tRNA-derived RNAs in hepatic diseases: From biological functions to clinical translation (Review)

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Abstract. In recent years, with the deepening understanding of the significant role of small non-coding RNAs in human health and diseases, transfer RNA-derived RNAs (tsRNAs) have gradually become a research focus. Existing evidence shows that tsRNAs not only participate in the regulation of liver gene expression, translation and stress response, but may also act as signaling molecules to mediate inter-organ communication. However, the specific pathological mechanism and clinical value in the occurrence and development of liver diseases remain unclear. Although tsRNAs show promising prospects as non-invasive biomarkers and are expected to be used in the diagnosis of liver diseases, screening of therapeutic targets, drug delivery and regulation of enzyme activity, the methodological limitations at present and the differences in performance among different disease types and populations have significantly restricted their clinical translation. The present article systematically reviews the pathological roles of tsRNAs in various liver diseases, with a focus on elaborating their roles in tumor evolution, metabolic disorders and immune regulation. Their clinical application potential was also evaluated, methodological challenges were discussed, and future directions were proposed to advance tsRNA-based clinical translation in hepatology.

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Contents

1. Introduction

Hepatic diseases, along with their complications such as cirrhosis and liver cancer, contribute significantly to mortality, morbidity and healthcare costs. According to data released by the World Health Organization, the global burden of hepatic diseases is substantial and continues to increase (1). Each year, hepatic disease contributes to ~2 million deaths, accounting for 4% of total mortality. Most deaths are attributable to cirrhosis or hepatocellular carcinoma (HCC), whereas acute hepatitis accounts for a smaller fraction. Globally, cirrhosis most frequently arises from viral hepatitis, alcohol abuse and non-alcoholic fatty liver disease (NAFLD) (2). However, addressing liver diseases is fraught with challenges because of the shortage of specific diagnostic markers and effective therapeutic targets, which result in inadequate access to diagnostic investigations and treatments (3). Although recognized biomarkers such as galectin-3 have been proposed as diagnostic and therapeutic targets for liver diseases, their sensitivity and specificity in early detection are limited, highlighting the necessity of developing novel molecular indicators to address this urgent need (4). Therefore, identifying more specific markers is essential to enhance the diagnosis and treatment of hepatic diseases.

Among the various influencing factors, transfer RNA (tRNA)-derived small RNA (tsRNA) plays a vital role in the pathogenesis of hepatic diseases. The existence of tRNA in all living organisms has established its status as one of the most

necessary and classic RNA types in nature (5). However, its functions are not limited to classical roles, existing research shows that tRNA can perform multiple non-classical functions either by itself or in the form of small fragments produced by specific cleavage (6). tsRNA generated from specific cleavage sites of tRNA/precursor tRNA is gradually growing into an important active category among small non-coding RNAs (sncRNAs) (7). With the increasing diversity of non-coding RNA types, machine learning-based classification tools that utilize intrinsic sequence features have emerged to facilitate transcriptome annotation across various species (8). A series of evidence no longer regards tsRNA as the product of tRNA degradation. They not only participate in transcription, translation and post-transcriptional regulation, but also widely influence cell type specialization during development by regulating cell proliferation, growth and metabolism (9). At the pathological level, abnormalities of tsRNAs are associated with diseases such as diabetes (10), infections (11) and cancer (12). It is worth noting that epigenetic transcriptome modifications profoundly affect the function of non-coding RNA by regulating their processing, stability and subcellular localization (13). tsRNAs inherit a wide range of post-transcriptional modifications from their parent tRNA, including m^5C, m^1A, and pseudo-uridine. These modifications not only protect tRNA from nuclease cleavage but also determine the biogenesis, half-life and target binding ability of tsRNAs. Therefore, this regulatory layer adds a crucial dimension to our understanding of the gene regulation mediated by tsRNAs in liver diseases.

The present review conducts a comprehensive examination of tsRNAs, covering their discovery, classification, biogenesis, detection methods, biological functions, and their regulatory roles in the gut-liver axis. The biogenesis and molecular functions of tsRNAs in hepatocytes are the primary focus of the present review. Their potential for clinical application was also highlighted, specifically in diagnosing, prognosticating, and treating hepatic diseases. Finally, current obstacles in tsRNA research were examined and future directions were discussed, with particular emphasis on liver diseases.

2. Biology of tsRNAs

Classification and nomenclature of tsRNAs. tRNA-derived small RNAs (tsRNAs) are a newly identified class of functional sncRNAs (14). The biogenesis of these molecules involves site-specific cleavage of either precursor tRNAs (pre-tRNAs) or mature tRNAs (14,15). Pre-tRNAs, which are transcribed by RNA polymerase III, possess a 5' leader sequence and a 3' tail sequence. The 5' leader is excised by the ribonucleoprotein enzyme RNase P, while RNase Z cleaves the 3' tail at the first unpaired base adjacent to the 3' end of the tRNA (16). Following this cleavage, tRNA nucleotidyl transferase adds a non-templated cholangiocarcinoma (CCA) sequence to the 3' end of the mature tRNA (7,17). Typically, mature tRNAs are composed of 70-90 nucleotides (nt). They assume a cloverleaf-like secondary structure featuring four loops: the dihydrouracil (D-loop), anticodon loop, variable loop and pseudo-uridine (TψC) loop (18).

The cleavage sites within pre-tRNAs or mature tRNAs serve as the basis for classifying tsRNAs into two main categories. The first is tRNA-derived fragments (tRFs), which are 14-30 nt in length. The second is tRNA halves (30-50 nt), which are also called tRNA-derived stress-induced RNAs (tiRNAs) because they are generated in response to stress (7,19-22). Stress-induced tiRNAs arise from the selective cleavage of the anticodon loop within mature tRNAs. This cleavage is carried out by various endonucleases, including angiogenin (ANG) (21), RNase L in vertebrates, Rny1 in yeast, and other enzymes not yet identified (7). Typical stressors that trigger tiRNA production include thermal shock, hypoxia and viral infection (23). Based on their sequence composition, tiRNAs are subdivided into 5'-tiRNAs (spanning the 5' end to the anticodon, including the D-loop) and 3'-tiRNAs (extending from the anticodon to the 3' end, encompassing the TψC loop) (24).

tRFs are ncRNAs that arise from mature or pre-tRNAs, generally 14-30 nt in length. Depending on the position of the cleavage site on either pre-tRNA or tRNA, tdFs are classified into tRF-5, tRF-3, tRF-2, tRF-1, internal tRF (i-tRFs) and other tRFs (25). tRF-5 molecules start at the 5'-end of the tRNA and stop at either the D-loop or the anticodon loop, and are subsequently categorized according to length as tRF-5a (14-16 nt), tRF-5b (22-24 nt), or tRF-5c (28-30 nt). tRF-3 molecules, on the other hand, run from the 3'-end to the T loop of mature tRNAs and are further separated into tRF-3a and tRF-3b based on size (18 or 22 nt) (22,26). The tRF-2 subclass is produced when enzymes cleave the anticodon loop, leaving the 5' and 3' terminal regions intact while preserving both the anticodon loop and part of the anticodon stem (27). The tRF-1 subclass arises from cleavage of the pre-tRNA's 3' terminus by RNase Z or ELAC2. Pre-tRNAs carry a 3' tail composed of a poly-U sequence, which corresponds to the RNA polymerase III termination signal (28). The i-tRFs, previously referred to as tsRNA-2s, represent a heterogeneous collection of tsRNAs that originate from the anticodon stem-loop region of mature tRNAs (29). Furthermore, i-tRFs can be classified into A-tRF, D-tRF, and V-tRF according to their cleavage initiation sites (15). Minor unannotated subgroups of tsRNAs have also been reported, but their biological roles and classification remain to be further clarified (30) (Fig. 1).

Early studies named these RNA fragments according to their position of origin, including 5'-tRF, 3'-tRF, 5'-tRNA half, and 3'-tRNA halves. In the present review, a clear grouping method was used that assigns fragments to five classes (tRF-5, tRF-3, tRF-2, tRF-1 and i-tRF) by where the RNA is cleaved. Different databases use different naming systems. For example, tRFdb uses names like tRF-3001b. MINTbase uses names like tRF-23-R9J89O9N9. tsRBase uses names such as hsa_tsr014055. However, functional studies often name fragments based on the parent tRNA. Examples are tRF-Gln-CTG-026 and 5'-tsRNA-Gly-GCC. As a result, one fragment can have multiple names. tRF-34, for instance, is the same fragment as 5'-tsRNA-Gly-GCC. i-tRFs have also been called tsRNA-2s. In the present review, tsRNAs are the authors' general term for all tRNA-derived small RNAs. It matches the tsRNAs used by some authors. When individual fragments were discussed, the name from the original study was used and its group was marked from the authors' scheme when it was first named (23,27).

Biogenesis and regulation of tsRNAs. Specific nucleases orchestrate the tightly regulated biogenesis of tsRNAs, while

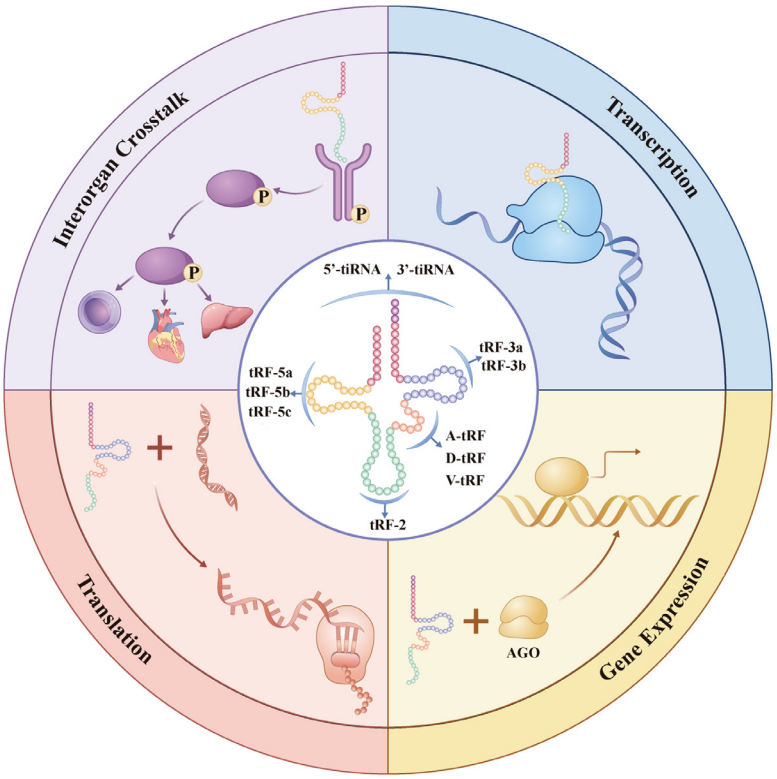

Figure 1. Overall review of tsRNAs classification and functional landscape. An overall summary of tsRNA biology is provided. One of the small non-coding RNAs owing to tRNA cleavage is called the tsRNA can be observed in the center of the picture. It is further divided into its subtypes based on cleavage. There are a few kinds of tRNA half which onsist of 5'-tiRNAs and 3'-tiRNAs. There are also tRNA-derived fragments. They include tRF-5a/b/c, tRF-3a/b, A/D/V-tRFs and tRF-2. Surrounding this core classification are five functional modules that capture the main biological roles of tsRNAs, spanning transcription, gene regulation, translation, signaling and clinical application. tRF, tRNA-derived fragments.

tRNA modifications further modulate this process. It takes place under conditions of physiological homeostasis as well as in response to stress stimuli, such as oxidative stress, heat shock, or nutrient deprivation (31-33). Mature tRNAs or precursor tRNAs (pre-tRNAs) serve as primary substrates, with cleavage events generating distinct tsRNA subtypes based on nuclease specificity and cleavage sites. In the process of tiRNA generation, angiopoietin (ANG) plays a key role as a ribonuclease in response to stress. Under physiological conditions, ANG binds to ribonuclease inhibitor 1 and is thus confined within the cell nucleus. After the activation of the stress signal, ANG translocates from the nucleus to the cytoplasm, where it preferentially cleaves the anticodon loop region of mature tRNA. It shows a significant preference for the purine-pyrimidine dinucleotide site (cleaving efficiency is CpA > CpG > UpA > UpG in sequence). In addition, ANG can occasionally generate short-chain tRF-5s (34-36).

