PIWI-Interacting RNAs (piRNAs) and Cancers

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The P-Element-induced wimpy testis (PIWI)-interacting RNAs(piRNAs) were first described in 2001 in experiments usingDrosophilamodels evidencing their role in fertility. Then, piRNAs were involved in the function of mammalian germ cells. During the last two decades, researchers have described piRNAs as small RNA molecules able to bind PIWI proteins to form piRNA/PIWI complexes, which act as mediators in several processes including transposon silencing, spermiogenesis, genome rearrangement, epigenetic regulation, protein regulation, and germ stem-cell maintenance in both normal and abnormal cells.

PIWI-interacting RNAs (piRNAs) | gastrointestinal cancers (GI cancers) | prognostic biomarkers | diagnostic biomarkers | therapeutic targets

1. The Functions of the piRNA/PIWI Complex

PiRNAs can bind PIWI proteins to form piRISCs, which can influence transposon silencing, genome rearrangement, epigenetic regulation of gene expression, and protein regulation. Therefore, these molecules can be involved in physiological processes such as germ stem-cell maintenance, gametogenesis, etc., and in pathological processes such as carcinogenesis.

1.1. Functions of the piRNAs/PIWI Complex in the Transcriptional Gene Silencing of Transposons and Other Genes

In mammalian cells, there are many repetitive pieces of DNA named transposons (also known as transposable elements (TEs)), which can move within the genome (a phenomenon called transposition), exerting a series of actions to modify the cell phenotype [1]. These TEs can be subgrouped into two major classes: (1) DNA transposons, which can excise themselves from the genome, move as DNA, and insert themselves into new genomic sites, and (2) Retrotransposons, which are DNA transposons originated from RNA intermediates that were first transcribed and then reverse transcribed to be finally inserted at new genomic locations [2].

In general, the uncontrolled expression and transposition of TEs are usually considered to be a threat to the genome integrity because these elements can be independently copied or moved from their original position to be then inserted into an atypical site of the genome, producing interference with normal sequences and significantly increasing the risk of developing gene mutations [3][4]. Since piRNA clusters harbor a large number and variety of transposons, it is easy to assume that piRNAs are able to regulate primarily the activity of these transposons. For this reason, the piRNA/PIWI complexes (or piRISCs) are usually considered as “the immune system of the cells” as they regulate the expression levels and transposition capability of TEs across the genome [5][6].

Since DNA transposons are currently not mobile in the human genome [2], the transposon silencing effect of piRNA/PIWI complexes is achieved mainly by repressing the expression of retrotransposon RNAs at both transcriptional and posttranscriptional levels [7]. Transcriptional silencing is mediated by nuclear piRISCs that contain PIWI proteins (primary piRNAs), whereas posttranscriptional silencing is mediated by cytoplasmic piRISCs that contain Aub or AGO3 proteins (secondary piRNAs) [6].

In transcriptional silencing, PIWI proteins of piRNA/PIWI complex combine with downstream effectors Panoramix (Panx/CG9754) and Asterix (DmGTSF1) to induce transcriptional gene suppression by recruiting silencing machinery components on the source locus [7]. Other downstream effectors are the protein Eggless (Egg) and its co-factor Windei (Wde), which add the repressive H3K9me3 marks to the target DNA region; meanwhile, lysine-specific demethylase 1 (Lsd1) removes the activating H3K4me2 marks from DNA promoter regions, causing the subsequent inhibition of RNA Pol II transcription [8]. Then, heterochromatin protein 1 (HP1) provokes heterochromatin formation in specific DNA sites that prevents the functioning of the transcription machinery. At the same time, piRNA/PIWI complex recruits DNA methyltransferase (DNMT) to methylate CpG sites of specific genes located in nearby regions that reduce their probability to be transcribed [9][10]. Probably, there are other simultaneous and synergic mechanisms but many of them are still unknown.

