HCC that compromise HR repair can potentially be exploited using PARP inhibitors.

Splicing/transcription modulators. Compounds that perturb RNA processing can induce R-loops by leaving nascent transcripts unprocessed. For example, spliceosome inhibitors such as sudemycin and H3B-8800 cause widespread splicing disruption, leading to R-loop accumulation and DNA damage in cancer cells (14). These inhibitors are proposed for splice-mutant cancers because such cells already have impaired RNA splicing, which increases baseline R-loop formation. Inhibiting relevant pathways exacerbates R-loop accumulation beyond a tolerable threshold, overwhelming DNA repair and promoting cancer cell death (14). In HCC, direct spliceosome mutations are rare; however, the use of a splicing modulator can artificially create an R-loop overload.

Gene editing and molecular strategies. In addition to conventional drugs, gene editing and molecular biology techniques offer ways to directly manipulate R-loops and their regulators in cancer cells. One innovative approach involves the use of engineered RNASEH to eliminate R-loops at specific genomic sites. Researchers have developed a CRISPR/dCas9-RNASEH1 fusion protein, which can be guided by single guide RNAs to genomic loci and locally resolve R-loops (42,43). This tool has been used experimentally to target tumorigenic R-loops; for instance, site-specific R-loop removal improved cellular reprogramming efficiency in cultured cells (42,43).

In principle, a similar strategy could be used to target a pathogenic R-loop in HCC; for example, if a particular R-loop silences a tumor suppressor gene, a targeted RNASEH1 might reactivate that gene. Conversely, gene editing can be used to create synthetic vulnerabilities in tumor cells. The CRISPR knockout of R-loop in HCC cells removes backup mechanisms that normally compensate for each other, exacerbating DNA damage (11). This heightened stress increases sensitivity to DNA-damaging agents, such as TOP inhibitors, ATR inhibitors and PARP inhibitors, which exploit defects in replication and repair pathways. Another gene-focused strategy targets non-coding RNAs that modulate R-loops. RNASEH1-AS1, an antisense lncRNA upregulated in HCC, may attenuate RNASEH1 function. Using antisense oligonucleotides to knockdown RNASEH1-AS1 could free RNASEH1 to better resolve R-loops, potentially reducing genomic instability in tumor cells.

Synthetic lethality and combination strategies. The concept of synthetic lethality, in which two non-lethal perturbations kill the cell when combined, is notably relevant to R-loops (11). The inherent DNA damage in cancer cells, arising from defects in HR repair or RNASEH function, activates compensatory pathways like ATR signaling. ATR responds to replication stress and R-loop-induced DNA breaks, stabilizing replication forks and coordinating repair to maintain cell survival. The present review has already discussed some synthetic lethal pairs, such as RNASEH2 loss and ATR inhibition (11) or splicing mutation and ATR inhibition (14).

One combined strategy for therapeutic application to HCC could be coupling ATR inhibitors with agents that increase R-loop formation, as aforementioned. Another notable possibility is the combination of R-loop targeting and immunotherapy. HCC treatment has included immune checkpoint inhibitors (ICIs) in recent years (44-46). There is evidence that tumors with increased DNA damage can be more immunogenic due to the activation of cGAS-STING and type I interferon responses (35). Excess R-loops can activate the cGAS-STING pathway, as displaced single-stranded DNA or R-loop-driven DNA breaks generate ligands for this pathway (1).

A transient burst of R-loop-induced damage may acutely increase the immunostimulatory signals via the activated cGAS-STING and type I interferon pathways. Therefore, a short course of R-loop-inducing therapy can enhance tumor immunogenicity by increasing DNA damage and immune signaling, making individual tumors, including HCC, more susceptible to immune checkpoint inhibitor-mediated targeting (13,47).

Another scenario using synthetic lethality could involve targeting metabolic pathways that become lethal when the number of R-loops is high. Single-cell analysis by Chen *et al* (1) highlighted a link between R-loops and lipid metabolism in HCC. The study identified an 8-gene signature of fatty acid metabolism-related R-loop regulator genes; inhibiting CLTC reduces fatty acid metabolism, decreasing lipid synthesis and accumulation in HCC cells. This metabolic disruption impairs tumor cell proliferation and overall tumor growth (1).

4. Challenges in targeting R-loops

Although targeting R-loop biology holds promise for HCC therapy, notable challenges must be addressed for such strategies to be effective and safe.

Detection and measurement of R-loops. The accurate detection of R-loops in cells and tissues is important. The primary method, DNA-RNA immunoprecipitation (DRIP) using the S9.6 monoclonal antibody, has known limitations in terms of specificity (48). Thus, a key challenge is the identification of patients with HCC who have high R-loop levels or dysfunctional R-loop regulation. Without reliable diagnostic assays, patient stratification for R-loop-targeted therapy is difficult.