In addition to its well-known role in miRNA processing, Dicer is also involved in generating shorter (14-30 nt) tRNA-derived fragments. By cleaving specific structural loops on the pre-tRNA precursors, Dicer can generate subtypes such as tRF-5a, tRF-5b, tRF-3a and tRF-3b. However, the degree of dependence of different tRF types on Dicer varies, and the generation of some tRFs does not require Dicer, which reveals the complexity of the related pathways (37,38). By contrast, the processing of the precursor tRNA is specifically carried out by RNase Z/ELAC2, which removes the 3' trailer sequence containing the RNA polymerase III-dependent poly (U) termination signal, generating tRF-1s during the classical tRNA maturation process (39,40). In lower eukaryotes such as yeast, members of the RNase T2 family (such as Rny1) can cleave the anticodon loop of mature tRNA under oxidative stress conditions to produce tiRNAs in an ANG-independent manner, thereby complementing the function of ANG (33).

Crucially, tRNA modifications play a central role by regulating the production process of tsRNA. Methyltransferases such as NSun2 and DNMT2 can catalyze cytosine-5 methylation (m5C) at specific positions of tRNA (for example, NSun2 acts on C48/49/50, and DNMT2 acts on C38). This modification can protect tRNA from nuclease cleavage, thereby inhibiting the excessive generation of tsRNA. Conversely, once these modifications are absent, the sensitivity of tRNA to ANG or Dicer increases accordingly, which in turn leads to abnormal accumulation of tiRNA or tRF (41-43). This indicates that the nuclease-driven cleavage event and the modifiers-mediated regulatory mechanism jointly determine the compositional characteristics of tsRNA, leading to the generation of subtypes with distinct functions that participate in various downstream cellular processes.

Distinctions and crosstalk with other non-coding RNAs. With the continuous improvement of next-generation sequencing technology, an increasing number of small RNA types with unique functions have been identified, among which tRNA-derived fragments have become one of the core research fields (44). Although tsRNAs share certain common features with other sncRNAs, including microRNAs (miRNAs or miRs), they are unique in their biogenic pathways and regulatory

mechanisms, thus standing out among the non-coding RNA family. Despite being of similar size and both being involved in post-transcriptional regulation, their generation pathways and functional mechanisms are significantly different. miRNAs are usually transcribed from independent genes and processed by Drosha and Dicer. tsRNAs are produced by cleavage from precursor or mature tRNAs by enzymes such as ANG or Dicer under specific conditions, particularly stress (36,45). At the functional level, miRNAs mainly bind to Ago2 in the Argonaute (Ago) protein family and inhibit target mRNA through complementary seed sequence pairing. By contrast, tsRNAs tend to bind to Ago1, Ago3 and Ago4, and can regulate gene expression through non-classical pathways, such as replacing the RNA-binding protein YBX1 or regulating translation initiation factors (24,46). Previous studies have suggested that there is crosstalk between two types of molecules: tsRNAs can compete with miRNAs for binding to Ago proteins, thereby affecting the silencing efficiency mediated by miRNAs, but it does not significantly change the abundance of miRNAs (47). These differences highlight the independent yet associated roles of tsRNAs and miRNAs in the ncRNA regulatory network.

In addition to miRNAs, tsRNAs can also functionally interact with various types of non-coding RNAs, including PIWI-interacting RNAs (piRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). These RNAs each have their own characteristics in biosynthesis and regulatory mechanisms (48). Among them, piRNAs are 23-32 nt in length and are mainly expressed in germline cells, exerting transposon silencing and genomic stability maintenance functions through the PIWI protein family (49,50). PiRNAs are processed from long single-stranded precursors, and their 3' ends undergo 2'-O-methylation modification catalyzed by HENMT1. This modification not only enhances their stability but also facilitates PIWI loading. By contrast, tsRNAs are derived from tRNA and are often induced to be generated under cellular stress conditions (51,52). Although both tsRNAs and piRNAs can bind to Ago family proteins (the former binds to AGO and the latter to PIWI), their functional pathways are largely different. Specifically, piRNAs mainly mediate transposon silencing at the transcriptional and post-transcriptional levels within the germline. By contrast, tsRNAs focus more on translation regulation and stress response in somatic cells (53). At present, research on the direct mechanistic crosstalk between the two remains insufficient. However, in specific physiological scenarios (such as sperm maturation and intergenerational inheritance), the two may converge through sharing RNA-binding proteins or epigenetic regulatory networks (43,54).

lncRNAs are defined as transcripts longer than 200 nt. This type of RNA can regulate gene expression through multiple pathways, such as participating in chromatin remodeling, causing transcriptional interference, and functioning as a molecular scaffold (55). In addition, certain lncRNAs can also act as competitive endogenous RNAs, isolating miRNAs by binding to them, thereby altering the pool of available miRNAs within cells and indirectly influencing the regulatory network mediated by tsRNA (56). There is already evidence suggesting that tsRNA may also reciprocally regulate the stability or function of lncRNA through direct base-pairing or competing with RNA-binding proteins. However, these mechanisms still need to be further clarified (57).

CircRNAs owe their high stability and their capacity to act as miRNA sponges, protein baits, or translation templates to their covalently closed loop configuration. Previous studies have confirmed that some circRNAs can interact with tsRNAs (58). For instance, circRNA-104075 can adsorb tsRNA-0009, thereby weakening the inhibitory effect of TsRNA-0009 on YBX1 and accelerating the progression of HCC (59). In addition, due to the lack of free terminals, circRNAs may tolerate exonuclease degradation and may affect the half-life or subcellular localization of tsRNA in specific cellular environments (60). However, the crosstalk mechanisms among tsRNA-piRNA, tsRNA-lncRNA, and tsRNA-circRNA have largely remained unexplored. In the future, integrating multi-omics methods to map these interaction networks and assess their physiological correlations in development and disease is an urgent task in this field.

3. Molecular functions of tsRNAs in hepatocytes

tsRNAs are widely involved in various physiological processes such as mRNA silencing, intercellular communication, gene expression regulation, post-transcriptional modification, and the balance between cell proliferation and death (61). Among the numerous molecular events regulated by tsRNAs, translation control is the most thoroughly studied process. A large amount of evidence indicates that under various stimulating conditions, tsRNAs mainly inhibit protein synthesis through different mechanisms (26). They can bind to ribosomes to regulate the translation process. For instance, tsRNA-mediated suppression of tiRNA-5s downregulates mitochondrial pyruvate carrier 1 by inhibiting translation, thereby weakening mitochondrial respiration (62). The 5' tRNA halves derived from tRNA-Pro specifically bind to 18S rRNA and then directly bind to 80S ribosomes to suppress translation (63). Furthermore, the ψ-mTOG, a tsRNA modified by pseudo-uridine, can bind to PABPC1, hindering the recruitment of PAIP1 and thereby selectively inhibiting the translation of mRNAs containing the 5' PES motif. After the deletion of this tsRNA, global translation is enhanced, cell differentiation is abnormal, and the long-term reconstruction function is defective in HSPCs (64). Another study has shown that tsRNA-1 (tRF-Gln-CTG-026) from tRNA-Gln-CTG inhibits global protein synthesis in mice, likely by weakening the interaction between the pre-rRNA processing protein TSR1 and the pre-40S ribosomal subunit (65). Notably, tsRNAs can also enhance translation, indicating that they have functional diversity in translation regulation. For example, a 22-nt tsRNA-3 originating from tRNA-Leu-CAG was shown to bind mRNAs that encode ribosomal proteins, including RPS28 and RPS15, thereby promoting their translation and subsequently increasing ribosome biogenesis (66). Follow-up investigations further revealed that two specific 5'tRNA halves, namely 5'-tiRNA-Pro-CGG-1 and 5-tiRNA-Cys-GCA-27, enhanced cap-dependent translation in granulocyte-monocyte progenitors (67). However, the molecular bases of these effects were not investigated.

Apart from their functions in translation control, tsRNAs also participate in transcriptional and post-transcriptional gene

regulation. One investigation demonstrated that 5' $tRF^{Glu/CTC}$ and 5' $tRF^{Gly/GCC}$ interact with their own DNA templates, forming R-loop structures that prevent the generation of tRNA-DNA hybrids. This, in turn, promotes the transcription of the genes encoding 5' $tRF^{Glu/CTC}$ and 5' $tRF^{Gly/GCC}$ (68). Subsequent research revealed that 5 tRF^{Cys}, a member of the 5' tRNA half family, facilitates the oligomerization of Nucleolin along with its associated metabolic transcripts Mthfd1l and Pafah1b1. This process leads to the assembly of a higher-order ribonucleoprotein complex that stabilizes these transcripts, thereby protecting them from exonucleolytic degradation (69). In fact, besides its roles in modulating transcription, tsRNA also exhibits a regulatory function in the process of reverse transcription. Of interest, tRFs can be detected in cells infected with HTLV-1. One such fragment, tRF-3019, was identified in virus particles and demonstrated the capacity to initiate HTLV-1 reverse transcription (70).

Similar to miRNAs, tsRNAs facilitate gene silencing via assembly of the RNA-induced silencing complex, resulting in post-transcriptional gene suppression (22). Additionally, dicer-dependent tsRNAs mediate nascent RNA silencing by guiding Ago2 cleavage toward introns of nascent transcripts (38). Multiple tsRNA types, tRF-1s, tRF-3s and tRF-5s, can bind AGO1-4 (71). Upon loading onto AGO proteins, which are core RISC components, tsRNAs direct the degradation of sequence-matched targets (38,72). One illustrative study by Green *et al* (73) showed that IL-1β stimulation of chondrocytes triggers tRNA-Cys-GCA cleavage and elevates tRF-3003a production. This tRF binds the AGO2/GW182 complex to assemble AGO-RISC, which then targets the JAK3 mRNA 3'-untranslated region (UTR), thereby silencing JAK3 and downregulating IL-6 via inhibition of the JAK/STAT pro-inflammatory axis (73). A parallel mechanism operates in OA anterior cruciate ligament cells, where tRF365 silences IKBKB through 3'-UTR targeting, as verified by dual luciferase reporter assays (74). However, only a limited number of tsRNAs combined with AGO have been identified, and numerous tsRNAs exhibit weak interactions with AGO proteins (19). Therefore, the specific molecular mechanisms underlying the function of tsRNAs within hepatocytes remain elusive (Fig. 1).

tsRNAs in the pathogenesis of hepatic diseases. Altered expression levels of tsRNAs have been observed across a wide range of hepatic diseases, suggesting their potential utility as both diagnostic markers and therapeutic targets. The present review consolidates recent progress in understanding the role of tsRNAs in various liver pathologies, while also examining the experimental approaches employed in tsRNA-related studies. Furthermore, it highlights the current limitations in existing research, with particular emphasis on challenges in data mining (75).

Liver cancer. Globally, HCC remains one of the most frequently diagnosed cancers and represents a major subtype of liver cancer. With an age-adjusted worldwide incidence of 10.1 cases per 100,000 person-years, this malignancy ranks as the sixth most common neoplasm and the third leading cause of cancer-related mortality (76). In China, the incidence and mortality rates reach 17.64 per 100,000 and 15.33 per 100,000, respectively, indicating that HCC continues to pose a significant public health burden for the Chinese population (77). Therefore, a thorough clarification of the molecular mechanisms involved in the occurrence and evolution of HCC is expected to provide support for early diagnosis and promote the discovery of new therapeutic targets.

tsRNA has been confirmed to be widely involved in the occurrence and evolution of various tumors. With the development of RNA sequencing technology, its expression profile and potential functions in cancer cells have been deeply analyzed. Existing studies have shown that abnormal expression of tsRNA exists in various tumors (27,78), especially in liver cancer tissues, where its expression pattern is significantly different from that of normal liver tissues (15). Moreover, tsRNAs can exist stably in abundant body fluids such as blood, serum and urine in a free form or encapsulated in extracellular vesicles (EVs), thereby participating in intercellular communication in the tumor microenvironment (TME) (15). This type of abnormally regulated tsRNA plays a key role in cancer-related biological processes. Previous study focuses on its function in promoting the proliferation and invasion of liver cancer cells.