1.2. Functions of piRNAs/PIWI Complex in the Post-Transcriptional Gene Silencing

In the post-transcriptional control of gene expression, piRNA/PIWI complexes can bind to coding RNAs (mRNAs) or non-coding RNAs (e.g., lncRNAs, pseudogenes, etc.) that are transcribed from DNA [10]. Here, the piRNA/PIWI complexes bind target RNA through effective piRNA:RNA interactions formed by a strict base pairing within 2–11 nt at the 5′-end of the piRNA that is part of the piRISC (perfect pairing) or by a less strict base pairing within 12–21 nt (imperfect pairing) [11], in a similar manner as occurs with the miRNA silencing mechanism. When base pairing is done, the proteins that are part of the RISC portion of piRISC, that is, comprised of different types of protein among cell types and species, work together in order to produce cleavage or deadenylation of the target RNA, which will result in target RNA decay [12][13].

This same mechanism of post-transcriptional silencing can be also exerted on TEs to maintain genome integrity [12][14]. In fact, the ping-pong mechanism for piRNA amplification uses a similar process to selectively detect and slice the RNAs from transposons, inducing a post-transcriptional silencing of TEs [15][5].

1.3. Functions of piRNAs/PIWI Complex in the Interaction with Proteins

The piRNA/PIWI complexes can also directly bind some proteins either through piRNAs or through the PAZ domain from PIWI proteins. This relationship between piRISCs and proteins can promote multi-protein interactions within cells, leading to the activation of a specific signaling pathway. For example, piRISCs can also influence importantly in the subcellular localization of the protein, producing unexpected loss or gain function [16][10][17]. Interestingly, as will be described next, these piRISC-protein interactions can directly or indirectly trigger the activation of pro-carcinogenic proteins or the inactivation of tumor-suppressor proteins, as occurs in most cancer tissues, via some post-translational modifications such as phosphorylation, among others [18][19].

2. The Role of piRNAs in Gastrointestinal (GI) Cancers

2.1. Gastric Cancer (GC)

Gastric cancer (GC) is a multifactorial disease, where certain environmental and genetic factors can increase the risk of developing this neoplasm, e.g., *Helicobacter pylori* infection (the most important risk factor), Epstein-Barr virus (EBV) infection, diet, alcohol consumption, smoking, and family history of GC (mainly linked to *CDH1* gene alterations) [20]. The poor prognosis of GC and the need for early diagnostic markers for this malignancy are evidenced in the fact that most of the patients are diagnosed in an advanced stage where the rate of median survival is less than 12 months [21].

2.1.1. The piRNAs as Prognosis Markers and Regulators of Gastric Cancer (GC)

Several piRNA-related studies have suggested that these transcripts can be implicated in the development and/or prognosis of GC. For example, Chen et al. described a microarray analysis carried out in four samples of GC tissues and four paired non-tumoral adjacent tissues (NAT), in which piR-651 was found to be upregulated in GC tissues. These results were validated in another sample cohort, evidencing those levels of piR-651 were even higher in those tumors with greater TNM stages. Interestingly, Chen et al. also found that piR-651 expression was higher in samples of colon, lung, and breast cancer, as well as in cell lines of hepatic carcinoma (HepG2), cervical cancer (HeLa), breast cancer (Bcap-37), mesothelioma (MSTO211H), lung cancer (NCI-H446), and gastric cancer (MGC-803 and SGC7901). Subsequently, the piR-651 expression was modulated via an inhibitor transfected into two GC cells lines (MGC803 and SGC-7901 cells) to evaluate how this transcript influences the cell growth and cell cycle in these cells. The results confirmed that piR-651 promotes cell growth in GC cells by inhibiting the cell cycle arrest at the G2/M phase. Therefore, piR-651 could be potentially involved in the development of GC and could be considered as a candidate diagnostic marker for GC [22].

