

Biological roles and clinical potential of piRNAs in cancer: Insights into glioblastoma

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ABSTRACT

Non-coding RNAs serve as key regulators of gene expression at both the transcriptional and post-transcriptional levels. Among them, piRNAs, a class of small non-coding RNAs, have emerged as significant mediators in cancer biology. PIWI proteins, members of the Argonaute family highly expressed in germline cells, interact with piRNAs to form piRNA/PIWI complexes, which play a key role in the development, recurrence, and metastasis of several malignancies, including gliomas. This review describes the biological functions of PIWI proteins and piRNAs, emphasizing their roles in cancer-related processes such as proliferation, migration, invasion, cell cycle regulation, epigenetic modification, immunity, DNA damage, and aging. Moreover, the clinical promise of piRNAs as biomarkers and therapeutic agents is discussed, with particular focus on glioblastoma, an area of growing research interest.

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Introduction

The global incidence of cancer has been steadily increasing over the past decades, reflecting both aging populations and changing lifestyles. In China, 2.81 million people die from cancer every year, while more than 4.29 million are found with malignancies annually. In most cancers, treatments are often ineffective due to the high frequency of both metastatic spread and post-treatment relapse, as well as late diagnosis of the disease, which highlights the need for new biological markers for malignancy prognosis and diagnosis, as well as new targets for proven therapy options (1). Recently, PIWI-interacting RNAs (piRNAs), a class of small non-coding RNAs (sncRNAs), have been identified as potential biomarkers and therapeutic targets across various cancers, especially glioblastoma (GB) (2).

SncRNAs are a subset of non-coding oligonucleotide regulators that have a variety of morphological and physiological roles. They are the main mediators of gene regulation at the transcriptional and post-transcriptional stages. Small nucleolar RNAs, small nuclear RNAs, small interfering RNAs (siRNAs), microRNAs (miRNAs), and piRNAs are the most studied members of the sncRNA family (3). Recently, the significance of piRNAs, a distinct

class of sncRNAs, in cancer biology has been recognized. In humans, over 30,000 piRNAs have been identified. These piRNAs have been described as guiding the chromatin-silencing machinery to transposon-encoding regions of the genome. Several studies have shown that a subset of piRNAs can also control DNA methylation in protein-coding genes, potentially impacting cancer development if the regulatory targets are cancer-relevant (4). PIWI proteins are members of the Argonaute protein family, which is widely expressed in the germlines of humans and other animals and plays a role in stem cell self-renewal and gametogenesis. PIWI-like proteins can identify malignancies associated with poor prognosis and serve as clinical indicators (5). PIWI proteins are closely associated with key malignant traits of tumor cells, including suppressed apoptosis, enhanced invasiveness, and rapid proliferation. Functionally, these proteins interact with a class of small non-coding RNAs known as piRNAs and play a critical role in regulating their biogenesis. In germline cells, PIWI proteins associate with transposon-derived piRNAs to assemble the piRNA-induced silencing complex (piRISC). This complex translocates to the nucleus, where it mediates the epigenetic suppression of transposable elements, thereby safeguarding genomic stability (6, 7).

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Following the well-characterized mechanistic framework established in *Drosophila*, human piRNA biogenesis operates through two interconnected pathways: the primary processing route and the ping-pong amplification cycle. In the primary pathway, long RNA molecules are first transcribed from specific genomic regions called piRNA clusters and then transported from the nucleus to the cytoplasm. On the outer surface of mitochondria, the enzyme Zucchini (Zuc/PLD6) cuts these transcripts into shorter fragments, generating intermediates with a 5' end. PIWI proteins bind these fragments, preferentially selecting those that begin with uracil (a feature known as 5'U bias), and the 3' ends are then trimmed to produce mature, functional piRNAs. The maturation process is finalized by HEN1, which adds a 2'-O-methyl group to the 3'-terminal ribose, producing a mature piRNA bound to a PIWI protein within the piRNA-induced silencing complex (piRISC). This piRISC complex then drives secondary piRNA production via the ping-pong pathway: it recognizes complementary primary precursor transcripts in the cytoplasm and, using its inherent slicer activity, cleaves them to generate additional mature piRNAs. This sequence-complementary cleavage mechanism thereby amplifies the cellular piRNA pool (8-10).

A previous study demonstrated that piRNAs, previously thought to exist only in germ cells, are abundantly expressed in the adult *Aplysia* brain and respond to serotonin, a key neurotransmitter involved in memory. The Piwi/piRNA complex promotes serotonin-dependent methylation of the CREB2 gene promoter, reducing its inhibitory effect on memory formation. These results reveal a small RNA-based mechanism that supports long-term synaptic changes required for memory storage (11). Furthermore, PIWI proteins contribute to the pathophysiology of gliomas (12). Recent investigations have increasingly focused on the roles of piRNAs and PIWI proteins in gliomas, with numerous studies already exploring their involvement. The pathophysiology of many malignancies, including gliomas, may be significantly influenced by these pathways (13). Notably, a large number of works have linked the piRNAs/PIWI complex to the development, incidence, metastasis, and recurrence of several malignancies, including gliomas, breast cancer, and prostate cancer (2).

GB is the principal malignant primary brain tumor in adults. Chemotherapy, radiation therapy, and surgical excision are the current standard of care. Recurrence of tumors, however, is almost inevitable. With a 5-year survival rate of less than 5%, the median overall survival is 12-15 months. The recommended course of treatment for GB has remained relatively unchanged over the past 20 years, despite extensive research that has advanced general oncological care; however, the overall survival of GB patients has not increased substantially (14-16). As a result, considerable effort has been devoted to elucidating the underlying pathophysiology of GB and to investigating new predictive biomarkers and potential treatment targets. piRNAs are among the most promising fields of study for promising biological signs and elements implicated in the molecular pathogenesis of GB (17). This review discusses the current evidence and examines the mechanistic roles that piRNAs and PIWI proteins may play in cancer, with a particular emphasis on glioblastoma (GB).

Biogenesis of piRNAs

A significant number of piRNA precursors originate

from specific genomic regions known as piRNA clusters, which can be categorized into uni- and dual-stranded. Uni-strand clusters generate precursor molecules that originate solely from one strand of the genome, whereas dual-strand clusters produce precursors corresponding to both genomic strands. Furthermore, specific piRNA precursors may also arise from the 3' untranslated regions (UTRs) of protein-coding genes or distinct transposons (18, 19). The transcription process of uni-strand clusters resembles that of canonical mRNA transcription, and these clusters exhibit the transcription-associated histone modification H3K4me2 at their promoters (20). Furthermore, piRNA precursors are characterized by a 5' methyl-guanosine cap and a 3' termination (21). In contrast, dual-strand clusters lack distinct markers of RNA polymerase II (RNA Pol II) promoters, such as H3K4me3 and RNA Pol II peaks, thereby producing non-polyadenylated piRNA precursors (22). Moreover, the conversion of dual-strand clusters into piRNA precursors is driven by the coordinated activity of several proteins: RNA Pol II, the RDC complex, Moonshiner, TRF2, and TREX (20).

Transcription of piRNA clusters is initiated by RNA Polymerase II, aided by the RDC complex, composed of Rhino (Rhi), Deadlock (Del), and Cutoff (Cuff). Rhino, a heterochromatin protein 1 (HP1) paralog, binds histone H3 trimethylated at lysine 9 (H3K9me3) (20). Although H3K9me3 is typically associated with transcriptional repression in heterochromatin, its presence is essential for the expression of piRNA precursors. Rhino further ensures proper transcript processing by suppressing aberrant splicing of dual-strand cluster transcripts and by blocking the use of canonical cleavage and polyadenylation signals. Concurrently, Cuff protects transcript integrity by preventing premature termination and protecting nascent precursors from degradation (20, 21). Furthermore, moonshiner, a paralog of the germline-specific transcription initiation factor IIA subunit, plays a key role in initiating transcription and in inhibiting the H3K9me3 mark through its interaction with the RDC complex and TRF2 (23). TREX is responsible for preventing the formation of R-loops, which are hybrids of nascent RNA and DNA (24, 25). The nuclear DEAD-box RNA helicase known as UAP56-associated protein 56 (UAP56) functions to suppress the splicing of precursor transcripts derived from dual-strand piRNA clusters (2, 26).

In this regard, two distinct pathways lead to the formation of mature piRNAs. Initially, precursor piRNAs are generated from genomic sources and subsequently transported from the nucleus to the cytoplasm. Following cleavage and modification, intermediate piRNAs associate with PIWI proteins to become mature piRNAs (2). The primary amplification cascade generates both sense and antisense transcripts from short nucleotide sequences within piRNA gene clusters. Following export to the cytoplasmic Yb body, these primary piRNAs are cleaved by the endoribonuclease Zucchini (27, 28). The resulting 5' fragment of the intermediate then associates with a PIWI protein, forming a piRNA/PIWI complex referred to as an intermediate piRNA. Subsequently, the 3' end of the pre-piRNA is trimmed to its mature length by the single-stranded RNA exonuclease PARN-like domain-containing protein 1 (PNLDC1, also known as Trimmer) (29). Maturation is completed when HEN1 methylates the 3'-terminal 2'-hydroxy group, yielding the final mature piRNA/PIWI complex (10, 30, 31).