One of the fundamental characteristics of cancer is the continuous proliferation and uncontrolled growth of cells, a process involving multiple genes and proteins, especially certain kinases and their receptors (79). After transfection with the Gly-tRF mimic, the mRNA level of Nedd4 family interacting protein 2 (NDFIP2) decreased. NDFIP2 is an activator of the Nedd4 family E3 ubiquitin ligases (80). Gly-tRF is highly expressed in HCC cell lines and tumor tissues and can promote the migration of HCC cells and epithelial-mesenchymal transition. Mechanistically, Gly-tRF inhibited the mRNA expression of NDFIP2 by directly binding to the 3'UTR of NDFIP2 and subsequently activated the AKT signaling pathway to accelerate the progression of HCC (81). Although numerous literatures have associated tsRNA with the proliferation of liver cancer cells, its downstream signaling pathways have long been unclear. For instance, LeuCAG3'tsRNA, which is critical for cell viability, has been demonstrated to promote the growth of HCC (66). With the development of molecular biology techniques, these pathways have gradually been clarified. A recent investigation has shown that a type of 5'-tsRNA-Gley-GCC (tRF-34) is upregulated in both HCC tissues and cell lines. The mechanism is to target the 3'-UTR of DAB2IP and downregulate its expression, thereby activating the MEK/ERK signaling axis and enhancing the secretion of VEGFA by HCC cells to the culture supernatant (82). Similarly, another 5'-tsRNA, namely 5'tRF-Gly, can also promote the progression of HCC. It exerts an oncogenic effect through direct interaction with carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1), thereby promoting disease progression (83). In addition to its known function of targeting nuclear genes, tsRNA can also drive the progression of HCC by regulating mitochondrial genes. For instance, hypoxia-induced $tRF\text{-}3^{Thr\text{-}CGT}$ has been reported to target human mitochondrial peptide deformylase, leading to mitochondrial energy metabolism remodeling and enhancing the migration and invasion abilities of HCC cells (84). It is worth noting that tsRNAs are implicated in both tumor-promoting and tumor-suppressing activities. HCETSR, a specific tRF

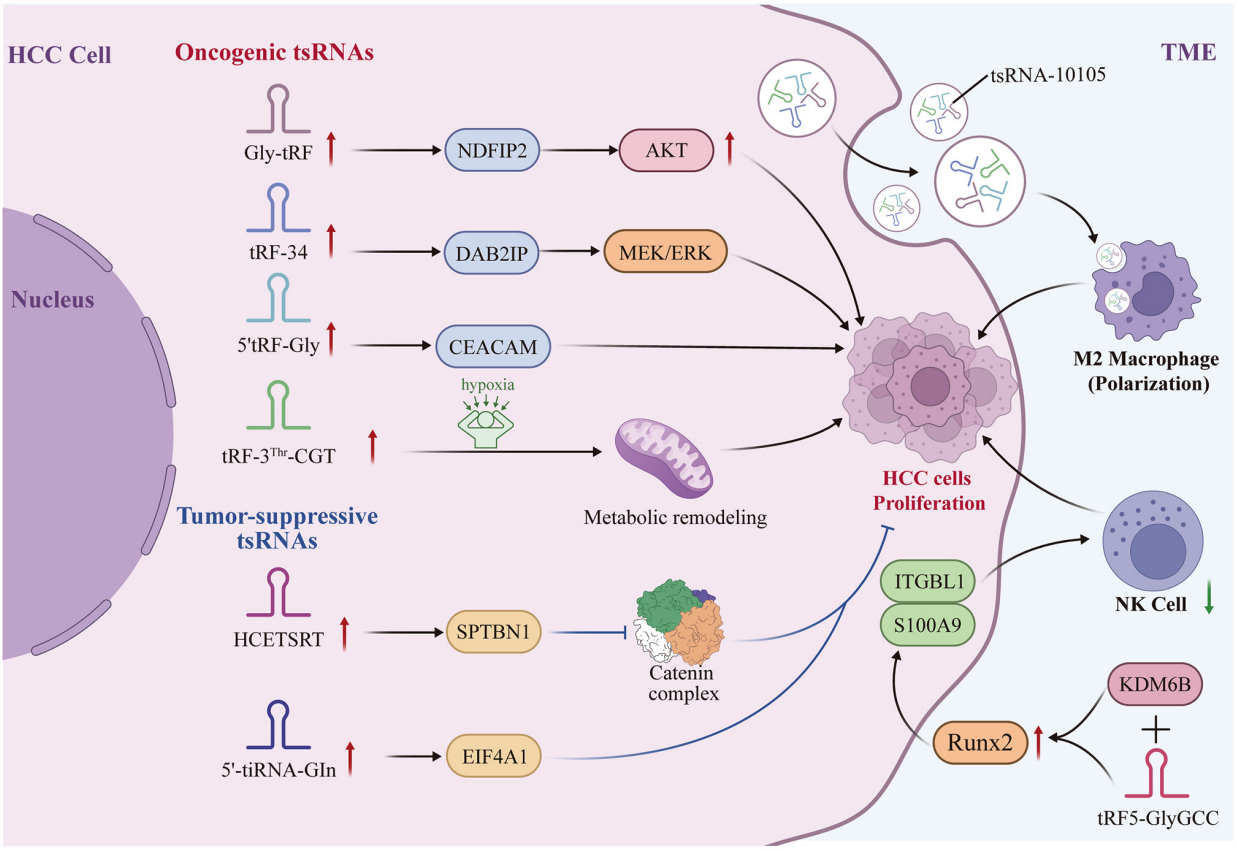

Figure 2. Oncogenic and tumor-suppressive tsRNAs orchestrate the TME in HCC. The different roles of oncogenic and tumor-suppressive tsRNAs regulating HCC progression and immunity are presented. The oncogenic tRNA-derived fragments found inside cancer cells (including Gly-tRF, tRF-34, 5'-tRF-Gly and tRF-3^{Thr}-CGT) activate tumor signaling pathways such as NDFIP2/AKT, DAB2IP/MEK/ERK and CEACAM1, and promote mitochondrial metabolic reprogramming to drive HCC progression. Tumor-suppressive tsRNAs (including HCETSR and 5'-tiRNA-Gln) can induce the depolymerization of the Catenin complex by down-regulating SPTBN1 and EIF4A1, thereby blocking the translation of oncogenic proteins. HCC-derived tsRNAs-10105 can drive the polarization of M2-type macrophages and simultaneously inhibit the cytotoxic activity of NK cells, thereby reshaping the tumor microenvironment. Meanwhile, oncogenic tRF5-GlyGCC can further weaken the anti-tumor immune response by upregulating Runx2 and inducing ITGBL1 and S100A9 expression. This figure comprehensively presents the intrinsic correlation between tsRNA expression imbalance, metabolic reprogramming, and immune escape in HCC. TME, tumor microenvironment; HCC, hepatocellular carcinoma; tsRNA, transfer RNA-derived RNA; CEACAM, carcinoembryonic antigen-related cell adhesion molecule.

that is significantly downregulated in HCC, shows a strong correlation with advanced tumor burden and increased HCC mortality. Mechanistically, HCETSR disrupts the interaction between SPTBN1 and the membrane-associated catenin complex (β-catenin, α-catenin and P120-catenin) by binding to SPTBN1, which in turn facilitates nuclear translocation of the complex. Moreover, HCETSR exerts a dual effect on β-catenin homeostasis: It stabilizes the protein by inhibiting proteasomal degradation while simultaneously reducing nascent β-catenin synthesis (85). Furthermore, Wu *et al* (86) has proved that 5'-tiRNA-Gln interacts with EIF4A1 to reduce related mRNA binding through the intramolecular G-quadruplex structure, and this process partially inhibits translation and HCC progression.

As a key immune interface, the liver employs its resident non-parenchymal cells (that is, Kupffer cells, liver sinusoidal endothelial cells and hepatic stellate cells) as primary sentinels for translocated molecular products (87). Consequently, investigating how tsRNAs interact with liver immune cells has become a key avenue for research into HCC pathogenesis. In patients undergoing hepatectomy for HCC, NK cell cytotoxicity is markedly impaired, a condition that strongly correlates with post-resection HCC recurrence and poor prognosis (88). Encouragingly, related study revealed that tRF5-GlyGCC interacts with KDM6B to epigenetically upregulate Runx2, which in turn transcriptionally activates ITGBL1 and S100A9 expression in HCC cells. This cascade directly reduces NK cell cytotoxicity while also attracting myeloid-derived suppressor cells to inhibit NK cell function, thereby promoting HCC progression (89). Furthermore, a recent investigation demonstrated that migrasomes enriched with tsRNA-10105 contribute to an immunosuppressive microenvironment in HCC by inducing M2 macrophage polarization. These migrasome-induced M2 macrophages can promote the proliferation and metastasis of HCC (90). However, the molecular interaction mechanism between tsRNA and liver immune cells remains unclear, which restricts the overall understanding of the regulatory network for the pathogenesis of HCC. Therefore, future research urgently needs to focus on the functional role and mechanism of action of tsRNA in liver immune cells, which will be an important direction for in-depth analysis of the pathogenesis of liver cancer (Fig. 2).

Metabolic dysfunction-associated steatotic liver disease (MASLD). In recent years, with the continuous increase in the burden of metabolic diseases, the prevalence of MASLD

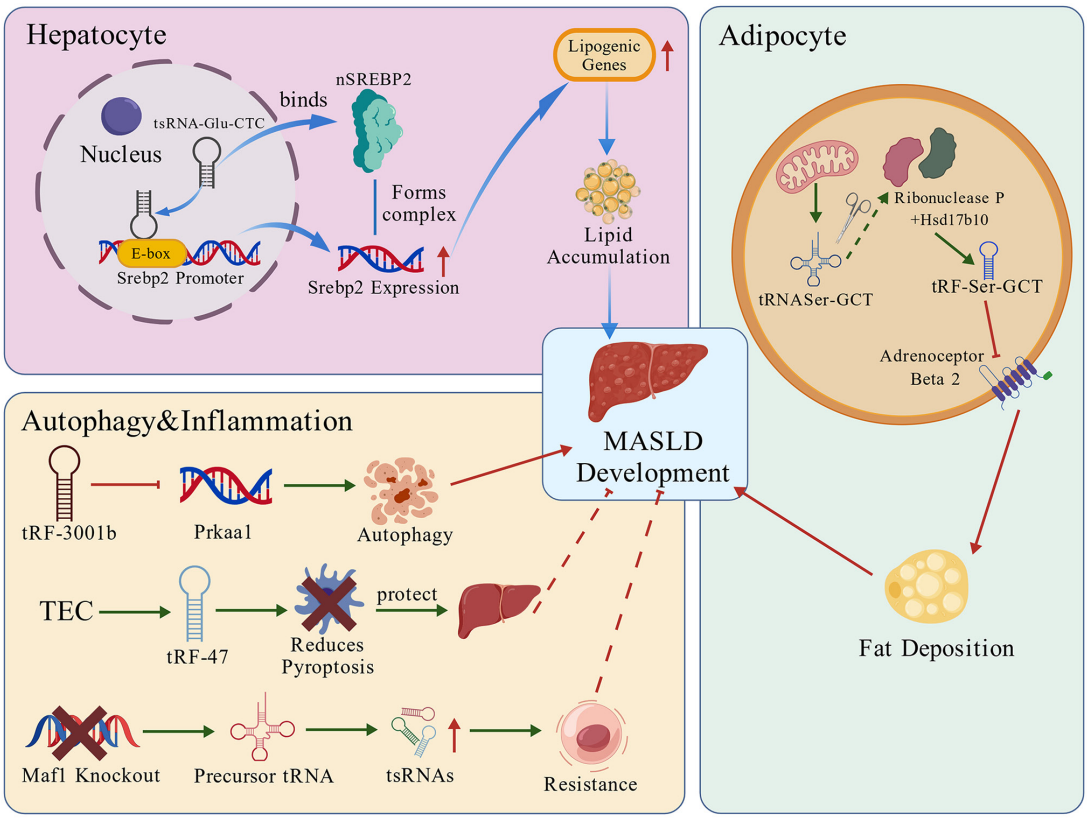

Figure 3. Schematic of tsRNA-related regulatory mechanisms in MASLD. This figure illustrates the mechanism by which tsRNAs promote MASLD pathogenesis through multiple pathways such as regulating liver lipid metabolism, mediating inter-organ signal communication, and participating in inflammatory responses under metabolic stress. Among them, tsRNA-Glu-CTC can bind to the SREBF2 promoter and form a complex with nSREBP2, thereby promoting the upregulation of SREBF2 expression and further activating downstream lipid synthesis-related genes, ultimately leading to pathological lipid accumulation in the liver. The tRF-Ser-GCT from adipocytes promotes fat storage via the β2-adrenoceptor. Furthermore, it also aggravates MASLD progression through inter-organ communication between adipose tissues and livers. Additionally, tRF-3001b inhibits autophagy by repressing Prkaa1, with autophagic dysfunction accelerating disease onset, while TEC upregulates tRF-47 to suppress pyroptosis, a key inflammatory pathway, and protect against liver injury. The removal of Maf1 increases the production of tsRNA from precursor tRNA and confers MASLD resistance. It highlights the critical role of tsRNAs in the development of MASLD and their potential as novel therapeutic targets. tsRNA, transfer RNA-derived RNA; MASLD, dysfunction-associated steatotic liver disease; TEC, tectorigenin.

has also risen significantly. MASLD covers a continuous disease spectrum ranging from simple fatty liver, metabolic dysfunction-related steatohepatitis, to liver cirrhosis and even HCC (91). As the main types, NAFLD and non-alcoholic steatohepatitis (NASH) pose a serious threat to human health and impose a heavy socio-economic burden. However, the exact molecular mechanisms of both have not yet been fully elucidated. Against this backdrop, tsRNAs offer a novel perspective for understanding the pathological processes related to NAFLD.