Specificity and off-target effects. Targeting R-loops can affect normal cells because they are not unique to cancer cells. All rapidly dividing cells generate R-loops (49). Therapies such as ATR inhibitors or TOP poisons also affect healthy proliferating tissues, causing side effects, such as bone marrow suppression (anemia, neutropenia), gastrointestinal toxicity (nausea, diarrhea) and hair loss (12). However, cancer selectivity remains a major challenge. Cancer selectivity is observed in cells with ~2-3 times higher R-loop levels than normal tissues, providing a therapeutic window for targeting R-loop-dependent vulnerabilities.

Resistance mechanisms. As with any cancer therapy, tumors may develop resistance to R-loop-targeting strategies. There are several plausible resistance routes: i) A tumor under ATR inhibitor pressure may upregulate compensatory pathways such as ATM or DNA-dependent protein kinase catalytic subunit (50); ii) a HCC cell may slow its proliferation or transcription rate to manage R-loop stress (51); iii) if a small molecule such as an ATR inhibitor is used, classical drug-specific resistance mechanisms can occur (52,53); and iv) the liver TME might shield HCC cells from treatments.

Therapeutic index and safety. A number of R-loop-targeting approaches run the risk of causing global DNA damage if not controlled. It is important to avoid therapies that could induce notable genomic instability in healthy tissues, resulting in secondary malignancies or organ failure.

Limitations in current knowledge. The mechanisms of R-loops in HCC remain yet to be fully elucidated. Most mechanistic insights have been obtained from cell-line studies or other cancer types such as breast cancer, colorectal cancer and leukemia. Care must be taken when suggesting that HCC has unique aspects that could influence R-loop dynamics in ways that have not yet been fully understood.

5. Future directions

The intersection of R-loop biology and cancer research is a rapidly advancing field (2,3). Several promising avenues of investigation are emerging:

Integrative multi-omics profiling of R-loops in HCC. A more comprehensive understanding of R-loop dynamics in HCC may be achieved by integrating multiple layers of data: Genomics, transcriptomics, epigenomics and proteomics. At single-cell resolution, it is possible to profile transcription and chromatin states, while R-loop mapping remains largely bulk; emerging methods like scDRIP-seq combined with single-cell ATAC-seq or RNA-seq enable integrated analyses. These investigative methods are capable of refining the current understanding of where R-loops form in HCC genomes, as well as their causes and consequences.

Identifying new therapeutic targets in the R-loop pathway. The present study has focused on known molecules that interact with R-loops, such as ATR and RNASEH; however, future studies may unveil HCC-specific interactions. The list of R-loop regulatory genes used in recent HCC studies (1) provides a starting point for identifying novel HCC-specific interactions that includes unexpected genes that are not previously linked to canonical R-loop regulation or cancer progression pathways.