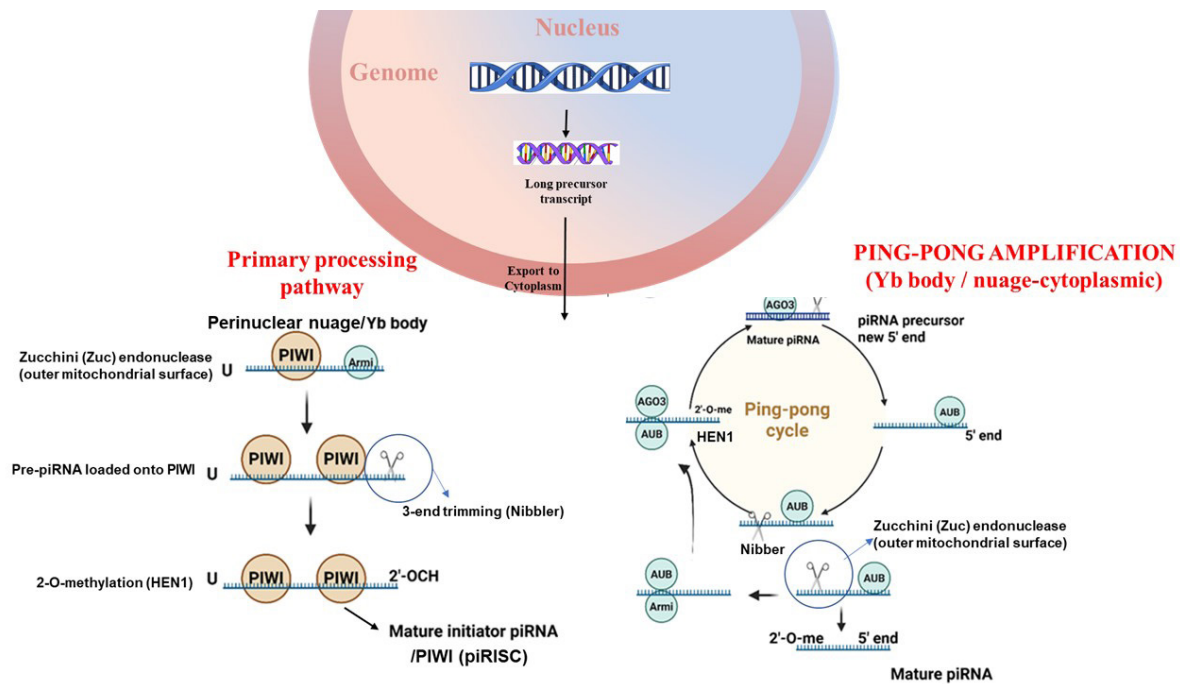


Figure 1. Primary processing and ping-pong amplification of piRNAs
Formation and maturation of piRNA/Piwi, piRNA/Ago3, and piRNA/Aub complexes

Ultimately, the complexes block transcription of target genes by relocating to the nucleus (32, 33). The 'Ping-Pong' amplification pathway represents a secondary mechanism for generating numerous piRNAs within the cytoplasm (10, 34). piRNAs associate with AGO3 or AUB proteins rather than PIWI proteins, resulting in the formation of piRNA/AGO3 or piRNA/AUB pairs that are within the cytoplasmic nuage. The piRNAs bound to AUB exhibit a 5' preference for U, while those associated with AGO3 display an A at the tenth position (35, 36). The two complex structures harbor mutually complementary piRNA sequences. As a result, the target RNA is cleaved by the piRNA/AUB complex in the cytoplasm, generating a novel sequence. This newly synthesized sequence subsequently serves as a substrate for the formation of a piRNA/AGO3 pair in the cytoplasm. Through a comparable mechanism, piRNA/AGO3 complexes facilitate cleavage of complementary cluster transcripts, which in turn promote the formation of new piRNA/AUB complexes (38). This notable amplification process is called the Ping-Pong amplification route (37, 38) (Figure 1).

Biological functions of piRNAs

Extensive investigations to date have detailed the functions of piRNAs within animal germ cells (39, 40). According to these findings, piRNAs protect the germ cell genome from damage by preventing transposable elements (TEs) from becoming active (41). In the context of human genetic disorders, which contain various cancers, transposable element silencing has also been documented (42, 43). Specific research has demonstrated that piRNAs can facilitate transposon inhibition, identify numerous complementary sequences, and mediate transcriptional silencing through epigenetic pathways (32, 44). Furthermore, piRNAs can induce posttranscriptional repression by forming piRNA/PIWI complexes and influence cancer progression across multiple omics dimensions (2) (Figure 2).

Given the diversity of PIWI proteins and their associated

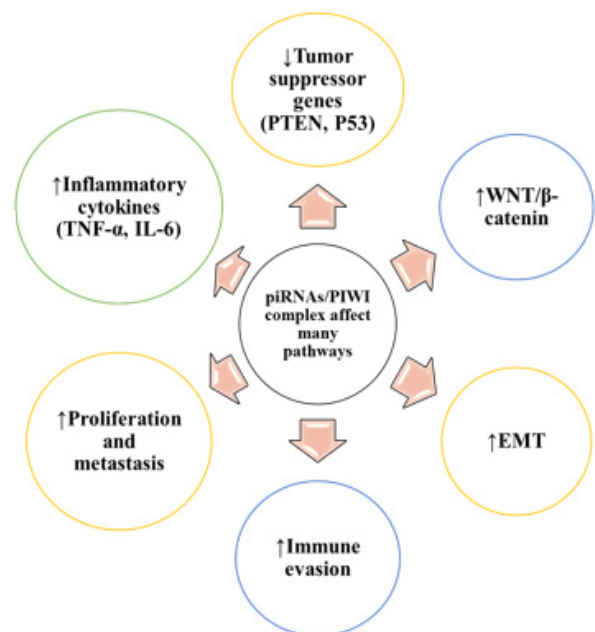


Figure 2. piRNAs/PIWI complex affect many pathways
EMT: Epithelial–mesenchymal transition; PTEN: Phosphatase and tensin homolog

piRNA effectors, along with the various regulatory patterns displayed by piRNA/PIWI complexes on target genes, several mechanisms exist through which these complexes can modulate gene transcription. Among these is transcriptional gene silencing (TGS), a pathway that primarily engages PIWI proteins with reduced catalytic activity (45, 46). In the TGS pathway, the piRNA/PIWI complex works with histone-modifying enzymes or DNA methyltransferases to silence chromatin, thereby blocking mRNA transcription (40, 47, 48). Epigenetic modifications play an essential role in tumorigenesis, with non-coding RNAs, especially piRNAs, contributing significantly to these

processes (49).

At the transcriptional level, piRNAs and PIWI proteins play a crucial role in altering chromatin architecture and histone proteins within the nucleus by modulating DNA methyltransferase (DNMT) activity. DNMT is responsible for the methylation of CpG islands located in promoter regions, which inhibits the commencement of transcription. PIWI proteins facilitate the binding of DNMT to TEs or specific target genes, leading to their silencing (31, 50). Nonetheless, the precise mechanisms by which piRNAs and PIWI proteins influence DNMT expression remain poorly understood. Additionally, piRNAs and PIWI proteins interact with the histone methylation machinery, thereby modulating the methylation of histone lysine residues, specifically H3K and H4K. These methylated histone marks recruit histone methyltransferases to target transcripts, ultimately resulting in transcriptional repression (51, 52). The transient transfection of PIWI-like 4 (PIWIL4), the sole member of the PIWI-like family that is expressed throughout human tissues, led to the methylation of histone H3 at lysine 9 within the p16Ink4a (CDKN2A) locus. This increase in histone methylation caused a down-regulation of the p16Ink4a gene. These findings indicate that PIWIL4 plays a significant role in the chromatin-modifying pathway in human somatic cells (52). Regarding piRNAs, they are capable of directly interacting with the 3' untranslated region (3'UTR) of mRNAs, thereby influencing their expression through mechanisms of posttranscriptional regulation (53, 54).

The miRNA-induced silencing complex (miRISC) is a multi-protein assembly that employs microRNAs to recognize mRNAs targeted for repression (55). Upon binding to its cognate mRNA, the miRISC triggers silencing through an intricate mechanism that is still being actively investigated. This silencing process involves both translational repression and mRNA degradation (56). Notably, there is no established upper limit regarding the size of target mRNAs that miRISC can repress. While the majority of microRNA binding sites are located within the 3' untranslated regions of target mRNAs, miRISC appears capable of mediating silencing from any position on an mRNA where stable binding can occur (57).