Later, Cheng et al. published another study in which piR-823 expression was found repressed in GC tumors and GC cell lines MGC-803 and SGC-7901 compared to the corresponding controls. In vitro analyses showed that the ectopic increase of piR-823 in GC cells reduced cell growth in both cell lines in a dose-dependent manner compared to controls. In vivo experiments also evidenced a significant decrease in tumor volumes and tumor weight in nude BALB/c mice xenografted with MGC-803 cells transfected with different doses of piR-823 mimics, which strongly suggests that piR-823 could act as a tumor-suppressor piRNA in the cell by avoiding the tumor formation [23].

In 2016, Martinez et al. analyzed the RNA-sequencing (RNA-seq) libraries of 320 gastric adenocarcinomas and 38 non-malignant tissues obtained from The Cancer Genome Atlas (TCGA) Project datasets in order to evaluate the expression of small RNAs and their association with clinicopathological variables. About 156 piRNAs were significantly deregulated in GC and most of them were overexpressed in GC, suggesting a potentially important role in GC. Interestingly, 70.6% of these piRNAs were not originated from known human piRNA clusters but they were mainly derived from protein-coding sequences, which have been associated with cis- and trans-regulatory effects on protein-coding transcripts in diverse species. The authors also found that lower piR-FR222326 expression was associated with poor overall survival (OS), whereas the higher expression of five of the selected piRNAs was significantly associated with lower recurrence-free survival (RFS). These RFS-associated piRNAs were then grouped into a multi-piRNA panel to evaluate the RFS prediction level in a GC cohort. Results showed that the piRNA panel composed of piR-FR290353, piR-FR064000, and either piR-FR387750 or piR-FR157678 was able to effectively stratify GC patients into low-risk and high-risk recurrence groups, demonstrating that certain piRNAs, as well as other non-coding RNAs, can be associated with GC patient outcome [24].

Further analyses evaluated the usefulness of this piRNA panel in the RFS data of nine additional tumor types. In this regard, the panel tended to behave similarly in colon cancer, suggesting conserved importance to digestive tract malignancies. Next, the authors assessed whether DNA copy number was associated with expression changes of the five piRNAs associated with RFS in the same TCGA cohort, confirming that, effectively, the alteration in the copy number observed in piR-FR381169, piR-FR290353, and piR-FR064000 loci was significantly associated with expression alterations, suggesting genetically selected mechanisms of deregulated piRNA expression in these cases. Finally, researchers validated expression levels of this RFS-related piRNA panel in an independent cohort composed of sequencing libraries from 25 GC belonging to the Gene Expression Omnibus (GEO) dataset, whose results were subsequently compared to those found in the TCGA cohort. The results showed that the piRNA panel composed of piR-FR290353 and piR-FR387750/piR-FR157678 was still useful to accurately predict RFS in GC cases [24].

2.1.2. The piRNAs as Diagnostic Markers in Gastric Cancer (GC)

A study by Cui et al. assessed the expression levels of piR-651 and piR-823 in the peripheral blood of 93 GC patients and 32 healthy volunteers, finding that both piRNAs were significantly lower in the blood of GC patients. In fact, piR-651 levels were even lower in the blood samples of patients with gastric signet ring cell carcinoma; meanwhile, the piR-823 levels were associated with cases of advanced T stage and presence of distant metastases. Interestingly, the levels of both piRNAs were found lower in the blood of postoperative patients than those from preoperative patients, which allows these piRNAs to be considered for the follow-up of patients after a determined treatment. Then, the diagnostic usefulness of these transcripts was assessed through the receiver operating characteristic (ROC) curves that showed “area under the curve“ (AUC) values of 0.841 for piR-651, 0.812 for piR-823, and 0.860 for the combination of both piRNAs, which were better than those AUC values obtained by the same group for other small RNAs (miR-106a and miR-17). In addition, comparing the positive detection rates, piR-651 and piR-823 showed to be more sensitive than serum measuring of routine tumor markers CEA and CA19-9. These robust data suggest that both piRNAs might be valuable blood biomarkers to distinguish GC patients from healthy subjects with high sensitivity and specificity [25].