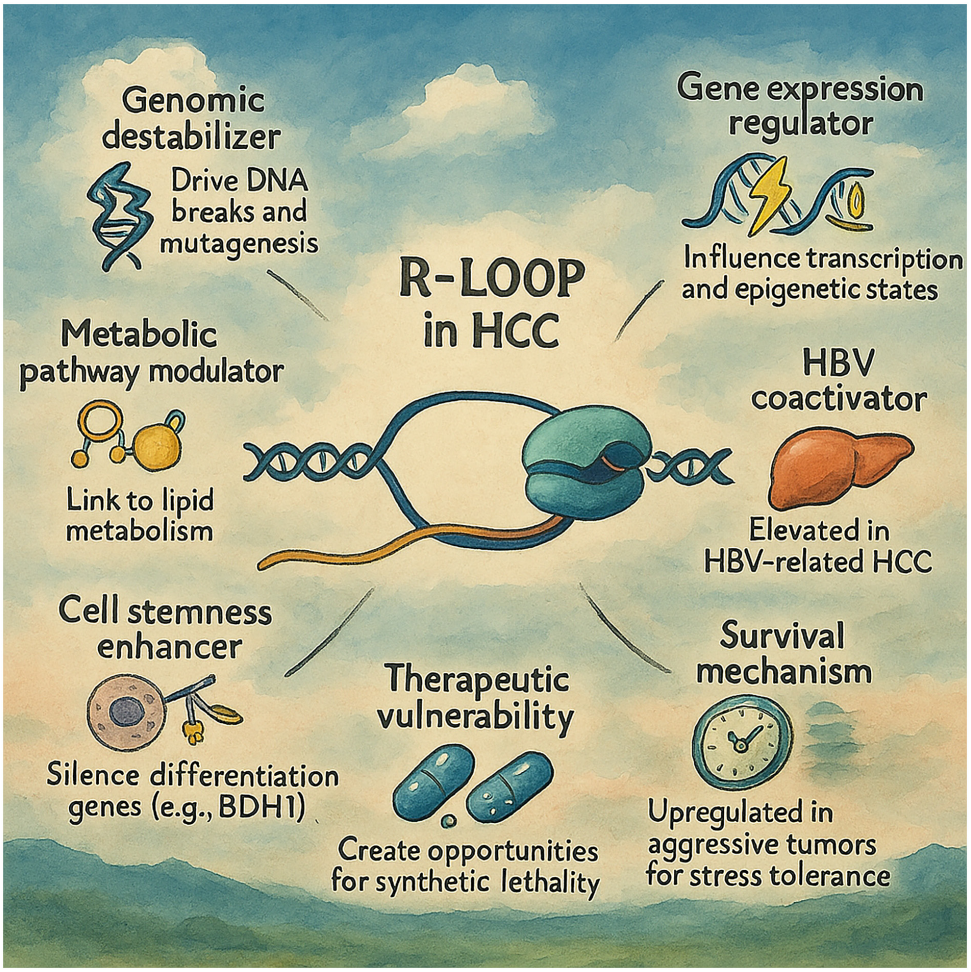

Figure 1. R-loops functions in HCC. The diagram depicts the R-loop structure (center) with its seven key roles in HCC pathophysiology: A genomic destabilizer inducing DNA damage; a gene expression regulator affecting transcription; a HBV coactivator in virus-related cases; a cell stemness enhancer silencing differentiation genes; an immune pathway activator triggering cyclic GMP-AMP synthase and stimulator of interferon genes signaling; a therapeutic vulnerability for synthetic lethality approaches; and upregulated survival mechanisms in aggressive tumors. These functions highlight R-loops as important mediators at the intersection of genomic instability and cancer progression. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; BDH1, β-hydroxybutyrate dehydrogenase 1.

R-loop biomarkers for patient stratification. As therapies that exploit R-loops are introduced to clinical settings, the parallel development of biomarkers is needed. Potential biomarkers could be the expression of an R-loop resolver, mutations in an R-loop-related gene or the direct measurement of RNA:DNA hybrids in tumor samples, if feasible.

Overcoming challenges: Improved delivery and combination regimens. Future research should aim to overcome the challenges outlined in the present review. Nanoparticle- or virus-based delivery of gene therapies may be refined for drug delivery. With small-molecule approaches, prodrugs that are activated in the TME can localize the R-loop-targeting effect (52,53). This refers to a subset of drugs, specifically those designed to target R-loops or exploit R-loop-associated vulnerabilities.

Exploring the R-loop-immune interface. The relationship between R-loops and the immune system is an area of study with direct implications for HCC (1,13). Future studies could clarify this by manipulating R-loops *in vivo* and observing immune responses.

Clinical translation and trials. Finally, the culmination of these future directions of study will be clinical trials that test R-loop-targeted therapies in patients with HCC. For instance, a trial of an ATR inhibitor in combination with an approved HCC therapy could be designed for patients whose tumors exhibit a predefined R-loop signature.

6. Study limitations

The present review had several limitations that warrant consideration. First, most mechanistic insights into R-loops in HCC have been derived from *in vitro* studies or non-HCC cancer models (6). Although previous data from single-cell and bulk transcriptomic analyses have suggested altered R-loop dynamics in HCC, these findings require validation using *in vivo* models and human tissue samples. Second, current methods for R-loop detection, such as DRIP-seq using the S9.6 antibody, suffer from limited specificity and potential cross-reactivity with double-stranded RNA, which may lead to the misinterpretation of hybrid prevalence and localization. Furthermore, the heterogeneity of HCC, driven by diverse etiologies such as HBV infection, metabolic syndrome and alcohol-related liver

disease, poses challenges in generalizing R-loop-associated mechanisms across all patient subsets. Finally, although the concept of targeting R-loops therapeutically, such as via ATR or PARP inhibition, is compelling, clinical evidence in HCC remains lacking of evidence of the efficacy of these inhibitors *in vivo* in HCC, and potential off-target effects or resistance mechanisms remain to be fully elucidated. Future studies should focus on refining detection techniques, validating key pathways in patient-derived models and integrating R-loop profiling into prospective therapeutic trials.

7. Conclusion

R-loops represent a notable interface between transcriptional regulation and genomic stability in cancer. Understanding R-loop biology is particularly relevant in HCC, a cancer characterized by genomic instability and complex molecular drivers. The present review provided an overview of R-loop formation and function (Fig. 1) and discussed the mechanistic impact of R-loops on transcriptional regulation and genome stability in HCC. The present review has discussed how HCC cells manage or mismanage R-loops and how this knowledge may provide the foundation for novel therapeutic strategies.

By integrating advanced genomic tools, identifying novel targets and biomarkers and addressing current challenges, therapies can be developed that either exploit the toxic potential of R-loops to destroy cancer cells or correct the underlying dysregulation of R-loops that contributes to HCC development. Ultimately, these efforts aim to improve the outcomes of patients with HCC by composing novel strategies of cancer therapy that target the fundamental intersection of transcription and genome stability.

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CHH was responsible for writing the original draft, conceptualization and funding acquisition. YWL revised the manuscript. PCC was responsible for editing the manuscript. Data authentication is not applicable. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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