The core pathological feature of NAFLD is the excessive deposition of lipids in liver cells, which induces chronic inflammation and promotes the progression of liver injury. The imbalance of lipid metabolism homeostasis is the direct cause of lipid accumulation in hepatocytes, and this process initiates hepatic steatosis and the emergence of NAFLD (92). The advancement of NAFLD is regulated by multiple factors, such as genetic background and environmental factors (93). Research has demonstrated that tsRNA-Glu-CTC plays a key role in hepatocyte lipid metabolism. Mechanistically, this tsRNA translocates into the nucleus and enhances the transcription of *Srebp2* by recognizing the E-box motif within its promoter region. Furthermore, it directly binds to the nSREBP2 protein to form a complex, jointly activating SREBP2 expression and consequently upregulating the expression of genes related to liver lipid synthesis. Unlike the common inhibitory regulation of miRNA, this tsRNA exhibits typical transcriptional activation. Silencing tsRNA-Glu-CTC can significantly alleviate diet-induced hypercholesterolemia and atherosclerosis, suggesting that, as an important activating molecule of SREBP2, it is the core regulatory factor of lipid metabolism (94) (Fig. 3).

In addition to the liver, the regulatory role of tsRNAs in adipose tissue should not be ignored either, as adipose metabolism is closely related to the occurrence and development of NAFLD. The generation of tRF-Ser-GCT depends on the processing of mitochondrial tRNASer-GCT by the ribonuclease P complex. Hsd17b10 is not only indispensable in this process but also participates in maintaining the normal lipid metabolism of adipocytes. At the mechanistic level, tRF-Ser-GCT directly targets the Adrenoceptor Beta 2, a key regulator of metabolic homeostasis, and thereby inhibits its expression. *In vivo* experiments further confirmed that restoring the expression of tRF-Ser-GCT in Hsd17b10-deficient mice could, to some extent, reverse the decline in adipose tissue mass and the reduction in adipogenic gene expression (95). Although previous studies have identified a large number of tsRNAs involved in fat deposition, most of these molecules

still lack clear regulatory mechanism analysis. These existing datasets have laid an important foundation for in-depth clarification of the epigenetic regulatory network of NAFLD (96) (Fig. 3).

Autophagy, as a key catabolic pathway within cells, maintains cellular homeostasis by degrading and recycling damaged organelles, misfolded proteins and biological macromolecules. Impaired function of this process is regarded as one of the important triggers for the development of NAFLD (97,98). During the process of NAFLD, autophagy in the liver has a bidirectional regulatory effect. Its protective functions of eliminating lipid droplets and alleviating cellular stress constitute the primary defense line against liver damage. Previous studies have shown that abnormal expression of tsRNAs is closely related to the progression of NAFLD regulated by autophagy. Distinct tsRNAs can influence the onset and severity of the disease by altering autophagic activity. For example, tRF-3001b directly binds to and suppresses Prkaa1 expression, with Prkaa1 being a critical gene involved in autophagy. Knockdown of tRF-3001b markedly worsens NAFLD disease progression and lowers hepatic triglyceride and cholesterol concentrations in NAFLD mouse models, and the established autophagy inhibitor chloroquine can dramatically abrogate the phenotypic effects induced by tRF-3001b deficiency (99) (Fig. 3).

A separate study demonstrated that tectorigenin (TEC) increases tRF-47 expression. Silencing of tRF-47 diminished the protective actions of TEC against NASH in cell-based assays, encompassing the blockade of autophagy, activation of pyroptosis, and discharge of pro-inflammatory factors. Correspondingly, *in vivo* suppression of tRF-47 promoted lipid-triggered liver damage and lipid accumulation (100). Of note, emerging evidence suggests that Maf1 knockout may enhance resistance to NAFLD via regulation of tsRNAs and autophagy: Within the livers of Maf1-deficient mice, precursor tRNA production is upregulated (despite unaltered mature tRNA levels), indicating enhanced precursor tRNA conversion into tsRNAs; in parallel, autophagy-generated amino acids and spermidine concentrations are also elevated (99,101) (Fig. 3).

In summary, tsRNA is deeply involved in the pathological processes of MASLD and NASH by regulating four core genetic programs: Lipid metabolism, apoptosis, inflammation and fibrogenesis. This mechanistic understanding provides a new opportunity for targeted intervention of tsRNA at the molecular level in the treatment of the aforementioned diseases. Additionally, tsRNAs can actively promote disease progression by mediating signal communication among damaged liver cells, hepatic stellate cells and immune cells, facilitating the transformation from simple steatosis to steatohepatitis and fibrosis (102).

Alcoholic-associated liver disease (ALD). Multiple lines of evidence indicate that various mediators such as the complement system, reactive oxygen species, neutrophils, macrophages and lipopolysaccharides play a key role in the pathogenesis of ALD (103). Previous studies have found that the deficiency of complement component 3 (C3) can alleviate ethanol-induced liver steatosis and inflammatory response. Further mechanism studies revealed that C3 activation upregulates the expression of a glycine tRNA-derived fragment (Gly-tRF) through its proteolytic fragments C3a and C3a-des-Arg via a CYP2E1-dependent pathway. After forming a complex with AGO3, the Gly-tRF can directly bind to the 3'-UTR of Sirtuin 1 (Sirt1), thereby inhibiting the expression of Sirt1. The downregulation of Sirt1 disrupts the lipid homeostasis of the liver, manifested as enhanced lipid synthesis and weakened fatty acid β-oxidation, ultimately promoting hepato-steatosis. Importantly, the C3-Gly-tRF-Sirt1 signaling axis has been verified in human ALD tissue samples, suggesting its clinical translational significance (104). In addition to the complement system, the role of immune cells in ALD is also worthy of attention. Recently, Wang *et al* (105) have observed that $C1Q^+$ macrophages may perform complex functions in ALD. A large number of apoptotic neutrophils have been detected in the liver of ALD, numerous of which are distributed adjacent to macrophages, suggesting that there may be an interaction between the two that affects the disease outcome (105) (Fig. 4). However, at present, the understanding of the functions of tsRNA in immune cells of patients with ALD remains relatively limited, and its specific molecular targets are also unclear. In addition, the cross-regulatory relationship between tsRNA and other known signaling pathways in the pathogenesis of ALD, such as arrestin domain-containing protein 3 (106) and the miR-155-5p/SIRT1/VDAC1 pathway (107), remains to be further clarified. The aforementioned findings provide potential directions for subsequent research.

Viral hepatitis. The key regulatory role of tsRNAs in the occurrence of HCC related to viral hepatitis has been confirmed by existing studies. Specifically, during the viral infection stage, the host generates the tRF_U3_1 fragment derived from the pre-tRNA 3'-trailer. This fragment acts as a molecular bait, binding the RNA chaperone La/SSB in the cytoplasm, resulting in a reduction in La/SSB reserves in the nucleus, inhibiting the expression of viral genes dependent on this protein, thereby forming an innate antiviral defense barrier (108). By contrast, under the condition of chronic hepatitis B virus (HBV) infection, proline tRNA-derived tRF-3a-Pro is specifically elevated in HCC tissues and peripheral circulation. It accelerates tumor proliferation by driving the G1/S phase transition of the cell cycle and can be used as a highly sensitive serum biomarker (109). The aforementioned findings reveal the spatiotemporal dynamic characteristics of tsRNAs in virus-host interactions and tumor evolution: They exert antiviral response functions during the acute infection period and transform into cancer-promoting factors in the chronic disease course stage. Thus, the dynamic recombination of non-coding RNA networks during the pathogenesis of liver diseases has obtained a novel explanatory framework (110).

4. Role of tsRNA in interorgan transport

It was generally considered previously that RNA only functions within the cells from which it originated. However, in recent years, this understanding has undergone a fundamental shift; multiple evidence has shown that ncRNAs can act on distant target cells and participate in specific regulatory networks. This transformation can be traced back to the key discovery in 2007-2008: Multiple research teams detected mRNA and miRNA in the extracellular environment (111,112).

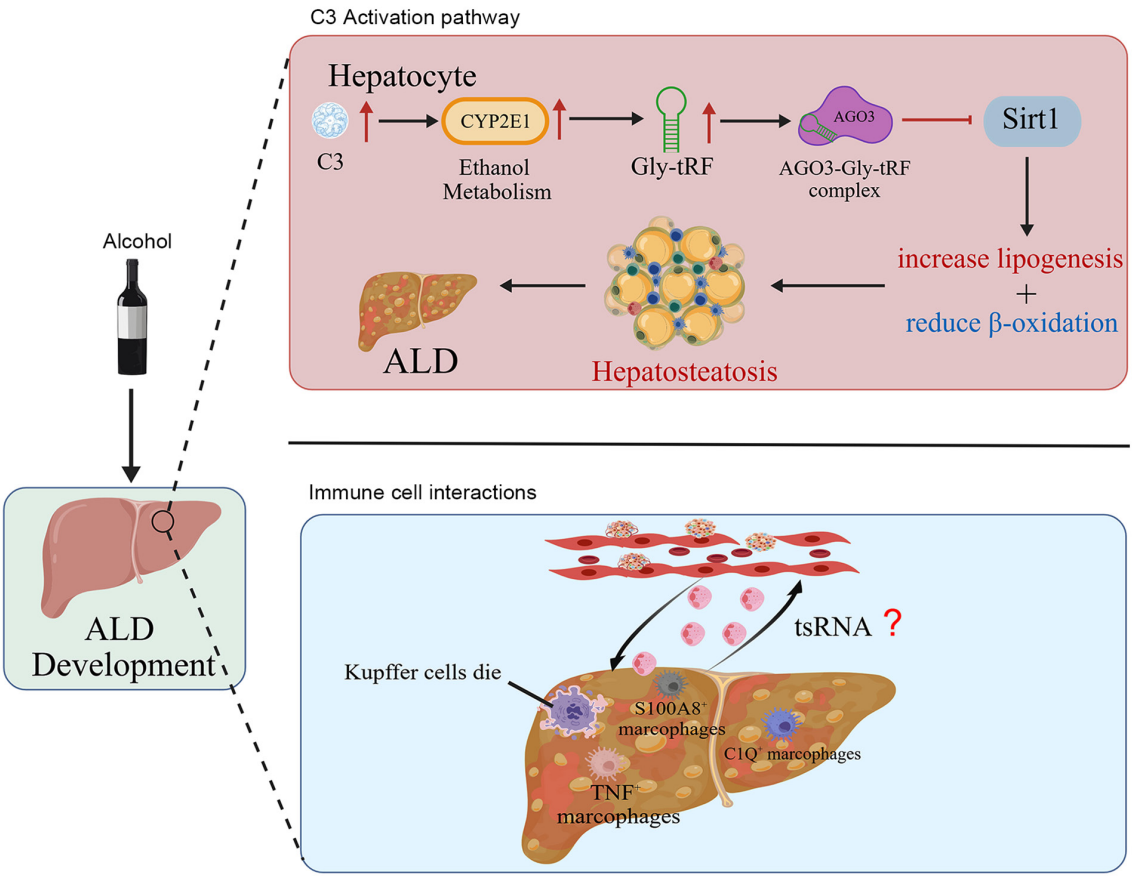

Figure 4. Pathological role of tsRNA in ALD. The two main pathogenic modules in ALD are combined here. The upper panel outlines the C3 activation-Gly-tRF axis. Ethanol can upregulate C3, which in turn induces CYP2E1-mediated ethanol processing. The latter can further induce Gly-tRF biogenesis. These tRFs travel to the nucleus and interact with AGO3 to suppress Sirt1 expression directly. The lipogenesis further impairs β-oxidation which results in hepato-steatosis a feature of initial ALD. The infiltrated C1Q+ macrophages in severe ALD contribute to functional cross-talk with hepatocytes as depicted in the lower panel. An important unanswered question is whether tsRNAs mediate signaling between apoptotic neutrophils and C1Q+ macrophages during ALD progression affecting liver inflammation and tissue repair. tsRNA, transfer RNA-derived RNA; ALD, alcoholic-associated liver disease.