Other piRNAs with a potential role as biomarkers are piR-018569, piR-004918, and piR-019308, which were evaluated in serum exosomes from 70 GC patients and 60 healthy donors, being found significantly elevated in the GC cases. In particular, piR-004918 and piR-019308 had significantly higher expression levels in the blood of patients with metastasis. The ROC curves evidenced that serum levels of piR-019308, piR-004918, and piR-018569 were able to distinguish between GC patients and healthy individuals, with AUC values of 0.820, 0.754, and 0.732, respectively. These AUC values were even better than those obtained by the routine tumor markers CEA, CA19-9, and AFP (AUC values of 0.689, 0.687, and 0.634, respectively). In fact, when piR-019308 or piR-004918 or piR-018569 were evaluated in combination with CEA and CA19-9, the AUC of each biomarker panel reached values of 0.914 ($p < 0.0001$), 0.859 ($p < 0.0001$), and 0.868 ($p < 0.0001$), respectively. Therefore, these data suggest that piR-019308, piR-004918, and piR-018569, mainly piR-019308, combined with CEA and CA19-9 can serve as reliable markers to detect GC [26].

2.2. Colorectal Cancer (CRC)

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide and the second cause of cancer deaths, with ~1.80 million new cases and ~880,000 deaths per year, respectively [20][27]. These CRC cases are mainly sporadic (70–80%) and have been associated with risk factors such as age and lifestyle without a family history of disease or genetic predisposition [28]. The evidence has demonstrated that CRC is a heterogeneous disease and its pathogenesis involves the activation of oncogenes and inactivation of tumor-suppressor genes, which are mostly the result of genetic mutations and epigenetic alterations, the latter including DNA methylation, histone modification, and non-coding RNAs (ncRNAs) [29].

2.2.1. The piRNAs as Prognosis Markers and Regulators of Colorectal Cancer (CRC)

Various piRNAs have been involved in the regulation of the colorectal carcinogenic process. One of these piRNAs is piR-823, which was found significantly upregulated in the CRC tissues compared to non-tumor adjacent tissues. This higher piR-823 expression was correlated with poorly differentiated tumors [30]. In vitro experiments showed that inhibition of piR-823 resulted in suppression of cell proliferation and colony formation, cell cycle arrest in the G1 phase, and induction of cell apoptosis in CRC cell lines HCT116 and DLD-1, whereas overexpression of piR-823 promoted cell proliferation in normal colonic epithelial cell line FHC. Interestingly, piR-823 was shown to increase the transcriptional activity of HSF1 (a common transcription factor of some members of the heat shock protein (HSP) family such as HSP27, HSP60 and HSP70) by inducing the phosphorylation of HSF1 at Ser326 [30]. Another study conducted by Sabbah et al. evaluated the piR-823 expression levels in tissues and serum of CRC patients. In the case of tissue samples, the authors confirmed the overexpression of piR-823 in CRC tissues and its association with those poorly differentiated tumors. In addition, they also found that higher piR-823 levels were associated with advanced TNM stages (III–IV) [31]. Therefore, both studies suggest that piR-823 can act as a potential prognostic marker and as a promising therapeutic target for CRC [30][31]. Sabbah et al. also studied piR-823 as a potential diagnostic biomarker in CRC and their results will be shown later.

In the case of Weng et al.'s study, they performed RNA-seq experiments and subsequent validations that resulted in the identification of piR-1245 as a significantly upregulated piRNA in CRC tumors. Interestingly, these elevated piR-1245 levels significantly correlated with poor differentiation, advanced T stage, and the presence of lymph node metastasis and distant metastasis in CRC patients. Furthermore, patients with higher expression of piR-1245 also showed significantly poor overall survival (OS). Functional experiments performed in CRC cell lines (HCT116 and SW480 cells) helped to determine that piR-1245 acts as an oncogene by promoting tumorigenesis via increasing colony formation and cell proliferation. The gene expression profiling and the analysis of piRNA:mRNA base-pairing interactions found that certain tumor-suppressor genes such as ATF3, BTG1, DUSP1, FAS, NFKBIA, UPP1, SESN2, TP53INP1, and MDX1 are direct targets of piR-1245. These targets were then validated through correlation analyses between the piR-1245 expression and the expression of each candidate gene, reaffirming the likely oncogenic role of piR-1245 and proposing this piRNA as a potential independent prognostic marker in CRC [32].