PIWI proteins, which belong to the RNaseH-related Argonaute superfamily, associate with small RNAs known as piRNAs to form piRISCs (31, 58, 59). Where piRNAs come from in the genome, their sequences, and their lengths vary a lot across animals. Yet some of the biological roles of piRISCs appear to be conserved across species. For example, piRISCs are required for reproduction and for silencing transposable elements (TEs) in organisms such as nematodes, *Drosophila*, and mice (38, 59, 60). An increasing body of research indicates that piRNAs and PIWI Argonautes may play a regulatory role in many, if not all, germline mRNAs (35, 61).

Furthermore, miRISCs and piRISCs typically engage mRNAs via precise nucleotide pairing in the seed region at the 5' end, exhibiting less stringent complementarity requirements towards the 3' end (53, 62). However, for piRNAs that are highly abundant in association with PIWIL1 or PIWIL2, perfect complementarity within the seed region is not strictly required (63). In one mechanism, piRISC binding can result in mRNA cleavage followed by degradation (64).

PIWI-piRISCs perform a protective role in animal germline cells by suppressing deleterious transposons

through either post-transcriptional degradation or transcriptional silencing (10). The process of piRNA biogenesis and the formation of the PIWI-piRISC complex is remarkably complex and, unlike the miRNA and siRNA pathways, occurs independently of a double-stranded RNA (dsRNA) intermediate (10). Instead, the long clustered piRNA transcripts are subject to endonucleolytic cleavage, resulting in intermediates that are subsequently loaded onto PIWI proteins. These intermediates then undergo further maturation at the 3' end, which is followed by 2'-O-methylation at the terminal nucleotide (31, 65).

Conversely, piRISCs may also enhance the stability of target mRNAs by facilitating their polyadenylation or by activating translation through interactions with initiation factors (66). An additional process is PTGS, which typically requires PIWI proteins for cleavage, as it relies on fragments of the target transcript (46, 67, 68). piRNAs modulate posttranscriptional networks through interactions with various RNAs, including mRNAs (68). Moreover, piRNA/PIWI complexes participate in proteomic-level interactions. Both piRNAs and the PAZ domains of PIWI proteins in the piRNA/PIWI complex can directly bind specific proteins, thereby facilitating multiprotein interactions (69).

piR-823 has the potential to partially inhibit PINK1-Parkin-mediated mitophagy, thereby helping preserve mitochondrial function, quantity, and dynamics. Parkin is known to ubiquitinate various mitochondrial proteins, including TOM20, mitofusins, and members of the VDAC family, facilitating the identification of impaired mitochondria (70, 71). Ubiquitinated mitochondrial proteins are subsequently recognized by p62/SQSTM1, which interacts with LC3 to form autophagosomes, a process integral to mitophagy (72).

As piRNAs can affect transcription and post-transcriptional processes, numerous aberrantly expressed piRNAs have been identified as contributing to cancer development and progression. Recent studies suggest that dysregulated piRNA expression is associated with cancer progression, highlighting their potential utility as diagnostic tools, therapeutic targets, and prognostic biomarkers (4, 73, 74).

piRNAs affect cancer through many mechanisms

Due to our limited understanding of piRNA function, they have historically been regarded as the dark matter of non-coding RNAs in the genome (9). Recent research indicates that abnormal expression of piRNAs, contributing significantly to these processes may serve as a distinctive marker for cancer and could correlate with the clinical features of tumor tissues. This points to the significant involvement of these molecules in numerous cancers, including liver, lung, breast, bladder, gastric, prostate, and colorectal tumors, as well as malignant melanoma (4, 49, 75). The dysregulation of piRNA expression implies that they may act as either tumor suppressors or oncogenes during tumorigenesis, depending on the roles of their target genes, mRNAs, or proteins, thereby acting as critical regulators of key cancer characteristics such as differentiation, proliferation, and metastasis (8, 76).

The roles of piRNA pathways have been demonstrated to correlate with various processes involved in cancer, including genomic instability, DNA methylation, aneuploidy, cellular metabolism, repetitive expression, and cell cycle progression (75, 77, 78). Studies indicate that piRNA pathway genes regulate genes that contribute to the development of stem

Table 1. Overview of piRNA subtypes and their functional roles in cancer

Type of piRNA	Effect	Ref.
piR-30188	piR-30188 functions as a tumor suppressor by regulating miR-367-3p, while upregulated of the typically down-regulated piR-8041 in GB suppresses tumor growth through MAPK signaling and heat shock protein modulation	(84, 214)
piR-28877, piR-28467, piR-27616	In breast cancer, piR-28877 (DQ598677) was found at higher levels, whereas piR-28467 (DQ598252) and piR-27616 (DQ597341) were found at lower levels. This matches what has been observed in GB	(215)
piR-27493, piR-7193	piR-27493 (DQ597218) and piR-7193 (DQ576872) were found at higher levels, and the same pattern has been seen in GB	(216)
piR-1849, piR-9491, piR-12487, and piR-12488	In GB, the levels of piR-1849, piR-9491, piR-12487, and piR-12488 are greatly reduced	(17)
piRNA complex	The assembly of a piRNA complex that includes PIWI, HP1a, and Su (var) 3–9 proteins. This complex can chemically modify histones, similar to the piRNA complexes found in U937 cells	(221)

This table summarizes the main categories of piRNAs, including oncogenic, tumor-suppressive, and diagnostic subtypes, along with their proposed mechanisms of action and implications for cancer biology and potential clinical translation.

cell characteristics (77, 79). Thus, identifying piRNAs associated with cancer and studying how their target genes function could help develop new ways to prevent and treat cancer (80) (Figure 2).

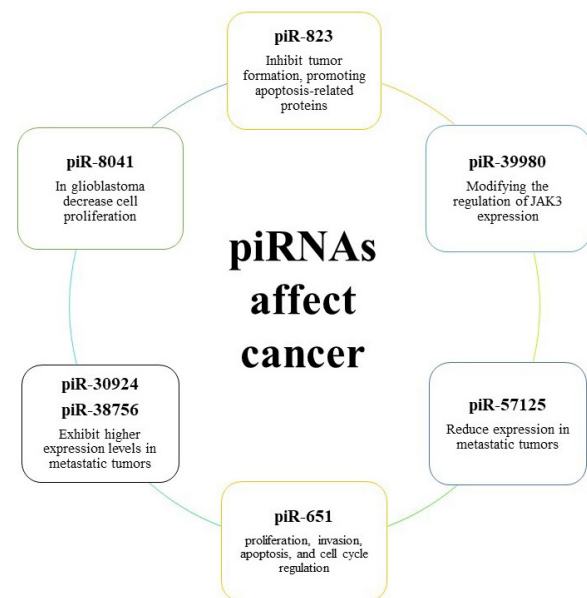
Table 1 provides an overview of the principal piRNA categories, namely, those with oncogenic, tumor-suppressive, and diagnostic functions, while also outlining their putative mechanisms of action, their relevance to cancer biology, and their prospects for eventual clinical application.

piRNAs affect proliferation, migration, invasion, and cell cycle arrest

One key feature of cancer is uncontrolled cell growth, and piRNAs have been shown to play a key role. Tumor cell proliferation often facilitates metastasis, as cancer cells detach from the primary tumor and establish colonies in distant sites, where metastatic cells continue to proliferate, migrate, and invade adjacent tissues (81, 82). This sequence of events is a primary contributor to unfavorable prognoses, and piRNAs are implicated at various stages of this progression. For example, in both metastatic and non-metastatic clear cell renal cell carcinoma, piR-30924 and piR-38756 were increased, whereas piR-57125 was decreased. Notably, the differential expression of these piRNAs was linked to tumor recurrence and overall survival, establishing them as independent prognostic indicators (83). The overexpression of piR-8041 in GB has also been shown to decrease cell proliferation, and treatment with piR-8041 in an *in vivo* model notably diminished the size of intracranial mouse xenograft tumors (84) (Figure 3).

Tumor metastasis comprises multiple interdependent stages, with epithelial–mesenchymal transition (EMT) being a critical phase in tumor progression (85). Following EMT, tumor cells gain invasive properties and penetrate the adjacent stroma, thereby creating a microenvironment conducive to tumor proliferation and metastasis (86). The identification of PIWI proteins associated with EMT markers could significantly enhance therapeutic strategies and the management of tumor metastasis. Additionally, piRNAs may play a crucial role in the EMT process (86–88) (Figure 2).