Subsequent pioneering research further confirmed that circulating miRNAs exist in the blood and their expression patterns are disease-specific (113). These findings jointly reveal that extracellular ncRNAs act as efficient regulatory factors for inter-organ communication in various physiological and pathological processes.

The anatomical and functional connection between the gut and the liver constitutes the gut-liver axis, which has significant clinical significance for disease pathogenesis. In this two-way interaction, the gut microbiota community occupies a core position. A healthy microbiome provides the host with a variety of metabolic products through the portal vein, which not only helps maintain intestinal homeostasis but also significantly regulates liver function (114). Clinical evidence shows that disorders of gut-liver communication are associated with the occurrence of NAFLD, alcoholic liver disease, and primary sclerosing cholangitis (115). The host and microorganisms have formed complex interactions in co-evolution to maintain a delicate balance. Increasing evidence indicates that, in addition to small molecules, peptides and proteins, small regulatory non-coding RNAs may also play an important role in cross-domain communication (116). Among very small RNAs (vsRNAs), tRFs such as Ile-tRF and Ala-tRF represent an important biological type and are selectively enriched in outer membrane vesicles (OMVs). These tRF-derived vsRNAs are thermodynamically stable and usually contain at least two G-C base pairs and one stem-loop structure. Bioinformatic analyses predicted that they could target various human host mRNAs with different functions (117). It has been previously found that *Escherichia coli* produces a special vsRNA called Ile-tRF-5X, which is selectively induced under environmental stress conditions and is not affected by transcriptional or translational inhibition. The tRNA fragment derived from this bacterium, after being released through OMV, can be transferred to human HCT116 cells and upregulate the expression of MAP3K4 (118). Bacterial-derived tsRNAs can regulate host cellular processes, while host-derived tsRNAs inversely modulate the composition of gut microbiota and immune responses. For example, *Fusobacterium nucleatum* (*F. nucleatum*) can stimulate normal human oral keratinocytes to release tsRNA-000794 and tsRNA-020498 through exosomes, both of which inhibit the growth of *F. nucleatum*, likely by interfering with protein synthesis. *Streptococcus mitis* (*S. mitis*), a health-associated oral bacterium, is not affected because its genome lacks sequences highly similar to these tsRNAs (116). Similar phenomena are also observed in tRF-His-GTG-1. The expression of this tsRNA is elevated in peripheral blood mononuclear cells of patients with systemic lupus erythematosus. It can directly bind to TLR7/8 to activate the ERK/p38 signaling pathway, thereby inducing the

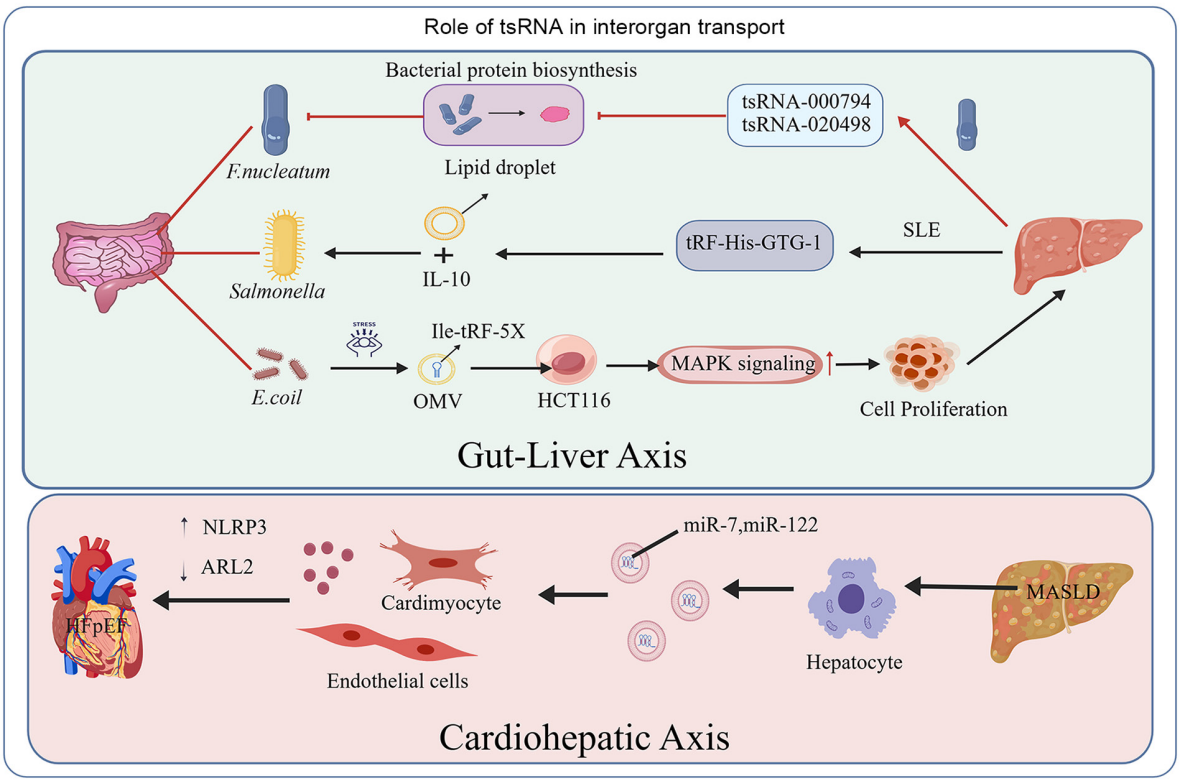

Figure 5. Role of tsRNA in interorgan transport. The roles of tsRNAs and related non-coding RNAs in mediating inter-organ communication to drive hepatic disease pathogenesis are illustrated, focusing on two key regulatory axes. In the gut-liver axis, hepatic tsRNAs (tsRNA-000794, tsRNA-020498, tRF-His-GTG-1) modulate gut microbiota (*Fusobacterium nucleatum*, *Salmonella*) and immune mediators (IL-10), while gut-derived Ile-tRF-5X, delivered via *Escherichia coli* OMVs, activates MAPK signaling to promote hepatocyte proliferation. In the cardio-hepatic axis, hepatocytes from metabolic MASLD secrete miRNAs (miR-7 and miR-122) that target cardiomyocytes and endothelial cells, upregulating NLRP3 and downregulating ARL2 to induce heart failure with preserved ejection fraction (HFpEF). Collectively, this diagram highlights tsRNAs as key mediators of inter-organ crosstalk, linking gut and cardiac dysfunction to hepatic disease progression. tsRNA, transfer RNA-derived RNA; MASLD, dysfunction-associated steatotic liver disease; OMVs, outer membrane vesicles; miR or miRNA, microRNA; SLE, systemic lupus erythematosus.

expression of PPARδ, promoting lipid droplet formation, and enhancing the phosphorylation of ERK/p38 and the production of IL-10. After inhibiting tRF-His-GTG-1, both lipid droplet accumulation and IL-10-dependent pathways are suppressed, thereby reducing the survival rate of *Salmonella* (119). These findings indicate that host tRFs can influence intestinal inflammation and barrier integrity by regulating the composition of gut microbiota, both of which are key factors in the progression of liver diseases. Host-derived tsRNAs can selectively inhibit the growth of pathogens (such as *F. nucleatum*) but have no adverse effect on symbiotic bacteria (such as *S. mitis*), suggesting that there is a precise regulatory mechanism that helps maintain microbial homeostasis. Therefore, tRF is not only a by-product of cellular stress but also an active mediating factor in the host-microbe co-evolution process. Disorder at this regulatory level may accelerate the vicious cycle among impaired barrier function, bacterial translocation and liver inflammation, thereby exacerbating conditions such as MASLD (Fig. 5).

Non-coding RNAs also serve an important role in the communication between the liver and the heart. The nanoscale EVs secreted by steatosis hepatocytes are rich in specific miRNAs (for example, miR-122) and proteins. These vesicles can be taken up by myocardial cells after entering the circulation. Previous studies have confirmed that EVs derived from fatty liver can directly damage the mitochondrial function of myocardial cells and inhibit key enzymes for energy production, ultimately leading to energy metabolism disorders in the heart. Therefore, the damaged liver can directly induce heart failure through intercellular substance transport without relying on the classic blood glucose or lipid metabolism pathways (120). This RNA-mediated cross-kingdom communication extends the understanding of the pathogenesis of liver diseases from the metabolite and cytokine levels to epitranscriptomic regulation, providing a potential new direction for RNA-targeted therapy (Fig. 5).

5. Clinical potential of tsRNAs as biomarkers

Diagnostic applications for tsRNAs. The rapid development of high-throughput sequencing technology has revealed multiple biological functions of tsRNAs, including their participation in gene expression regulation and stress signal activation. Accumulating evidence indicates that ncRNAs such as miRNAs, lncRNAs, tsRNAs and circRNAs (102) play a critical role in the occurrence and progression of hepatic diseases. Furthermore, the expression levels of tsRNAs show significant fluctuations during the development of liver diseases (121). Consequently, tsRNAs are regarded as potential biomarkers of human liver diseases (122).

Given the high heterogeneity of the clinical manifestations of liver cancer, molecular markers are expected to enhance

its diagnostic and prognostic efficacy, especially the tsRNA studies that have emerged in recent years, which have provided a new perspective (122). In circulating blood, tRNA-derived fragments can be protected from RNase degradation by being encapsulated in vesicles or binding to extracellular RNA carriers. Exosomes, as membranous nanovesicles released by normal and pathological cells (123), carry proteins, lipids, and various nucleic acids, and play a key role in intercellular communication. Previous studies have confirmed that exosomes are rich in sncRNAs, such as miRNAs, lncRNAs and circRNAs, and can regulate the initiation and progression of diseases through interaction with target cells. Recent evidence further indicates that tsRNA is also highly enriched in microvesicles and exosomes (14). Zhu *et al* (124) demonstrated that the levels of four specific tsRNAs, namely tRNA-ValTAC-3, tRNA-GlyTCC-5, tRNA-ValAAC-5 and tRNA-GluCTC-5, are significantly elevated in plasma exosomes from individuals with HCC, suggesting their potential utility as novel diagnostic markers for this malignancy, pending validation in independent cohorts. In a parallel observation, tsRNA-20 (derived from tRNA-Gly-CCC-1-1) and tsRNA-46 (derived from tRNA-Glu-CTC-1-1) exhibit specific differential expression in the plasma EVs of patients with hepatitis B virus-related liver failure. Notably, these tsRNAs were reported to show 100% specificity and 94.80% sensitivity in distinguishing patients from healthy individuals in that single cohort, although such performance estimates require confirmation in larger, multi-center studies (125). Moreover, distinguishing between hepatocellular adenoma (HCA) and HCC can be difficult. Nevertheless, an accurate diagnosis is essential because the treatment and prognosis for these tumors differ considerably. Thus, relevant diagnostic indicators are urgently needed. Those identified in dogs may also have translational value for human medicine, given the similarities between human and canine HCA and HCC. Previous findings demonstrate that tRNA-Val expression is markedly lower in HCC tumor tissue than in HCA tumor tissue or normal liver tissue. A comparable pattern is observed in plasma EVs and HCC cell lines relative to healthy controls. These results suggest that tRNA-Val could serve as a promising detection marker for distinguishing canine HCC from HCA (126) (Table I).