Other piRNAs also evaluated in CRC cases are piR-18849, piR-19521, and piR-17724, which have been found upregulated in tumors. No associations were found between piR-17724 expression and clinicopathological features. However, the increased piR-18849 expression was associated with poorly differentiated tumors and the greater presence of metastasis in lymph nodes; meanwhile, the higher expression of piR-19521 was associated with poorly differentiated CRC tumors, suggesting the probable usefulness of piR-18849 and piR-19521 as prognostic biomarkers for CRC patients [33].

Recently, Iyer et al. conducted a systematic transcriptomic discovery based on RNA-seq followed by a validation of piRNAs using two different clinical cohorts. This study identified piR-24000 as a small RNA markedly overexpressed in CRC. Interestingly, the high expression of piR-24000 was significantly associated with moderate and poor tumor differentiation, presence of distant metastases, and advanced tumor stage (mainly stage IV) in CRC cases. Moreover, piR-24000 overexpression had a positive but not significant association with advanced nodal metastasis and with advanced tumor invasion. These data suggest that piR-24000 may constitute an oncogene in CRC that could act as a diagnostic or prognostic biomarker or as a therapeutic target in this malignancy [34].

Although most studies evaluate the expression of piRNAs and their relationship with clinicopathological variables or their interaction with signaling pathways, other studies such as the one carried out by Chu et al. aimed to analyze the presence of single nucleotide polymorphisms (SNPs) in the sequences of certain piRNAs. These authors evaluated the expression of all known piRNAs in a cohort of CRC patients and cancer-free individuals, identifying seven common SNPs present in nine known piRNAs frequently deregulated in CRC. The results, based on an additive model, revealed that reference SNP rs11776042 in piR-015551 had a significant protective effect on the risk of developing CRC. However, this protective effect was not considered as significant after correction for multiple comparisons. Furthermore, the authors suggested that piR-015551 might be also generated from the genomic sequence of lncRNA LNC00964-3, whose expression was also significantly lower in tumors versus normal tissues; therefore, both transcripts may be involved in the development of CRC [35].

2.2.2. The piRNAs as Diagnostic Markers in Colorectal Cancer (CRC)

The usefulness of piRNAs as diagnostic markers in CRC has been assessed in several studies. For instance, Sabbah et al. evaluated the serum levels of piR-823 as a non-invasive diagnostic biomarker to detect CRC cases, finding that this piRNA was significantly increased in the serum of CRC patients compared to those samples from healthy donors. The ROC curve showed an AUC value of 0.933 for piR-823 (83.3% sensitivity and 89.3% specificity) [31], which reaffirms the idea that this piRNA not only could be considered a promising prognostic marker and therapeutic target for colorectal neoplasia but also could be used as a biomarker to detect this malignancy in the future.

Another study by Vychytilova-Faltejskova et al. carried out an RNA-seq screening to determine piRNA profiles in serum samples of CRC patients (before surgery) and healthy donors. They proportionally divided the samples into different sets, based on the TNM stage, for each investigation phase: screening (144 cases and 96 controls), training (80 cases and 80 controls), and validation (179 cases and 100 controls). As the different phases of the study progressed, piR-5937 and piR-28876 were selected as transcripts enable to differentiate CRC cases (even in stage I of disease) from healthy cases with higher sensitivity and specificity than routine CRC biomarkers CEA and CA19-9. PiR-5937 and piR-28876 were found downregulated in blood samples of CRC cases compared to healthy individuals and were inversely correlated with advanced clinical stage, in a significant manner. Interestingly, when piR-5937 and piR-28876 expression was assessed in the blood of these patients 1 month after the surgical resection, the levels of both piRNAs were increased significantly. These results indicate that although the role of piR-5937 and piR-28876 in colon carcinogenesis is not well defined, their serum levels could be useful to diagnose CRC even in early stages and to potentially carry out a postoperative follow-up of patients with resected CRC [36].