Moreover, numerous regulatory factors play a crucial role in tumor cell cycle regulation. Anomalies in any regulatory factor can hinder the transition between stages, leading to cell cycle arrest (89). Furthermore, cyclin D1 plays a critical role in cell cycle progression and has been shown to regulate the release of two piRNAs, piR-016658 and piR-016975, in

**Figure 3.** Different types of piRNAs/PIWI complex affect cancer IL: Interleukin

breast cancer (90).

piRNAs serve as sequence-specific guides for PIWI proteins, and the PIWI–piRNA pathway is believed to play a crucial role in the silencing of TEs through DNA methylation, thereby preserving genomic integrity in germline stem cells (50, 91). Furthermore, recent studies indicate that this silencing mechanism may extend beyond the germline to encompass additional genomic regions in cancer, including those associated with tumor suppressor genes. This expansion may contribute to the development of an aberrant ‘stem-like’ state, ultimately leading to tumorigenesis (49, 92).

A study provided insights into the biological effects of piRNA-823 in multiple myeloma (MM) (93). When piRNA-823 was silenced in MM cells, cell death increased, and the cell cycle was disrupted. As a result, MM cells stopped multiplying in both lab experiments and living organisms. Similar effects were seen with piRNA-823 and piRNA-651 in gastric cancer cells (80, 94). However, research into how piRNAs actually work remains limited. Some studies show that PIWIL2, a protein from the PIWI family,

acts as an oncogene. It prevents cell death (apoptosis) and promotes cell growth by activating the STAT3/Bcl-xL and STAT3/cyclin D1 signaling pathways in tumors (95, 96). In this regard, several apoptosis regulators and signaling-related genes were affected by piRNA-823. Among them, the p16INK4A/cyclin D1/CDK4/Rb pathway stood out as being modulated by this piRNA. This pathway is essential for controlling the cell cycle as it moves from the G1 phase to the S phase. It is also a common target during cancer development. In multiple myeloma (MM), alterations in at least one component of this pathway have been observed, and these changes influence disease progression and patient outcomes (93, 97). Therefore, this pathway is likely a key player through which piRNA-823 affects how MM cells grow and survive (93) (Figure 3).

Moreover, in a study, a novel piRNA (piR-YBX1) was identified as down-regulated in triple-negative breast cancer (TNBC) tissue compared with corresponding normal breast tissue (98). Scientists studied how piR-YBX1 stops TNBC cells from growing and spreading. They did this using lab experiments and tests on living organisms. Further results showed that piR-YBX1 targets a gene called YBX1 (Y-box binding protein 1), which in turn shuts down the MAPK signaling pathway. The RAF/MEK/ERK signaling axis is activated in more than 40% of human cancers and plays a major role in cancer cell behavior (99). In this study, the researchers used KEGG pathway enrichment analysis to pinpoint the MAPK signaling pathway. They then confirmed that when piR-YBX1 levels were increased, MEK and ERK1/2 phosphorylation was blocked (98). Experiments have shown that piR-YBX1 binds directly to YBX1 mRNA and reduces YBX1 levels. Next, the researchers wanted to understand how YBX1 contributes to TNBC cell growth and spread, using piR-YBX1 as a tool to control it. Their findings show that piR-YBX1 strongly limits TNBC cell proliferation, migration, and invasion by keeping YBX1 in check (98).

Furthermore, another study demonstrated that elevated expression of piR-651 in non-small cell lung carcinoma cells led to significant increases in cell viability and metastasis, along with a decreased proportion of cells in the G0/G1 phase (100). In alignment with prior research, the findings corroborated that piR-651 was increased in breast cancer cell lines, specifically MDA-MB-231 and MCF-7 (101). When piR-651 levels were increased in breast cancer cells, the cells grew and moved more quickly. Flow cytometry analyses showed that piR-651 promotes proliferation and invasion while inhibiting apoptosis and cell cycle arrest. Mechanistically, piR-651 recruits DNMT1 via PIWIL2 to methylate the PTEN promoter, leading to PTEN silencing and increased oncogene expression. Therefore, piR-651's biological roles are closely tied to breast cancer cell proliferation, invasion, death, and cell cycle regulation. Moreover, piR-651 appears to regulate the activity of tumor-related genes, further supporting this idea (101) (Figure 3).

Investigations have demonstrated a marked decline in piR-017724 levels across hepatocellular carcinoma specimens and cultured cell models. Lower expression of this piRNA was closely associated with more advanced tumor stages and unfavorable patient outcomes. Additionally, experimental depletion of piR-017724 significantly accelerated the proliferative, migratory, and invasive capacities of HCC cells (102).

piRNAs affect epigenetic modification

The main types of epigenetic changes include DNA methylation, histone modifications, and chromatin remodeling, is significantly associated with cancer, and methylation of CpG islands can lead to transcriptional silencing of tumor suppressor genes (49, 103, 104). DNA methylation is significantly associated with cancer, and methylation of CpG islands can lead to transcriptional silencing of oncogenes, while it can also silence oncogenes in certain contexts (105, 106). DNA methylation is mainly carried out by enzymes called DNA methyltransferases. These enzymes fall into two main groups: DNMT3 (which includes DNMT3a and DNMT3b) and DNMT1. DNMT1 is responsible for maintaining DNA methylation over time (103, 104). Recent investigations indicate that piRNAs play a role in CpG island methylation (107). In addition to DNA methylation, piRNAs and PIWI proteins are closely associated with histone modifications (108). In *D. melanogaster*, piRNAs have been shown to enhance histone modifications (22, 45, 109).

Moreover, studies have shown that PIWI proteins directly alter histone modifications at piRNA-binding sites (45, 47, 52, 110, 111). Ubiquitination is an important type of histone modification. It helps control where proteins go, how they are broken down, and how they are regulated. The connection between ubiquitination and non-coding RNAs, such as piRNAs, has attracted considerable attention (112). The involvement of piRNAs in cancer frequently intersects with multiple hallmarks of cancer; for instance, in MM, silencing piRNA-823 reduces tumorigenicity, decreases DNA methyltransferases DNMT3A and DNMT3B, lowers global DNA methylation, and restores expression of the tumor suppressor p16(INK4A). These findings suggest that piRNA-823 plays an oncogenic role in MM and may serve as a potential therapeutic target (93). These varied functions may involve intricate mechanisms warranting further investigation.

Current evidence indicates that piRNAs mediate transcriptional-level epigenetic regulation in somatic cells primarily through aberrant sequence-specific DNA methylation and histone modifications (34). The crosstalk between piRNAs and DNA methylation significantly impacts genomic stability and gene expression, potentially disrupting cellular signaling pathways and contributing to disease development (93). As a key epigenetic mechanism, DNA methylation silences transposons and repetitive elements while stably repressing certain transgenes and endogenous genes (50, 113).

In both MCF-7 and T-47D breast cancer cell lines, piRNA-823 increased cell growth, colony formation, and tumor regenerative capacity. It also encouraged cancer cells to acquire stem cell-like properties and proliferate, promoting breast cancer stem cell (CSC) proliferation. It did so by altering DNA methylation and activating Wnt signaling pathways (114). In a xenograft model where MCF-7 cells were implanted, a nanoparticle-based gene therapy designed to target piRNA-823 successfully blocked tumor formation and slowed tumor growth (114). Wnt/ β -catenin pathway activity is frequently modulated by DNA hypermethylation in the promoter regions of its regulators, including APC, which undergoes CpG island hypermethylation during tumorigenesis (115, 116). Ding *et al.* demonstrated that piR-823 overexpression in MCF-7 breast cancer cells up-regulates DNMT1, DNMT3A, and DNMT3B, leading to APC promoter hypermethylation and subsequent

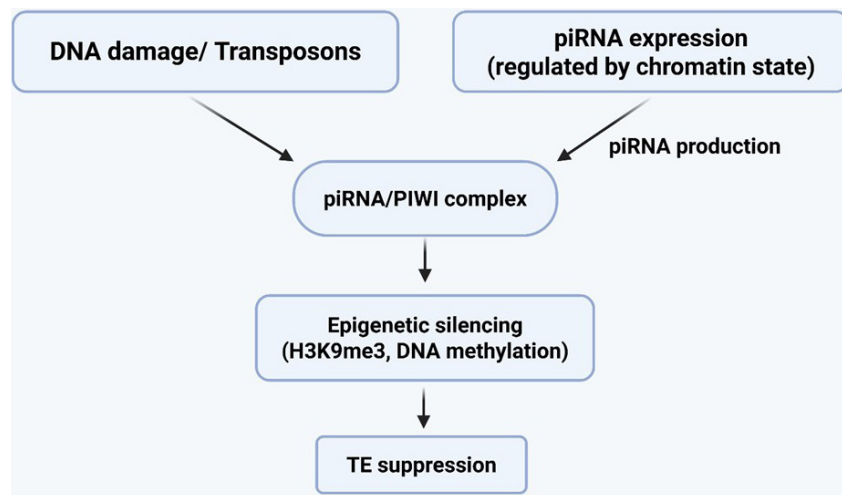


Figure 4. piRNAs/PIWI complex affects epigenetic modification
TE: Transposable element

Wnt pathway activation, thereby promoting cancer cell stemness (114). This piR-823-induced APC promoter hypermethylation was attenuated by the DNMT inhibitor 5-AzaDC, and the resulting reduction in APC expression was consistent with the observed hypermethylation (114) (Figure 4).