However, most tRNA-derived fragments circulating in the bloodstream are not encapsulated within EVs. Evidence suggests that the majority of extracellular tRNA-derived fragments in serum are instead associated with extra-vesicular components or bound to ribonucleoproteins (127). Recently, liquid biopsy targeting circulating nucleic acids has gained significant attention in the field of early tumor screening. Therefore, numerous specific tsRNAs with diagnostic value and biological functions have been found. In HCC, elevated expression levels have been observed for hsa_tsr014055 (128), tRF-23-R9J89O9N9 (129), tsRNA-Thr-5-0015 (130) and tRF-33-RZYQHQ9M739P0J (131). Importantly, combining these tsRNAs with alpha-fetoprotein (AFP), prothrombin induced by vitamin K absence-II (PIVKA-II), and des-γ-carboxyprothrombin (DCP) enhances diagnostic accuracy. Their prognostic value has been evaluated using Kaplan-Meier curves (128-131). By contrast, tsRNA-Asp-5-0002 is downregulated in HCC, and its reduced expression correlates with TNM stage, tumor differentiation and lymph node metastasis. Receiver operating characteristic (ROC) curve analysis has suggested that tsRNA-Asp-5-0002 may have favorable diagnostic performance for HCC in the studied cohort (132). Besides, Zhan *et al* (133) has identified tRF-Gln-TTG-006, which is significantly upregulated in HCC serum. During the validation phase, it was found that the tRF-Gln-TTG-006 signature differentiated HCC patients from healthy individuals with high sensitivity and specificity even at early disease stages; its generalizability awaits external validation (133) (Table I). However, some limitations should be considered despite these encouraging results. One is that nearly all of the aforementioned signatures came from single retrospective cohorts. Estimating accuracy in the same cohort used for discovery is prone to overfitting. Therefore, current guidelines require validation in independent patients before a model can be considered clinically useful (134). The other limitation is that most studies compared diagnosed patients with healthy controls. This setup tends to enroll the most severe cases and the healthiest controls. That inflates sensitivity and specificity compared with real use (135). External validation across multiple centers and populations is thus still needed for each of the candidate tsRNAs described here. Circulating tsRNA levels shift with age, sex, diet and circadian rhythm; however, discovery cohorts remain small and single-center. Whether a signature established in one population remains robust in another, with different genetic backgrounds, lifestyles, or disease etiologies, has rarely been tested. Systematic evaluation across diverse cohorts is therefore essential before any tsRNA biomarker can be considered generalizable.

Notably, tsRNAs can even elucidate the precise etiological origins of HCC. For example, a previous study has characterized the expression profile of tsRNAs in HBV-related HCC and confirmed the diagnostic potential of serum tRF-3a-Pro. Data from both animal models and cell-based assays have corroborated that tRF-3a-Pro represents a candidate molecular marker for identifying HBV-related HCC. This tsRNA is present at elevated levels, and its diagnostic value needs confirmation beyond the discovery cohort. In addition, integrating this candidate with the conventional detection markers AFP led to enhanced diagnostic value for this cancer type (109). Equally important, other noteworthy molecules include the mt-tRFs tRHalve3-His-CAU and tRNAleader-Gln-UUG, which are linked to an improved prognosis, alongside the ge-tRF tRFmisc-Tyr-GTA, which predicts worse clinical outcomes. These RNA fragments exhibit stable but diverse expression profiles in tumor samples and still have independent prognostic value after adjusting for clinical confounding factors. Therefore, they are regarded as candidate biomarkers, but their specificity and clinical utility require further validation in independent populations (136). In the diagnosis of liver cancer, tsRNA can also be applied to the detection of CCA. The results of high-throughput RNA sequencing showed that, compared with the adjacent normal tissues, the expression of a large amount of tsRNA in CCA tissues presented abnormal changes. Among them, the downregulation of tRF-34/tRF-38/tRF-39 and the upregulation of tRF-20 were validated by reverse transcription-quantitative PCR (RT-qPCR), and the differences reached a significant level. These tsRNAs are enriched in key cancer-related signaling pathways such as Notch, Hippo and cAMP, suggesting their functional importance in the

Table I. Key tsRNA biomarkers for hepatic diseases.

tsRNA subtype	Expression pattern	Clinical relevance
tRNA-ValTAC-3, tRNA-GlyTCC-5, tRNA-ValAAC-5, tRNA-GluCTC-5	Increase in HCC	Herald their utility as biomarkers for the diagnostic process of this malignancy
tsRNA-20, tsRNA-46	Increase in HBV-Related Acute-on-Chronic Liver Failure	Potential clinical significance for the diagnosis and treatment of related hepatic diseases
tRNA-Val	Decrease in HCC tumor tissue	A promising novel biomarker to distinguish canine HCC from HCA
hsa_tsr014055, tRF-23-R9J89O9N9, tsRNA-Thr-5-0015, tRF-33-RZYQHQ9M739P0J	Increase in HCC	Has potential diagnostic value for HCC
tsRNA-Asp-5-0002	Decrease in HCC	Is related to TNM stage, differentiation, and lymph node metastasis
tRF-Gln-TTG-006	Increase in HCC	Distinguish HCC cases from healthy subjects even in the early stage
tRF-3a-Pro	Increase in HBV-related HCC	Serve as a novel biomarker for HBV-related HCC
mt-tRFs tRHalve3-His-CAU and tRNAleader-Gln-UUG	Increase in colorectal liver metastases	Specific biomarkers for early detection, prognostication, and therapeutic targeting in colorectal liver metastases
tRF-34-JJ6RRNLIK898HR, tRF-38-0668K87SERM492V, tRF-39-0668K87SERM492E2	Decrease in CCA	Serve as a novel biomarker for CCA
tRF-20-LE2WMK81	Increase in CCA	Serve as a novel biomarker for CCA
tRF-Val-CAC-005, tiRNA-His-GTG-001, tRF-Ala-CGC-006	Increase in MASLD	Elevated levels in MASLD compared to non-MASLD; levels associated with MASLD activity score

HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; MASLD, dysfunction-associated steatotic liver disease; tsRNA, transfer RNA-derived RNA; HBV, hepatitis B virus.

pathogenesis of CCA. The distinct expression profiles of these tsRNAs in CCA tissues highlight their significant potential as a non-invasive diagnostic marker and provide a promising direction for improving the early detection and clinical management of this highly aggressive malignant tumor (137) (Table I).

Apart from liver cancer, tsRNAs also play the role of biomarkers in the diagnosis of other liver diseases. Dynamic monitoring of ncRNA may help track the progression of diseases. Similarly, the levels of tRF-Val-CAC-005, tiRNA-His-GTG-001 and tRF-Ala-CGC-006 were upregulated in patients with MASLD with more advanced fibrosis, suggesting that they may be used to assess the risk of fibrosis progression (138). However, this speculation still needs to be verified by subsequent studies. Existing reviews show that research on tsRNA markers mainly focuses on liver cancer (HCC), while their value in early-stage liver diseases such as MASLD and liver fibrosis is rarely reported. Given that early intervention is of decisive significance for the prognosis of liver diseases, filling this gap should become a key direction for future research. Furthermore, there is existing evidence indicating that tsRNAs are closely related to the severity of liver diseases, which is expected to provide a reference for the risk of disease progression and thereby make up for the deficiencies of conventional detection methods and existing markers. Therefore, one of the main focuses of future research lies in screening for diagnostic markers of tsRNAs with higher specificity from various liquid biopsy sources.

Detection technologies for tsRNAs. In the exploration of non-invasive biomarkers, tRNA-derived RNA has become a key breakthrough due to its detectable and analyzable properties in body fluids. The methodological framework of this field relies on two complementary paradigms: High-throughput sequencing is used for unbiased discovery, while targeted quantitative detection serves clinical applications.

Although traditional small RNA sequencing was once an important cornerstone of discovery research, the widespread RNA modifications can block linker junctions and reverse transcription, fundamentally undermine its accuracy and ultimately leading to biased and incomplete modified tsRNA maps obtained. This limitation is being addressed by a new generation of methods. PANDORA-seq employs combinatorial enzymatic treatments to strip key RNA modifications, thereby unveiling an abundant and previously hidden landscape of specific tsRNAs and ribosomal RNA-derived small RNAs,

revolutionizing our view of the small RNA repertoire (139). Of note, the choice of sequencing pipeline should be guided by the research objective. PANDORA-seq is particularly suited to global characterization of tsRNA repertoires in tissues and biofluids, as its sequential enzymatic treatment removes RNA modifications and terminal structures that otherwise preclude adaptor ligation and reverse transcription, thus markedly improving the detection of highly modified tsRNAs (140). However, modification information is erased during enzymatic treatment, and modified tsRNAs are over-represented relative to conventional small RNA-seq, which precludes simultaneous modification analysis. These features should be considered when PANDORA-seq data are interpreted and different results across pipelines are compared. Unlike PANDORA-seq, nanopore-based direct RNA sequencing reads native RNA molecules directly, without reverse transcription or amplification. RNA modifications are therefore preserved and remain detectable in the raw current signal. Because tsRNAs inherit the modifications of their parental tRNAs, the capacity of this platform to quantify RNA abundance and modification status in a single experiment is of particular interest for tsRNA research. Recent methodological advances in nanopore sequencing of tRNA populations have demonstrated the feasibility of such integrated analysis (141). Nevertheless, several limitations remain. Standard settings should exclude a large fraction of short RNA reads and favor longer transcripts. And reprocessing of raw signals is required to recover accurate abundances (141). Moreover, its high error rate and large input requirement remain practical obstacles to routine clinical application, and the accuracy of modification detection still depends heavily on computational models (142). In particular, the inability to efficiently capture molecules shorter than 100 nt restricts its direct use for most tsRNAs (141). Mim-tRNAseq can complement the aforementioned methods. Its principle lies in utilizing the modification-induced misincorporation patterns to achieve high-resolution quantitative analysis of the abundance and modification status of tRNA, thereby demonstrating the significant heterogeneity and modification interdependence in human cells. However, this method also has several limitations that cannot be ignored. The initial RNA amount needs to be no less than 0.5 μg, thus making it difficult to adapt to extremely low input or single-cell samples. Some modifications cannot generate identifiable misincorporation signals during the reverse transcription stage. In addition, its detection targets are limited to mature tRNAs, and the precursor tRNA-derived fragments are still outside the coverage range (143). Overall, PANDORA-seq has the strongest sensitivity in discovery; mim-tRNAseq is suitable for the construction of quantitative modification maps of full-length tRNA, while nanopore sequencing can maintain the native modification information. The final technology selection should comprehensively consider the availability of samples and whether there is a need for modification analysis.

Through targeted strategies, the biomarker value of tsRNAs has surpassed the basic discovery stage. The specific tsRNA signatures have been verified in the early screening, prognosis prediction and therapeutic effect monitoring of diffuse large B-cell lymphoma and can be used for high-precision identification of forensic body fluid sources, thus demonstrating practical efficacy at the clinical and application levels (144,145). These technological advancements not only broaden the detectable range of tsRNAs but also enhance the resolution of RNA modification profiles, providing crucial support for elucidating their biological functions and evaluating their potential as biomarkers.