Serum samples were also used to evaluate the role of piR-54265 as a diagnostic biomarker. Mai et al. found that high levels of piR-54265 observed in serum of CRC patients significantly correlated with those values observed in CRC tumor tissues. Particularly, the piR-54265 overexpression was significantly associated with advanced tumors, the presence of metastases, and poor survival in CRC cases. Since piR-54265 was involved in the chemotherapeutic response, the authors also evaluated the correlation between serum piR-54265 levels and the curative efficacy of preoperative neoadjuvant chemotherapy composed of 5-fluorouracil and oxaliplatin in 317 CRC patients. In this regard, patients with low serum piR-54265 levels had a significantly better chemotherapeutic response than those with high serum piR-54265 levels, indicating a remarkable association between serum levels of this piRNA and disease progression or control rate of chemotherapy in CRC patients. Additionally, the results of this study evidenced that serum levels of piR-54265 accurately discriminate between those subjects whose CRC is progressive from those individuals whose CRC is non-progressive, including those clinical beneficiaries of a complete response, partial response, or stable disease after neoadjuvant chemotherapy [37].

The diagnostic usefulness of piR-54265 in serum was evaluated in more depth later by analyzing the blood of 725 CRC patients, 209 cancer-free healthy controls, 1303 patients with other types of digestive cancers, and 192 patients with benign colorectal tumors. The outcomes evidenced that serum piR-54265 levels were significantly elevated only in subjects with CRC compared to individuals from other groups analyzed, with an AUC value of 0.896 (sensitivity of 85.7% and specificity of 65.1%) to recognize CRC cases. Interestingly, serum piR-54265 levels declined substantially in those patients who underwent surgery but increased again in those cases whose tumors relapsed. On the other hand, prospective case-control analysis showed that the prediagnostic serum piR-54265 levels were significantly associated with future CRC diagnosis, with odd-ratio (OR) values of 7.23, 2.80, 2.45, and 1.24 for those CRC cases diagnosed within 1, 2, 3, and >3 years, respectively. Therefore, as a conclusion, serum piR-54265 analysis was found to be more sensitive than other CRC markers evaluated in blood, constituting a promising biomarker for CRC screening, early detection, and clinical surveillance [38]. However, a recent study by Tosar et al. suggests that piR-54265 could be actually a fragment of a full-length sequence belonging to a snoRNA called SNORD57 found in the serum of CRC patients; thus, methodological considerations must be taken to reliably analyze a determined piRNA or other small ncRNAs [39].

Another interesting study by Qu et al. also performed a three-phase study to establish a serum panel of piRNAs that may have potential clinical value in CRC cases. The screening phase consisted of an RNA-seq analysis carried out in the serum samples of 10 CRC patients with TNM I-II stage, 10 CRC patients with TNM III-IV stage, and 10 healthy controls, in which 16 downregulated piRNAs were selected to be evaluated in the training phase. This second phase allowed the selection of five piRNAs (piR-001311, piR-004153, piR-017723, piR-017724, and piR-020365) as significantly repressed in CRC cases; thus, researchers constructed two diagnostic panels to compare their screening performance during the validation phase: a piRNA-based panel (panel I) and CEA-based panel (panel II), which were evaluated separately (I vs. II) and in combination (I + II) using another independent cohort of 100 CRC patients and 100 healthy controls. Results demonstrated that the piRNA-based panel was better than the CEA-based panel to detect CRC, with an AUC value of 0.867 for this panel I (sensitivity of 78.3% and specificity of 74.2%). Accordingly, Kaplan-Meier analysis showed that patients with low serum piR-017724 levels had worse survival (OS and PFS), which was consistent with the multivariate Cox regression analysis that indicates that serum piR-017724 was an independent prognostic factor for OS and PFS. Therefore, the piRNAs that constitute the mentioned serum piRNA panel (panel I), especially piR-017724, have a promising utility not only as biomarkers for CRC detection but also as prognosis predictors at the time of diagnosis [40].