Recent works have highlighted the important role of epigenetic regulation in the biological behaviors associated with cancer (117, 118). Among the various forms of internal epigenetic modifications, N6-methyladenosine (m6A) in RNA has emerged as a prominent factor in both physiological and pathological processes associated with various cancers (119). Proteins regulating m6A RNA modification fall into three categories: methyltransferases (“writers”), demethylases (“erasers”), and m6A-binding proteins (“readers”). Writers, mainly METTL3, METTL14, and WTAP, catalyze m6A addition, while erasers such as FTO and ALKBH5 remove this modification. Readers, predominantly YTH domain family proteins (YTHDF1/2/3) and YTH domain-containing proteins (YTHDC1/2), determine target RNA fate (120-122). A functional link between m6A modification and piRNAs has been reported in myocardial infarction (123).

A connection was identified between piRNA-30473 and aberrant m6A methylation in DLBCL. Inhibiting piRNA-30473 significantly reduced WTAP expression at both mRNA and protein levels and decreased global m6A levels, triggering downstream signaling pathways (124). m6A modification regulates key biological processes, including primary microRNA processing (125) alternative splicing (126), stem cell regeneration (127), and mRNA translation (128), and its dysregulation disrupts RNA metabolism, promoting various cancers. Growing evidence highlights its interplay with non-coding RNAs such as piRNAs in tumor progression (129), and m6A dysregulation has also been linked to leukemia (130, 131). M6A-seq analysis identified HK2 as the main WTAP target; WTAP knockdown reduced HK2 m6A levels and down-regulated HK2 expression (124). HK2, a key glycolytic kinase, drives tumor proliferation via enhanced aerobic glycolysis (132, 133). Studies on hypoxia-regulated DLBCL progression strongly implicate HK2 in B-cell lymphoma development, positioning it as a major metabolic driver of the DLBCL

phenotype (134). Collectively, these findings suggest that the piRNA-30473/WTAP/HK2 axis contributes critically to DLBCL pathogenesis (124).

piRNAs affect immunity

Investigations into piRNAs have also extended to other domains, including immune regulation. A study investigation has identified aberrantly expressed serum small extracellular vesicle piRNAs in patients with pancreatic neuroendocrine neoplasms. It has elucidated the role of sEV piR-hsa-30937 in modulating CD276+ macrophages and T-cell immunity within the tumor microenvironment of pancreatic neuroendocrine neoplasms (135). The binding partners of CD276 are unknown, and its role in immune evasion remains unclear. Inhibiting CD276 enhances IFN γ production by T cells and can eradicate cancer stem cells in head and neck squamous cell carcinoma (136, 137). CD276 is overexpressed in tumor angiogenesis and can be targeted with therapies like monoclonal antibodies and CAR T cells (138). CD276-targeted therapies show promise in pancreatic neuroendocrine neoplasms, with low risk of toxicities due to its low expression in normal tissues. Evaluation of CD276-targeting CAR T cells for diffuse intrinsic pontine glioma suggests potential for repeated dosing and localized immune activation. Overall, CD276-targeted therapies could be effective and safe options (139, 140).

Focusing on lipid metabolism, particularly cholesterol, is crucial for preventing tumor development and addressing treatment resistance. A growing body of clinical trial evidence suggests that statins, which suppress cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase, can improve survival and clinical outcomes when used as part of combination therapy for metastatic pancreatic, lung, and breast cancer, as well as several other malignancies (141-143). Alternative cholesterol-targeting agents, such as the SREBP inhibitor fatostatin and the NPC-1L1-targeting drug ezetimibe, have shown tumor-suppressive effects and hold promise for anti-cancer therapy. A recent study highlighted the critical role of piRNA-137463 in cholesterol metabolism-driven tumor growth, suggesting its relevance for identifying LUAD patients who may benefit from cholesterol-directed therapies (144). Cholesterol promotes immune evasion through multiple pathways: it induces

CD8⁺ T cell exhaustion in the tumor microenvironment via an ER stress/XBP1-dependent mechanism that up-regulates PD-1 and other immune checkpoints (145), while XBP1 further drives immunosuppression by activating MDSCs. Cholesterol accumulation disrupts TCR dimer formation and signaling, blocks T cell activation, triggers phosphorylation of the T cell activation linker, and enhances PCSK9 secretion, which binds LDLR and prevents LDLR/TCR recycling to the plasma membrane, ultimately impairing cytotoxic T lymphocyte effector function (146). Cancer cells also secrete oxysterols that down-regulate C-C chemokine receptor type 7 on dendritic cells, reducing tumor antigen presentation (147). The present study confirmed that piRNA-137463 upregulates PD-L1 in LUAD cells by inhibiting its ubiquitination and proteasomal degradation, thus enhancing its stability (144). Prior findings suggest piRNA-137463 drives cholesterol biosynthesis, promoting tumor growth, EMT, metastasis, and immune evasion, while also contributing to apoptosis resistance, cancer stem cell modulation, angiogenesis, genomic instability, tumor-promoting inflammation, and microbiome alterations (144). Future studies should explore whether piRNA-137463 facilitates immune evasion via additional cholesterol-mediated mechanisms, potentially enhancing the efficacy of PD-1 inhibitors.

piRNAs affect DNA damage and aging

DNA damage refers to a permanent alteration in the nucleotide sequence of DNA that occurs during replication. SNPs, which are variations in the DNA sequence caused by a single nucleotide mutation, can also result in DNA damage. These SNPs contribute to an increased risk of cancer through two primary mechanisms. Firstly, SNPs in genes associated with the piRNA biogenesis pathway may disrupt piRNA production, thereby affecting their expression and maturation (148, 149).

Conversely, SNPs may not influence piRNA maturation and expression; however, they can alter interactions between piRNAs and other RNAs or proteins, thereby affecting the likelihood of tumorigenesis (150). Furthermore, the accumulation of DNA damage contributes to the aging process, diminishing the efficiency of normal cells and ultimately leading to various diseases, including cancer. The modulation of TEs by piRNAs can influence the rate of DNA damage associated with aging (151). Previous research has indicated that piR-39980 inhibition can trigger cellular senescence in neuroblastoma by modulating JAK3 expression through a mechanism independent of traditional apoptosis pathways (152) (Figure 3).

RNA-binding proteins (RBPs) regulate gene expression by engaging in diverse mRNA processes, including transcription, capping, splicing, stability, nuclear export, and translation. Recent findings further indicate that RBPs help prevent detrimental DNA structures such as R-loops (RNA-DNA hybrids) and participate in the DNA damage response (153). Bamezai *et al.* found that PIWIL4, an RNA-binding protein linked to germ stem cells and a member of the RNase H-like superfamily, is present at high levels in patients with acute myeloid leukemia (AML). This protein is essential for leukemic stem cell function and AML growth. However, healthy human hematopoietic stem cells do not need PIWIL4 to function (154). In AML cells, PIWIL4 interacts with a limited set of recognized piRNAs. Instead, it primarily engages with mRNA associated with protein-

coding gene regions and with enhancers enriched for cancer-linked genes and human myeloid progenitor gene markers (154, 155).

In contrast to normal hematopoietic stem cells, AML leukemic stem cells and leukemic masses exhibit a preferential reliance on PIWIL4, which enzymatically resolves R-loops. Loss of nuclear PIWIL4 in AML cells leads to R-loop buildup, accumulation, DNA damage, and activation of the ATR pathway, effects that are reversed by restoring PIWIL4 expression (154). Supporting evidence from prokaryotic PIWI, archaeobacterial PIWI, and the human PIWI domain of AGO2 further confirms that PIWIL proteins possess R-loop resolution capabilities (156-158).

PIWIL4 binds to RNAs associated with the CMP/GMP lineage, hypoxia, TNF α /NF- κ B signaling, and LSC maintenance factors, regions marked by R-loop accumulation, active transcription, and open chromatin in AML cells. It uses its R-loop-resolving function to suppress R-loop formation and promote transcription at CD93 and TRIB1, two genes critical for LSC function and AML progression (154). Recent insights highlight a non-canonical, piRNA-independent role for PIWIL4 in AML, where it resolves R-loops at loci essential for leukemia stem cell identity, and its expression is dynamically lost during myeloid differentiation (159). Cycling LSCs likely rely more heavily on PIWIL4-mediated R-loop resolution, as transcription-replication conflicts during S-phase are a major source of R-loop-induced damage in proliferating cells (160). Notably, CD93, a surface marker that regulates LSC self-renewal and identifies cycling LSCs in the MLL-AF9 model, is a direct PIWIL4 target (161).