Although certain progress has been made in related technologies, the detection and quantitative analysis of tsRNA in body fluids still face multiple technical bottlenecks. One of the core challenges lies in how to efficiently isolate EVs, which are the main carriers of tsRNA in biological fluids. Previous studies have shown that to obtain high-purity EV samples, the separation process must be optimized, as different operation methods have a significant impact on both EV yield and RNA composition (146). In the reported validation studies of tsRNA biomarkers based on RT-qPCR, both diffuse large B-cell lymphoma and forensic body fluid identification have been used as typical application scenarios. However, such methods rely on preset targets and are difficult to discover unknown types of tsRNA, such as sequencing technology (144). In addition, tsRNA generally has highly modified and fragmented characteristics, especially tsRNA derived from mitochondrial tRNA, which further increases the difficulty of accurate quantification and functional interpretation (147). Although existing detection techniques have significantly enhanced the detection capacity of tsRNA, issues such as sample pretreatment, modification preferences, and insufficient standardization remain key challenges hindering tsRNA from becoming a reliable clinical biomarker.

Therapeutic strategies and translational perspectives of tsRNAs. Given the significant therapeutic potential of newly identified functional small RNAs in liver diseases, related research is increasingly focusing on their specific roles in liver diseases (122). Standard treatments for end-stage liver disease which are surgical resection, liver transplantation and standard pharmacological treatment have a poor clinical outcome, damaging tissue and toxic effects. Compared with traditional methods, RNA-based therapy, as an advanced alternative, uses functional small RNAs as therapeutic agents to alleviate pathological conditions and has been increasingly pursued for managing hepatic diseases. The key prerequisite for the success of this therapy lies in the preparation of highly efficient, safe and highly targeted specific functional RNA molecules.

In hepatic diseases, tsRNAs can exert therapeutic effects by directly regulating pathological signaling pathways. As a key regulatory factor of cellular stress response (6), this type of RNA is deeply involved in core cellular events in liver injury and liver fibrosis, and thus is regarded as a potential direct therapeutic target. When the methyltransferase NSun2 is exhausted, cells produce tRF-Gln-CTG-026. This molecule inhibits excessive overall protein synthesis by blocking the binding between TSR1 and 18S rRNA, thereby promoting the proliferation and survival of hepatocytes after liver injury. These results support the feasibility of tRF-Gln-CTG-026 as an RNA-based therapeutic agent for liver injury intervention (65). In addition, the tsRNAs regulated by NSun2 also significantly regulate the capillarization of hepatic sinusoids. A recent study has found that reduced NSun2 expression alters the functional

Table II. List of selected tsRNAs involved in treating hepatic diseases with their molecular mechanism.

Authors, year	tsRNA subtype	Source	Delivery system	Molecular/cellular targets	Hepatic disease indication	(Refs.)
Gonskikh *et al*, 2020	tRF-Gln-CTG-026	Loss of NSun2	None	TSR1, 18S rRNA	Liver injury	(63)
Katopodi *et al*, 2025	tRF-1-S25, tRF-5-V31	Reduced NSun2 expression	None	DUSP1/FAK/ p-FAK signaling axis	Liver fibrosis	(142)
Qu *et al*, 2025	5'-tiRNA-Gln	Endogenous tumor suppressor	None	EIF4A1 (G-quadruplex structure binding)	HCC	(84)
Rao *et al*, 2025	tRF-3040b	Downregulated by TEC	None	PINK1/PRKN-mediated mitophagy	NASH	(144)
Li *et al*, 2026	tRF-31R9J	Upregulated by TEC	None	histone deacetylase 1, histone H3K18 lactylation/acetylation, pro-ferroptosis genes	NASH	(145)
Cheng *et al*, 2022	tRF5-GlyGCC	Endogenous oncogenic tsRNA	Radiotherapy + tRF5-Gi@HOP nanocomposite *in situ* hydrogel	KDM6B/Runx2/ ITGBL 1/S100A9, NK cell cytotoxicity	HCC	(87)
Yang *et al*, 2024	tsr-019759 (tRNAVal-AC-2-1)	Endogenous oncogenic tsRNA	PEI-modified PLGA nanoparticles coated with HCC CCM	Reciprocal crosstalk between HCC cells and M2 TAMs	HCC	(146)

HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; tsRNA, transfer RNA-derived RNA; TEC, tectorigenin.

profiles of tRF-1-S25 and tRF-5-V31, which regulate sinusoidal capillarization by targeting key proteins including DUSP1 and FAK (148). Importantly, systemic administration of inhibitors directed against these tsRNAs rescues liver fibrosis in mice, indicating that NSun2-dependent tRNA modifications give rise to tsRNAs that suppress sinusoidal capillarization. Thus, modulating the DUSP1/FAK/p-FAK axis represents a novel therapeutic strategy for liver fibrosis (148). In HCC, 5'-tiRNA-Gln serves as a tumor suppressor by interacting with eukaryotic initiation factor 4A1 (EIF4A1) through its G-quadruplex structure, inhibiting its RNA unwinding function. This suppresses translation of key oncoproteins ARAF, MEK1/2 and STAT3, thereby blocking tumor-promoting pathways. As a result, the overexpression of 5'-tiRNA-Gln inhibits the proliferation, migration and invasion of HCC cells. The expression significance of 5'-tiRNA-Gln in HCC was found to be negatively related with both these oncoproteins in HCC tissues thus indicating its therapeutic potential (86). Furthermore, 5'-tRF-GlyCCC, which serves as the essential functional component of human bone marrow mesenchymal stem cell-derived EVs (BMSC-EVs), directly binds to the 3'-UTR of FoxO3. This interaction leads to the downregulation of gluconeogenic genes as well as lipogenic genes, leading to the mediation of the anti-MAFLD effects of BMSC-EVs (149) (Table II).

Besides being direct therapeutic targets, tsRNAs also act as key regulatory targets for natural bioactive compounds in hepatic disease treatment. In NASH, the natural compound TEC demonstrates therapeutic potential. On one hand, TEC mitigates hepatocyte injury by downregulating tRF-3040b, which promotes PINK1/PRKN-mediated mitophagy and subsequently inhibits the GSDME/NLRP3 inflammasome-activated pyroptosis pathway, thereby alleviating inflammatory damage (150). On the other hand, TEC upregulates tRF-31R9J. This fragment directly binds to histone deacetylase 1, reducing histone H3K18 lactylation and acetylation levels, which in turn suppresses the transcriptional activation of ferroptosis-promoting genes (ATF3, ATF4 and CHAC1) and ultimately leads to decreased ferroptosis in hepatic cells (151). Collectively, TEC can target different tsRNAs and synergistically regulate pyroptosis and ferroptosis, the two programmed cell death patterns. This dual regulation, on the one hand, blocks hepatic lipid accumulation, and on the other hand, alleviates inflammatory responses and enhances hepatocyte survival, thereby demonstrating multi-dimensional effects in the treatment of NASH. The aforementioned results not only deepen the understanding of tsRNA-mediated regulatory mechanisms in liver lesions but also lay a new theoretical foundation for multi-target combined intervention strategies centered on tsRNA (Table II).

Most patients with HCC present with advanced disease that is unamenable to curative surgical resection or localized percutaneous tumor ablation, creating an urgent clinical need to develop effective combinatorial therapeutic strategies. Studies

have confirmed that tsRNAs can be combined with various therapeutic approaches to significantly enhance the efficacy of hepatic disease treatment. Among these, the combination of radiotherapy and targeting tRF5-GlyGCC has been proven to be a promising optimized adjuvant therapy for preventing post-operative HCC recurrence. Radiotherapy plus tRF5-GlyGCC targeting may optimize postoperative HCC adjuvant therapy. tRF5-GlyGCC binds KDM6B, activating Runx2 to drive ITGBL1 and S100A9 expression. ITGBL1 directly, and S100A9 indirectly (via myeloid-derived suppressor cells), suppress NK cell elimination activity, weakening antitumor immunity. A tRF5-GlyGCC inhibitor reverses this, restoring NK cell cytotoxic capacity and synergizing with radiotherapy to prevent HCC recurrence (89).

The aforementioned studies have demonstrated that tsRNA-targeted therapy has considerable potential for the treatment of liver diseases; however, its clinical translation still faces major challenges. Functional small RNA molecules are readily degraded by nucleases *in vivo* and lack efficient tissue-targeted delivery strategies, thereby limiting their therapeutic efficacy. Although conventional nanomedicine delivery systems have improved drug stability to a certain extent, they remain associated with immune clearance and toxicity, which hinders precise and sustained intervention in hepatic lesions. Therefore, the development of novel, highly efficient and safe tsRNA-specific delivery systems is essential to promote the clinical translation of tsRNA-targeted therapy (152). Recently, biomimetic nanotechnology and stimuli-responsive material design have been employed to construct various engineered nano-delivery platforms for tsRNA. These platforms not only effectively address the *in vivo* stability and targeted accumulation of tsRNA, but also enable precise temporal regulation of specific pathological signaling pathways, thereby providing innovative strategies for personalized combination therapy of liver diseases (153). Gong *et al* (89) developed a tRF5-Gi@HOP nanocomposite powder that forms a water-responsive hydrogel for sustained local liver release, blocking the tRF5-GlyGCC/KDM6B/Runx2 pathway to reverse NK cell immune suppression and inhibit abdominal adhesion, providing a targeted and safe tsRNA strategy for postoperative HCC. Delivery efficiency was verified by drug loading and 24-day *in vitro* release; local retention by rheological, adhesive, and morphological tests; and *in vivo* efficacy by radiosensitization, tumor recurrence, and immune infiltration. Biosafety was assessed by hepatocyte viability, hemolysis, blood biochemistry, multi-organ pathology, subcutaneous degradation and wound staining (89). A second system, in-tsr/PEI/PLGA@CCM (HCC cell membrane-coated, PEI-modified PLGA nanoparticles), was shown to disrupt HCC cell-M2 TAM crosstalk and suppress HCC progression in both subcutaneous and orthotopic mouse models. Delivery efficiency was evidenced by tumor accumulation, while biosafety was assessed by serum biochemistry (ALT/AST), and histopathological examination of major organs (154) (Table II). Collectively, these tsRNA-based delivery systems illustrate the potential of personalized treatment. However, their translational prospects depend on systematic evaluation of delivery efficiency and biosafety using the aforementioned standardized indicators. Future studies should report quantitative biodistribution, hepatocyte uptake, target knockdown, ALT/AST, liver histopathology and immunogenicity to enable meaningful cross-study comparisons.

6. Challenges and knowledge gaps

tsRNAs are an emerging class of regulatory sncRNAs with diverse molecular functions, but their translational application in hepatology remains in its infancy. In the present review, evidence indicating that tsRNAs are not merely degradation products of tRNA turnover but functional molecules involved in hepatic gene regulation, stress responses and inter-organ communication, was consolidated. Although high-throughput sequencing, bioinformatics and cell-based experiments have been the principal strategies used to interrogate tsRNA-liver disease associations (155), critical appraisal of the current literature reveals that, while certain foundational principles are becoming established, substantial controversies, conflicting evidence, and unresolved mechanistic gaps continue to impede clinical translation.

A broad consensus now exists that tsRNAs are differentially expressed across the spectrum of hepatic diseases, including HCC, MASLD, ALD and viral hepatitis, and that they participate in core pathological processes such as lipid metabolism, immune evasion and fibrogenesis. Similarly, the regulatory role of tRNA modifications, particularly m5C methylation by NSun2 and DNMT2, in governing tsRNA biogenesis is well accepted across multiple tissues (156). Nevertheless, when these general principles are applied to specific hepatic contexts, the evidence becomes markedly contradictory.

The most striking contradiction concerns the tissue-specific consequences of tRNA modification loss. In the liver, reduced NSun2 activity generates protective tsRNAs, such as tRF-Gln-CTG-026, which promotes hepatocyte survival after injury, and other NSun2-dependent fragments that suppress sinusoidal capillarization and fibrosis. Conversely, in epidermal tissues, NSun2 deficiency suppresses tumor formation (157), whereas ALKBH3-mediated removal of m1A and m3C accelerates ANG-dependent cleavage and yields pro-tumorigenic tsRNAs (158). These opposing outcomes suggest that identical epitranscriptomic modifications can be protective in one cellular environment and pathogenic in another. Whether this discrepancy reflects differences in baseline enzyme expression, substrate tRNA availability, cell-type-specific downstream targets, or divergent signaling landscapes remains unexplored in hepatic systems. Crucially, nearly all liver-derived evidence originates from hepatocyte-centric or bulk-tissue analyses, despite growing recognition that tumor-derived tsRNAs can reprogram tumor-associated macrophages and that lineage-specific PUS7 circuits operate in stem cells. Whether NSun2, DNMT2, ALKBH3 and PUS7 exert consistent or opposing activities across hepatic parenchymal and non-parenchymal cell populations, and whether the same modification event can elicit divergent effects depending on the recipient cell, is a fundamental unresolved question.