Recently, Iyer et al. also evaluated the expression of piR-24000 in blood samples of CRC patients, demonstrating a strong diagnostic accuracy of this piRNA to differentiate CRC patients from normal subjects. However, when researchers subgrouped the patients into early-stage (stages I and II) and late-stage (stages III and IV) groups, the early-stage group had lower AUC, sensitivity, and specificity values compared to those obtained from the late-stage group. These results indicate that piR-24000 may serve as a biomarker that strongly discriminates between CRC patients and control subjects [34]; however, its usefulness in early diagnosis of CRC could have a lower performance.

2.3. Pancreatic Cancer (PC)

Pancreatic cancer (PC) has ~458,918 new cases and causes ~432,242 deaths per year, being considered as the seventh leading cause of cancer-related deaths worldwide [27]. Histologically, pancreatic ductal adenocarcinoma (PDAC) constitutes 90% of PC cases and, due to the broad heterogeneity of genetic mutations and dense stromal environment, this neoplasm is considered one of the most chemoresistant cancers [41]. Certain risk factors have been associated with PC development, including tobacco smoking, diabetes mellitus, obesity, dietary factors, alcohol abuse, age, ethnicity, family history and genetic factors, *Helicobacter pylori* infection, non-O blood group, and chronic pancreatitis [42]. Unfortunately, PC patients seldom exhibit symptoms until an advanced stage of the disease, which induces a late diagnosis. For this reason, this malignancy evidences a 5-year survival rate of only 9%, becoming one of the most lethal malignant neoplasms in terms of prognosis [42]. Moreover, regarding diagnosis, some studies have stated that screening of large groups is not considered useful to detect PC at an early stage; therefore, newer techniques and the screening of tightly targeted groups, especially individuals with family history, are being evaluated [42]. In this regard, new early-detection markers and therapeutic targets could be useful to help to overcome the multiple troubles that PC has in relating to diagnosis and treatment.

In a similar manner to GBC, few studies have been involved in evaluating the expression profiles of piRNAs and their biological role in PC. An example is a study by Miller et al., who performed a massive analysis of cDNA ends (MACE) along with an RNA-seq analysis to characterize the complete transcriptome (mRNA, miRNAs, snoRNAs, lncRNAs, and piRNAs) in tissues from six PDAC patients and five non-tumoral controls. Among the many transcripts differentially expressed between PDAC cases and normal controls, the authors found that piR-017061, a piRNA transcribed from the sequence of snoRNA HBII-296A, was significantly downregulated in PDAC compared to normal pancreas tissues [43].

Later, Xie et al. continued investigating the implication of piR-017061 in PDAC. They confirmed that this piRNA was significantly downregulated in both PDAC samples and cell lines (PANC-1 and BxPC-3 cells) compared to controls (non-tumoral adjacent tissues and HPDE6-C7 cell line). In addition, those PDAC patients with higher piR-017061 expression levels had significantly better overall survival. In vitro experiments modulating the piR-017061 expression in PANC-1 and BxPC3 cells demonstrated that piR-017061 normally inhibits cell growth and clonogenicity and promotes pancreatic cell apoptosis. These results were validated on in vivo experiment using BALB/c nude mice xenografted with PANC-1 cells previously transfected with piR-017061 mimic, which developed smaller tumors with a greater number of apoptotic cells compared to controls. Conversely, in vivo experiments using piR-017061 inhibitors evidenced the opposite effects. Then, bioinformatics and molecular analyses revealed a direct interaction between piR-017061 and PIWIL1 to bind and subsequently degrade the mRNA of the *EFNA5* gene, which encodes an ephrin localized in the membrane of cells. Therefore, the loss of piR-017061 observed in PDAC cases results in the accumulation of EFNA5, which facilitates PDAC development. Hence, these data provided novel insights into PIWI/piRNA-mediated gene regulation in PDAC and provide background about a novel therapeutic strategy for this malignancy [44].