Doxorubicin (DOX), recognized as the standard chemotherapy agent for soft tissue sarcoma, was first introduced in the 1970s and has consistently ranked among the most effective drugs for treating this condition (162). This is an anthracycline medication that inhibits DNA replication by blocking the action of topoisomerase II after intercalating into DNA (163). Multiple mechanisms may function together to induce resistance against DOX. Typical molecular alterations driving DOX resistance include decreased drug uptake, metabolic changes affecting the drug, elevated efflux, enhanced DNA damage repair, inhibition of programmed cell death, and disruption of redox equilibrium (164, 165). Alongside reduced DNA damage and increased DNA repair capacity, the failure of therapeutic agents to accumulate within malignant cells is a major contributor to drug resistance, potentially leading to treatment failure. Diminished drug import and increased drug export, both of which lower intracellular drug levels, are well-documented processes in the context of combating chemoresistance. In addition, researchers have proposed that faster drug metabolism may be one way cells reduce the amount of drug that builds up inside them. This, in turn, lowers chemoresistance (166). Studies on how DOX is broken down in the body have shown that when drug metabolism reduces the amount of DOX inside cells, it greatly reduces the treatment's effectiveness (167).

A current study revealed a notable increase in the cytochrome P450 enzyme, cytochrome P450 1A2 (CYP1A2), in DOX-resistant HT1080 cells (168). CYP1A2, a member of the CYP1 family within the cytochrome P450 superfamily, is a crucial enzyme involved in drug metabolism (169).

It was shown that increased levels of piR-39980 in HT1080 cells suppressed CYP1A2 (168). Previous studies

have reported that piR-39980 is expressed at lower levels in HT1080 fibrosarcoma cells than in IMR90 normal fibroblast cells. This piRNA was found to act as a tumor suppressor by inhibiting proliferation and migration while also promoting apoptotic cell death (170).

Transposable elements (TEs), or transposons, are mobile genetic sequences that constitute roughly 45% of the human genome. While they have been predominantly investigated in germline cells and are generally regarded as dormant in differentiated somatic epithelial tissues, their reactivation can introduce regulatory or protein-coding changes upon genomic reintegration, thereby contributing to various diseases (171, 172). Moreover, recent evidence links increased TE activity to genomic instability, oncogene expression, and elevated mutation rates in roughly 50% of tumors (173). Known germline regulatory mechanisms of TEs include DNA methylation and histone modifications at LINE-1 loci, as well as small RNA-mediated interference via the PIWI pathway (174, 175).

Multiple studies have reported PIWI protein overexpression in somatic tumors, where they act as oncogenes, consistent with the broader re-expression of stem cell and germline markers in cancer. However, other research has shown that PIWI proteins can also function as tumor suppressors, revealing a lack of consensus on their role in somatic tissues and tumors (176, 177). This ambiguity is particularly evident for PIWIL2, the principal endoribonuclease that collaborates with primary piRNAs within the piRISC complex to target and degrade TE RNA transcripts such as LINE-1 (178).

piRNAs as potential diagnostic/prognostic Biomarker and therapeutic target

A reliable screening biomarker should be capable of identifying cancer in its early stages to lower morbidity rates and improve survival rates. Studies have shown that irregular piRNA expression is associated with various traits in cancer patients (179). As initial products of multiple regulatory and signaling pathways, piRNAs could theoretically play a crucial role in the early detection and management of cancers (9, 75, 180). Because piRNAs are expressed only in certain tissues, they are considered promising biomarkers for diagnosing cancer and predicting its course from tissue samples (107).

Additionally, piRNAs are noninvasive biomarkers that can be detected in exosomes or as free circulating entities in serum, plasma, saliva, and stool. Techniques such as microarray, sequencing, and quantitative real-time PCR (qRT-PCR) can be employed to detect piRNAs (73, 181). Variations in circulating piRNA expression levels may serve as valuable cancer biomarkers, exhibiting higher specificity and sensitivity than circulating miRNAs and lncRNA-based biomarkers (182).

Elevated piRNA-823 expression is linked to survival outcomes in cancer patients (183, 184). In MM, G-MDSCs promote cancer stemness by activating piRNA-823, thereby increasing DNA methylation and enhancing tumorigenic potential (185). Mechanistically, piRNA-823 upregulates DNMTs, causing hypermethylation of tumor suppressor promoters such as APC and activating Wnt signaling, a mechanism conserved across multiple cancers (114). In RCC, loss of piRNA-823 correlates with genomic instability, and patients exhibit higher serum piRNA-823 levels than healthy individuals, a finding associated with adverse

clinical outcomes (183, 184). Taghizadeh *et al.* identified distinct piRNA signatures in RCC and CRC and proposed a piRNA-823 diagnostic kit for prognosis and patient monitoring (183).

These small RNAs cross cell membranes without degradation in circulation and remain stable in serum and plasma despite repeated freeze-thaw cycles or prolonged exposure at room temperature (186, 187). This stability underscores the need to identify novel piRNA biomarkers for early cancer diagnosis and to validate them in larger, independent cohort studies and extended clinical trials. Integrating circulating miRNAs and piRNAs may thus improve diagnostic specificity and sensitivity, offering new strategies for developing targeted panels for early detection and prognosis.

In addition siRNA or miRNA pathways, the piRNA synthesis pathway does not require Dicer or related dsRNA-processing enzymes, making synthetic piRNAs with improved target specificity advantageous (75). Nonetheless, the efficacy of piRNAs as therapeutic targets depends on effective *in vivo* delivery methods, and comprehensive studies must be conducted before any therapeutic application of piRNAs can be considered (188).

Emerging evidence supports the potential utility of circulating piRNA signatures as diagnostic and prognostic biomarkers for colorectal cancer. A five-piRNA panel comprising piR-001311, piR-004153, piR-017723, piR-017724, and piR-020365, termed Panel I, demonstrated markedly superior detection capability compared to the conventional CEA-based Panel II (189).

Recent findings suggest that the piRNAs hsa-piR-23246, hsa-piR-32858, and hsa-piR-9137 may serve as potential biomarkers for distinguishing pancreatic malignancy (190). Another study demonstrated for the first time that urinary extracellular vesicle piRNAs, specifically novel_pir349843, novel_pir382289, novel_pir158533, and hsa_piR_002468, are upregulated in prostate cancer (PCa) patients, highlighting their potential as promising diagnostic biomarkers (191). According to research, piR-13643 and piR-21238 were markedly upregulated in human papillary thyroid carcinoma. piRNAs may function as potential new biomarkers for the precise identification of papillary thyroid carcinoma (192). Scientists concluded that serum piR-54265 could potentially act as an essential biomarker for CRC screening, facilitating early detection and ongoing clinical monitoring (193).

PIWI and glioblastoma

GB represents the most aggressive form of glial malignancy and is associated with a dismal prognosis (194, 195). GB is a tumor of astrocytic lineage that is believed to arise from neural stem cells or glial progenitor cells within the nervous system (196, 197). Two distinct types of GB can be identified based on their genetic pathways, including primary GB, which arises *de novo*, and secondary GB, which develops from a pre-existing low-grade glioma. Primary GB is more prevalent among older people, while secondary GB is typically observed in younger individuals (198). Key prognostic factors include the patient's age at diagnosis, tumor size and location, Karnofsky performance status, and the tumor's genetic characteristics (199). Currently, the standard way to treat GB is to remove as much of the tumor as possible through surgery. After that, patients receive radiotherapy together with chemotherapy using a

drug called temozolomide (TMZ) (200). Despite advances in general oncological care over the past two decades, the recommended treatment for GB has remained largely unchanged, and overall survival rates have not significantly improved. Consequently, considerable efforts are underway to deepen the understanding of GB biology and identify novel predictive biomarkers and therapeutic targets.

PIWIL1, also known as HIWI, is a human PIWI family member that is crucial for stem cell self-renewal and division (201). This gene has been identified as an oncogene across multiple tumor types, including endometrial, breast, lung, and GB cancers (202). Numerous studies have indicated that PIWIL1 significantly contributes to tumor aggressiveness, including proliferation and invasion, and its expression may serve as a prognostic marker for poor outcomes in hepatocellular carcinoma, gastric cancer, colorectal cancer, and GB (203). The reduction of PIWIL1 expression in human lung and gastric cancer stem cells leads to inhibited cell proliferation, which is attributed to cell cycle arrest and apoptosis, alongside changes in key molecular markers related to proliferation, such as p21 and Cyclin D1, as well as apoptosis markers like Bax and Bcl-2 (203). Therefore, it is evident that PIWIL1 is integral to cell proliferation and the progression of various cancers.

PIWI proteins are critical for preserving germline stem cell stemness. In glioblastoma, all four human PIWIL proteins (PIWIL1–PIWIL4) are dysregulated (17), with PIWIL1 mRNA and protein markedly overexpressed; the latter is detected in 88% of tumor samples. PIWIL1 supports GSC self-renewal and tumorigenic capacity, preferentially localizing to Sox2-, Olig2-, and Nestin-positive GSCs within the perivascular niche (204). A conserved function for PIWIL1 in somatic stem cell maintenance beyond GB has recently been demonstrated in colorectal cancer, where it localizes to centrosomes during mitosis and diminishes upon differentiation, suggesting PIWIL1 may act as a broad stemness factor across malignancies (205). Knockdown of PIWIL1 impaired GSC self-renewal and extended survival in GSC-derived mouse models, with PIWIL1 shown to orchestrate the expression of multiple genes governing differentiation and cell-cycle progression (204, 206).