A second area of contradiction lies in the dualistic role of tsRNAs in tumorigenesis. Some fragments, such as Gly-tRF, tRF-34, and 5'-tRF-Gly, act as oncogenic drivers by activating AKT, MEK/ERK and CEACAM1 signaling (79-81), whereas others, including HCETSR and 5'-tiRNA-Gln, exert

tumor-suppressive effects by disrupting β-catenin complex stability or inhibiting EIF4A1-dependent translation (83,86). This functional dichotomy is not merely a matter of different tsRNAs having different roles; it raises a deeper controversy about whether tsRNA-mediated regulation follows predictable rules based on sequence, subcellular localization, or AGO-loading preference, or whether biological output is predominantly context-dependent. Notably, numerous proposed oncogenic or tumor-suppressive mechanisms have been validated only in single cell lines or patient cohorts, leaving it unclear whether contradictory findings reflect true biological heterogeneity or methodological artifacts such as cell-line-specific expression backgrounds and off-target effects of tsRNA mimics.

Beyond conflicting experimental results, several conceptual controversies persist. First, the relative importance of AGO-dependent vs. AGO-independent mechanisms remains unresolved. Early studies emphasized tsRNA-mediated gene silencing through AGO1-4 loading and RISC assembly, analogous to miRNAs (20). However, subsequent work demonstrated that numerous tsRNAs bind preferentially to AGO1, AGO3 and AGO4 rather than AGO2, and that a substantial fraction exerts regulatory effects through non-canonical pathways, such as displacing RNA-binding proteins (for example, YBX1) or modulating translation initiation factors (22,44). The critical unresolved controversy is whether AGO-associated tsRNAs represent the primary functional pool in hepatocytes or whether protein-displacement and ribosome-interaction mechanisms dominate in liver pathophysiology. The limited number of tsRNAs with validated AGO interactions, combined with evidence that numerous tsRNAs exhibit weak AGO binding (17), challenges the assumption that tsRNA function is predominantly miRNA-like.

Second, the physiological significance of circulating tsRNAs remains contentious. Although their stability in serum and plasma, whether encapsulated in EVs or bound to ribonucleoproteins, has fueled enthusiasm for liquid biopsy applications, it is debated whether these circulating fragments are actively secreted as intercellular signaling molecules or passively released as byproducts of cellular injury and tRNA turnover. This distinction is not merely semantic: If circulating tsRNAs are primarily damage-associated molecular patterns, their therapeutic value is limited, whereas active secretion would support their role as druggable mediators of inter-organ crosstalk. The observation that bacterial-derived tsRNAs in outer membrane vesicles can regulate host gene expression, and that host tsRNAs can modulate gut microbiota composition, supports an active signaling role in the gut-liver axis. However, the reverse pathway, whether liver-derived tsRNAs actively shape the intestinal microenvironment (159,160), remains entirely speculative, and system-level evidence for tsRNA-mediated liver-heart or liver-brain communication is virtually absent.

Several knowledge gaps must be addressed to move the field from descriptive association to mechanistic understanding and clinical utility.

First, at the cellular level, the cell-type-specific tsRNA modification landscape in the liver is unknown. The liver comprises hepatocytes, Kupffer cells, hepatic stellate cells and sinusoidal endothelial cells, each with distinct epitranscriptomic machinery and stress responses. Current bulk-tissue or hepatocyte-centric studies cannot resolve whether observed tsRNA alterations originate from parenchymal cells, immune cells, or stromal components, nor can they distinguish cell-autonomous effects from paracrine signaling. Furthermore, although tsRNAs are implicated in immune regulation, such as tRF5-GlyGCC-mediated NK cell suppression and tsRNA-10105-driven M2 macrophage polarization (87,88), the precise molecular targets and receptor systems on immune cells remain unidentified. At the inter-organ level, the gut-liver axis has emerged as a key frontier, yet major gaps persist. While bacterial tsRNAs delivered via outer membrane vesicles can activate host MAPK signaling (116), and host tsRNAs can selectively inhibit pathogen growth (for example, *F. nucleatum*) without harming commensals (for example, *S. mitis*) (114), the bidirectional regulatory logic remains opaque. Whether hepatic tsRNAs are secreted into bile or systemic circulation to modulate gut microbiota, and whether gut-derived tsRNAs contribute to extrahepatic organ dysfunction beyond the liver, are critical unanswered questions. Similarly, the cardio-hepatic communication axis, in which steatotic hepatocyte-derived EVs deliver ncRNAs to cardiomyocytes, has been demonstrated for miRNAs (118) but not for tsRNAs, representing a significant cognitive gap at the systems level.

Second, with respect to technical gaps, the methodological limitations of tsRNA detection constitute a major barrier to both discovery and clinical translation. Standard small RNA sequencing systematically under-represents heavily modified tsRNAs because m1A, m1G, m22G and m3C modifications block reverse transcription and adapter ligation. Although PANDORA-seq and mim-tRNAseq have begun to address this bias, these platforms are not interchangeable: PANDORA-seq erases modification information during enzymatic treatment, precluding simultaneous quantification of abundance and modification status, whereas mim-tRNAseq requires substantial RNA input (≥0.5 μg) and is restricted to mature tRNA-derived fragments, excluding pre-tRNA species. Nanopore-based direct RNA sequencing preserves native modifications but suffers from high error rates, large input requirements, and inefficient capture of molecules shorter than 100 nt, rendering it impractical for most tsRNAs in routine clinical settings (139,143). Consequently, no single platform currently provides a standardized, modification-aware, and clinically feasible workflow for tsRNA profiling. Moreover, EV isolation methods significantly impact both yield and RNA composition, yet no consensus exists on optimal protocols for hepatic biofluids. The majority of circulating tsRNAs are not EV-encapsulated but associated with extra-vesicular ribonucleoproteins, yet most biomarker studies have focused exclusively on exosomal fractions, potentially introducing systematic bias. The lack of standardized blood processing protocols, reference ranges for healthy populations, and accounting for confounding variables further undermines the reliability of circulating tsRNA signatures.

Third, regarding translational gaps, perhaps the most critical gap is the near-universal absence of large-scale, multicenter, prospective validation. Nearly all diagnostic tsRNA signatures for HCC, such as hsa_tsr014055 (128), tRF-23-R9J89O9N9 (129) and tRF-Gln-TTG-006 (133), have been derived from single retrospective cohorts with small

sample sizes. Estimating diagnostic accuracy within the discovery cohort risks substantial overfitting. Furthermore, most studies compare diagnosed patients against healthy controls, a design that artificially inflates sensitivity and specificity by excluding patients with early-stage disease, competing liver pathologies, or non-malignant liver lesions. Whether these signatures maintain performance in real-world scenarios, where distinguishing HCC from cirrhosis, CCA, or hepatic adenoma is clinically essential, remains unproven. An equally important translational gap lies in establishing causality vs. correlation. Although numerous studies demonstrate that tsRNA levels correlate with disease severity, prognosis, or histological grade, few have rigorously established that manipulating a specific tsRNA alters disease trajectory *in vivo* through a defined molecular target. Several proposed diagnostic tsRNAs still lack validated downstream targets, raising the possibility that their altered expression reflects epiphenomenal tRNA turnover rather than causal pathogenic drive. Without target validation and mechanistic clarity, the rationale for tsRNA-targeted therapeutics remains speculative.

Addressing these controversies and gaps requires a coordinated research agenda. At the mechanistic level, cell-type-specific and spatially resolved tsRNA profiling, ideally combined with single-cell epitranscriptomics, is needed to resolve the contradictory roles of modification enzymes across hepatic cell lineages. Manipulating NSun2, DNMT2, or ALKBH3 in specific cell populations (for example, hepatocytes vs. Kupffer cells) followed by functional rescue experiments would clarify whether tissue-specific effects reflect intrinsic cellular wiring or paracrine crosstalk (39-41). At the validation level, the field must move beyond descriptive biomarker discovery toward rigorous external validation. Future studies should adopt prospective cohort designs, include clinically relevant comparator groups (for example, cirrhosis without HCC and mixed-etiology liver disease), and standardize pre-analytical variables including fasting status, time of collection and EV isolation protocols. Integration of tsRNA signatures with established markers (AFP, PIVKA-II and DCP) should be evaluated in independent populations to assess additive diagnostic value rather than isolated performance (126-131). At the therapeutic level, mechanistic studies must prioritize target identification and causal validation. For tsRNAs with therapeutic potential, such as 5'-tiRNA-Gln in HCC (86) or tRF-Gln-CTG-026 in liver injury (65), defining their direct binding partners (RNA, protein, or ribonucleoprotein complexes) and resolving their subcellular sites of action is essential. The development of tsRNA-specific delivery systems, such as the HCC cell membrane-coated PLGA nanoparticles (in-tsr/PEI/PLGA@CCM) or the water-responsive hydrogel tRF5-Gi@HOP (89), represents promising progress. However, systematic evaluation of hepatocyte uptake efficiency, target knockdown specificity, off-target effects on physiological tsRNA networks, immunogenicity, and long-term biosafety, quantified by standardized metrics including biodistribution, ALT/AST profiles and liver histopathology, remains incomplete. Without such standardized benchmarking, cross-study comparison and regulatory approval will remain elusive.

Finally, the inter-organ dimension of tsRNA biology demands focused investigation. Bidirectional tsRNA trafficking along the gut-liver axis, and the potential role of liver-derived tsRNAs in remote organ dysfunction (for example, heart failure with preserved ejection fraction in MASLD), should be mapped using multi-omics integration and organoid co-culture systems. Such approaches will determine whether tsRNAs are merely biomarkers of inter-organ disease or active mediators amenable to therapeutic interception.

7. Conclusion and future perspectives

In conclusion, tsRNAs have emerged as biologically active molecules with considerable, but currently unrealized, potential in hepatology. The functional effects of tsRNA do not occur randomly; rather, they stem from a precisely regulated biological production process. tRNA and its precursors are cleaved at specific sites by nucleases, including angiogenin, Dicer and RNase Z/ELAC2. This process is further regulated by epigenomic transcriptome modifications, such as m^5C and pseudo-uridine, resulting in the generation of distinct tsRNA fragments with specific regulatory characteristics. These fragments subsequently exert specific biological functions through various molecular mechanisms, including classic AGO-dependent gene silencing, ribosome binding, RNA-binding protein displacement, and transcriptional feedback regulation. In the liver, tsRNA can enter liver cells to regulate gene expression, translation and stress responses. Increasing evidence indicates that fluctuations in tsRNAs expression are closely related to the occurrence and progression of liver diseases: In HCC, tsRNA drives tumor progression through oncogenic signal transduction and immune escape; in dysfunction-associated steatotic liver disease, tsRNA disrupts lipid homeostasis and autophagy; in ALD, the interaction between complement and tsRNA amplifies pathological damage. In viral hepatitis, the RNA cross-dialogue between the host and the pathogen determines the disease outcome. The diversity of these mechanisms makes tsRNAs not only a promising non-invasive biomarker for early diagnosis and prognostic assessment but also a potential drug target that can be intervened through therapeutic inhibition, stimulation, or engineered nano-delivery. Although their differential expression across liver diseases and their mechanistic versatility in gene regulation are increasingly well documented, the field is hampered by conflicting evidence on the tissue-specific consequences of tRNA modifications, unresolved controversies regarding canonical vs. non-canonical mechanisms, and substantial translational gaps in validation, standardization, and delivery. Moving tsRNAs from promising research findings to trustworthy clinical markers and therapeutic targets will require integrated, multicenter, mechanism-driven investigations that explicitly address these contradictions and gaps, rather than further single-study descriptions.

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Authors' contributions

YCa was chiefly responsible for the composition, review, and revision of the manuscript. YCh organized the references and generated the tables and figures. FY established the structural outline of the manuscript. YY prepared the original manuscript. TJ participated in the review and critical editing of the manuscript for significant intellectual content. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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