Furthermore, silencing PIWIL1 decreased Olig2 expression, whereas Olig2 overexpression rescued the phenotype induced by PIWIL1 knockdown. These results suggest that PIWIL1 helps maintain stemness, at least partly, by interacting with Olig2 (206). Additionally, silencing PIWIL1 decreased BTG2 expression, a factor whose loss is known to facilitate tumorigenesis in conditions such as oligodendroglioma, medulloblastoma, and leukemia by influencing differentiation (207–211). In mouse models of GB, tumors with silenced PIWIL1 showed elevated BTG2 expression, reinforcing the notion that PIWIL1 is a pivotal regulator of the undifferentiated state of GSCs (206).

In GSCs, PIWIL1 regulates the G1/S transition in part through transcriptional modulation of CDKN1B, CCND2, FBXW7, and its downstream target c-Myc, indicating that PIWI-like proteins affect distinct cell cycle components across different cancers (206). Additionally, PIWIL1 depletion up-regulated MCL1 and triggered cellular senescence. Collectively, PIWIL1 governs a network of key regulators involved in cell-cycle progression, apoptosis, senescence, proliferation, and stemness, thereby promoting GSC expansion and maintenance (206).

In GB, PIWIL1 inhibition diminished GSC self-

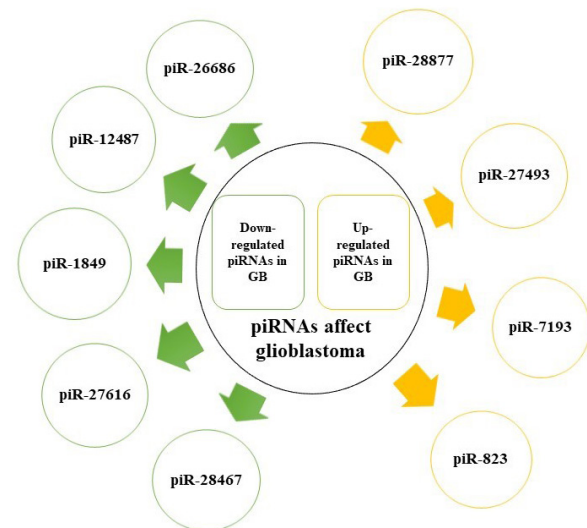


Figure 5. piRNAs/PIWI complex affect glioblastoma (GB)

renewal capacity, leading to senescence or apoptosis. This phenomenon contributed to reduced tumor proliferation and extended survival in murine models derived from GSCs. In mouse embryos, PIWIL1 localized to specific brain regions, suggesting its involvement in the development of newborn neurons and neocortico-genesis. Nevertheless, PIWIL1 knockout mice remain viable and do not display significant neurological impairments (206). Consequently, targeting PIWIL1 in patients with GB could enhance tumor management while minimizing neurological complications.

The dysregulation of short non-coding RNAs has been identified as a significant factor in the progression of various tumors. Furthermore, sncRNAs play a vital role in numerous cellular functions; hence, any disruption in their regulation is a pivotal event in cancer development. A specific category of sncRNAs-piRNAs, along with their corresponding PIWI proteins, has been noted for their abnormal expression in several cancers, including GB (17). This dysregulation is frequently associated with worse prognosis, suggesting its functional involvement in cancer development and highlighting its promise as a diagnostic or prognostic biomarker and potential therapeutic target (212). Most tumors arise from epigenetic alterations, accumulated mutations, and genomic instability. The piRNA/PIWI pathway is a key regulator of genome stability. PIWIL3 expression correlates directly with GB aggressiveness, affecting tumor growth and metastasis, and its down-regulation has been shown to suppress gastric cancer progression via the JAK2/STAT3 pathway (213).

Figure 5 presents a list of piRNAs/PIWI complexes that could affect GBM. piR-30188 functions as a tumor suppressor by regulating miR-367-3p, while up-regulation of the typically down-regulated piR-8041 in GB suppresses tumor growth through MAPK signaling and modulation of heat shock proteins (84, 214).

piRNAs as Biomarkers and Therapeutic Targets in Glioblastoma

Through a multi-cohort small RNA-sequencing and validation strategy, a study identifies 58 deregulated piRNAs in GB, confirms four candidates (piR-1849, -9491, -12487, -12488), demonstrates that piR-9491 and -12488 impede clonogenicity, and establishes piR-23231 as a prognostic

marker for patients on the Stupp regimen, collectively positioning piRNAs as promising diagnostic, prognostic, and functional players in gliomagenesis (17). For instance, a study reported piR-28877 (DQ598677) as up-regulated, while piR-28467 (DQ598252) and piR-27616 (DQ597341) were noted as down-regulated in breast cancer, aligning with findings in GB (215). In addition, another study carried out a detailed analysis of piRNAs in breast cancer. The researchers found that piR-27493 (DQ597218) and piR-7193 (DQ576872) were increased. This finding aligns with observations in GB (216). Similar patterns of dysregulation among these piRNAs may indicate conserved cellular functions across diverse cell types (217).

Furthermore, another study successfully confirmed that piR-1849, piR-9491, piR-12487, and piR-12488 are significantly down-regulated in GB. In the context of GB, piR-823 was up-regulated, consistent with MM, suggesting potential implications (17). Notably, piR-1849, a molecule of particular interest, has been identified as encapsulated within extracellular vesicles released from GB cells, suggesting its potential as a biomarker for the disease (218). In the previously mentioned study on piRNAs and prognosis, the data were analyzed to find correlations between piRNA expression patterns and overall survival in GB patients. Kaplan-Meier analyses revealed that only piR-23231 (DQ592953) showed a significant association with prognosis ($P=0.0071$), with lower levels linked to worse outcomes. This matched earlier next-generation sequencing (NGS) results, which indicated that piR-23231 was significantly down-regulated in GB and may contribute to disease aggressiveness and lower survival rates. However, this requires validation through *in vitro* and *in vivo* studies. Cox regression analyses were also performed to evaluate the prognostic potential of piR-23231. Ultimately, piR-23231 expression levels correlated with overall survival, suggesting that certain piRNAs could serve as prognostic biomarkers in GB (17).

Among the piRNAs tested in GB, restoring expression of piR-8041, which is markedly down-regulated in tumors relative to normal brain, produced the strongest anti-proliferative effect, while sparing normal human astrocytes. These findings indicate that the antitumor activity is influenced not solely by tumor specificity but also by the degree of piRNA underexpression, potentially reflecting variability in piRNA targets, their accessibility or abundance, and PIWI protein levels across different cell types. In an intracranial xenograft model, piR-8041 administered before tumor implantation significantly suppressed tumor growth for approximately 2 weeks compared with controls, after which tumor progression resumed. This indicates that sustained treatment will be required to maintain tumor-suppressive piR-8041 levels, which clinically will depend on drug-delivery systems capable of crossing the blood-brain barrier for effective tumor-site delivery (84) (Figure 3).

Another study found 625 differentially expressed piRNAs and 335 DE miRNAs in GB, which may influence target gene expression and contribute to malignancy. Both miRNAs and piRNAs targeted 128 genes, of which 9 were significantly associated with cancer-related pathways and diseases. In contrast, 399 targets were exclusively targeted by miRNAs, and only 6 were exclusively targeted by piRNAs. Among the shared targets, pathway analysis and existing literature indicate that HRH1 is involved in GPCR signaling and atxn3 in Akt signaling. Furthermore, braf (targeted

only by piRNAs) and ccnd1 (targeted only by miRNAs) are also enriched in the GPCR and Akt pathways (219). Based on these observations, this study suggests that when both miRNAs and piRNAs are reduced in GB, HRH1 levels go up. HRH1 then interacts with Gas/GTP proteins. This interaction turns on BRAF through a signaling pathway involving cyclic adenosine monophosphate (cAMP). Subsequently, BRAF activates ERK, which in turn up-regulates CCND1 due to the failure of miR-365-3p to bind its 3' UTR and regulate its expression (219, 220). Similarly, atxn3 overexpression may result from the loss of small RNA binding to its target regions. In summary, dysregulation of these miRNAs and piRNAs disrupts target gene expression, affecting pathways such as GPCR and Akt signaling, thereby promoting GB cell proliferation (219).

In U251 MG glioblastoma cells, transient transfection was used to overexpress selected piRNAs, and successful up-regulation was verified by qRT-PCR. Functional assessment demonstrated that enforced expression of piR-hsa-9491 and piR-hsa-12488 significantly suppressed the colony-forming potential of GB cells. These observations indicate that the two piRNAs can influence cellular behavior, affirming their identity as biologically active entities rather than nonfunctional byproducts of RNA turnover (17).

Unlike LV-hsa_piR_014637, the piRNA LV-has_piR_011186 appears to mediate DNA and histone H3 methylation within the CDKN2B promoter, thereby regulating CDKN2B expression and ultimately promoting cell proliferation while suppressing apoptosis. The proposed mechanism involves piRNA binding to a complementary sequence, thereby recruiting DNA- and histone-methylating proteins to the CDKN2B locus. Consistently, a piRNA complex containing PIWI, HP1a, and Su(var)3-9 proteins capable of catalyzing covalent histone modifications has been reported, similar to complexes identified in U937 cells. In summary, LV-has_piR_011186, rather than LV-hsa_piR_014637, plays a key role in directing DNA and histone H3 methylation at the CDKN2B promoter, thereby significantly impacting CDKN2B expression and driving proliferation while inhibiting apoptosis, likely

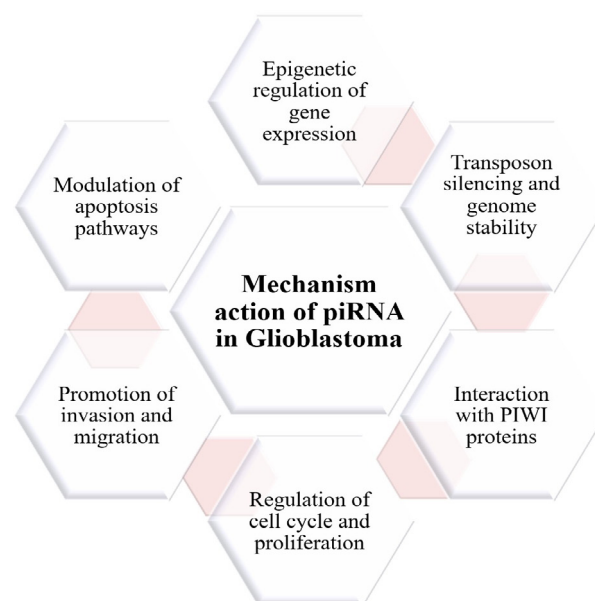


Figure 6. Mechanism of action of piRNA in Glioblastoma (GB)

through sequence-specific piRNA binding and subsequent recruitment of methylating proteins (Figure 6) (221).

The role of artificial intelligence and machine learning (ML) in advancing piRNA research

Numerous research articles have been released that utilize ML techniques for diagnostics, employing small non-coding RNAs as biomarkers. For instance, Kang *et al.* applied ML approaches to investigate miRNA–disease correlations across three cancer types. They established a set of descriptors to facilitate disease classification (222). Similarly, Xu *et al.* leveraged target genes and pathways to develop ML models for diagnosing Alzheimer's disease (223). The application of ML to identify biomarkers for various diseases using small non-coding RNAs has seen significant interest in recent years (224). In recent research, scientists employed comparable methodologies for CRC diagnostics using piRNAs (224).

Moreover, another study investigated the prediction of CRC using ML techniques and specific biomarkers to assist in diagnosing future patients (224). Their data were collected from a study that identified potential biomarkers for CRC using qRT-PCR. The investigators isolated piRNAs from mucus, saliva, blood, and/or tissue specimens obtained from patients. Using this information, they developed a machine learning (ML) model for CRC diagnosis. When this ML model was applied to previously unseen data sharing the same descriptor features employed during training of the piRNA dataset, it enabled estimation of a patient's probability of having CRC. It facilitated recommendations for subsequent diagnostic evaluation (224). They proposed a descriptor system that employs piRNA parameters and sequence descriptors to develop ML models for colorectal cancer. Each ML model achieved an accuracy exceeding 90%, and most models classified independent datasets with over 70% accuracy.

Additionally, data unrelated to CRC yielded significantly lower accuracies, indicating that their model can be highly selective in elucidating CRC. With this evidence, they have developed an ML model to explore the correlation between piRNA and CRC. The findings suggest that this model can serve as an effective tool for diagnosing CRC (224).

The significance of piRNA in cancer diagnosis has garnered increasing attention in the research community. Understanding the complex relationship between piRNAs and cancer may provide the foundation for innovative diagnostic strategies, enabling earlier and more accurate detection of malignancies (107, 225). In an investigation, a methodology was introduced to predict the correlation between piRNAs and breast cancer (226). They developed comprehensive descriptors that encapsulate piRNA sequence data and employed ML techniques to model and forecast breast cancer occurrences. Their models achieved high accuracy, demonstrating the robust predictive power of the piRNA descriptors for breast cancer. They proposed a groundbreaking piRNA descriptor system derived from piRNA sequences to train ML models for breast cancer prediction. Initially, they trained and evaluated a logistic regression model, which successfully classified breast cancer cases with an accuracy exceeding 90%. Subsequently, they implemented additional classifiers, including Random Forest, LMT, and SMO, all of which yielded accuracies surpassing 80%. SHAP analysis was conducted to pinpoint the five most pertinent descriptors for predicting breast

cancer, validate their models across independent datasets, and demonstrate their proficiency in identifying piRNAs with an accuracy greater than 85% (226). Furthermore, additional analyses to assess the specificity of these models were performed, revealing that they exhibit heightened sensitivity to breast cancer compared to two other cancer types. Lastly, the predictive capabilities of advanced ML algorithms were investigated, and it was noted that these sophisticated models did not show significant improvements, underscoring the need to customize algorithms to the dataset's characteristics (226).

These findings suggest that this methodology is robust and indicate that piRNA-based ML models may be a significant asset for enhancing breast cancer diagnostics in the future. In contrast to conventional diagnostic techniques, such as imaging and pathological analysis, the piRNA-based ML strategy does not require invasive procedures or specialized equipment. It presents the potential to create innovative, non-invasive, and data-driven alternatives. Furthermore, this approach demonstrates potential for consistent, accurate diagnosis while minimizing the variability in interpretation associated with traditional methods (226).

Future perspectives

The future of piRNA research in GB holds significant promise for transforming diagnostic and therapeutic paradigms. As high-throughput sequencing and single-cell technologies advance, comprehensive piRNA expression atlases specific to GB subtypes and tumor microenvironments will become feasible, enabling the discovery of highly specific diagnostic and prognostic biomarkers. Therapeutically, piRNAs' extracellular stability makes them attractive candidates for non-invasive liquid biopsies. Furthermore, exploiting the endogenous RNA interference pathway by developing synthetic piRNA mimics or antisense oligonucleotides (piRNA-targeting ASOs) could allow precise targeting of oncogenic networks or the reactivation of tumor-suppressive pathways that are currently undruggable. Looking ahead, integrating piRNA signatures with existing genetic, epigenetic, and imaging data using artificial intelligence could yield powerful predictive models for patient stratification, treatment response monitoring, and personalized therapy selection, ultimately advancing a more precise oncology framework for GB management.

Challenges

Despite the exciting potential, several formidable challenges must be overcome to translate piRNA biology into clinical applications for GB. A primary hurdle is the incomplete functional annotation of the vast majority of piRNAs; their mechanisms of action in cancer cells, particularly within the complex GB tumor ecosystem involving glioma stem cells and immune interactions, remain largely elusive. Technical challenges also persist, including the need for standardized, cost-effective detection methods for low-abundance piRNAs in clinical samples and the difficulty of distinguishing true signals from noise in sequencing data. From a therapeutic development standpoint, achieving efficient, specific, and safe *in vivo* delivery of piRNA-based agents to infiltrative GB cells across the blood-brain barrier presents a major obstacle. Finally, the significant heterogeneity of GB, both between patients and within a single tumor, means that single piRNA

biomarkers or therapies may have limited applicability, necessitating combinatorial approaches and rigorous validation in large, diverse clinical cohorts.

Conclusion

PiRNAs are a class of sncRNA molecules that have recently been found to be relevant to the biology of cancer. The piRNA/PIWI complex can affect a variety of malignancies, including gliomas, through pathways such as migration, proliferation, cell cycle arrest, immunity, and epigenetic alterations. Furthermore, piRNAs are likely essential for the early detection and management of various cancers, and their tissue-specific expression patterns make them strong candidates as diagnostic and prognostic biomarkers in oncology.

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Authors' Contributions

A N, A O, F AM, E GH, and F D drafted the manuscript; A N and F D prepared the figures; M JN and A G critically revised and edited the article, conceived the study design, and the final version of the article has been approved by all authors.

Conflicts of Interest

The authors declare no competing interests.

Declaration

We would like to disclose that the authors used ChatGPT (OpenAI) for language editing purposes. This included grammar correction, sentence rephrasing, and improving overall readability. No AI tool was used for generating scientific content. All AI-assisted text was carefully reviewed, verified, and edited by the authors

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