2.4. Hepatocellular Carcinoma (HCC)

Primary liver cancer is the sixth most common cancer in the world and the fourth most common cause of cancer deaths [20]. Primary liver cancer is a group of pathologically heterogeneous malignancies, including mainly hepatocellular carcinoma (HCC), as well as other less frequent neoplasms such as intrahepatic cholangiocarcinoma, mucinous cystic neoplasms, intraductal papillary biliary neoplasms, hepatoblastoma in children, angiosarcoma, etc., all of them with different underlying etiologies and carcinogenic mechanisms [20]. Hepatocellular carcinoma (HCC) constitutes ~90% of primary liver cancer, with the infections by Hepatitis-B virus

(HBV) and hepatitis-C virus (HCV) the contributors of ~80% of HCC worldwide [45]. Other risk factors are aflatoxin exposure, alcohol consumption, obesity, and non-alcoholic steatohepatitis (NASH), associated with metabolic syndrome or diabetes mellitus [46].

Few studies about piRNAs in liver cancer have been performed and these studies have focused primarily on piR-Hep1 and piR-823. Law et al. performed RNA-seq in HCC cell lines (HKCI-4 and HKCI-8 cells) and immortalized hepatocyte cell line (MIHA cells), discriminating about 171 differentially expressed piRNAs in HCC cell lines. The most interesting piRNA within this set of transcripts was piR-Hep1, which not only was confirmed as upregulated in HCC cell lines but was also found highly expressed in HCC tissues compared to normal livers and non-tumoral adjacent liver tissues. Moreover, increased piR-Hep1 expression was detected in the non-malignant adjacent tissues compared to normal liver tissues. In vitro assays in HKCI-8 and MIHA cells evidenced that piR-Hep1 overexpression promotes an increment in cell viability and cell migration and invasion, accompanied by increased AKT phosphorylation. This evidence shows that piR-Hep1 may act as an oncogenic small non-coding RNA in the hepatic tissue through the activation of AKT and its associated signaling pathways. More interestingly, since cirrhosis and chronic hepatitis are often considered precancerous lesions of HCC, and the expression of piR-Hep1 in these lesions is higher than normal liver tissues, the elevated piR-Hep1 levels may indicate the risk to develop liver cancer in an individual [47].

In another study by Rizzo et al., RNA-seq analysis was also performed in a total of 55 samples composed of 14 cirrhotic nodules (CN), 9 low-grade dysplastic nodules (LGDN), 6 high-grade dysplastic nodules (HGDN), 6 early HCC (eHCC), and 20 progressed HCC (pHCC) collected from 17 patients in order to determine piRNA expression profiles along the hepatic carcinogenic progression. This study found a pattern of 125 piRNAs capable of discriminating between HCC tissues from matched CN tissues, which also showed a correlation with the presence of microvascular invasion in HCC. Additional functional and predictive bioinformatic analyses showed that these deregulated piRNAs have interesting RNA targets, most of them genes that belong to recognized signaling pathways involved in hepatocarcinogenesis and HCC progression, on which these piRNAs could exert their function. Interestingly, 24 piRNAs showed specific expression patterns in dysplastic nodules compared to cirrhotic liver and/or pHCC. These findings suggest that certain piRNAs can be used as differential diagnostic biomarkers to determine both preneoplastic and neoplastic liver disease [48].

Among those piRNAs that Rizzo et al. found gradually increased in the progression from cirrhosis to dysplastic nodules and towards HCC, piR-823 deserves to be highlighted [48] because this piRNA has been also implicated in other cancer types including gastric and colorectal cancer and may play an important role in HCC as well. In this regard, Tang et al. investigated the role of piR-823 in the activation of hepatic stellate cells (HSCs), a cell type that usually is activated after infections, long-term alcohol consumption, cholestasis, and chronic metabolic disorders, becoming the main source of myofibroblasts that synthesize the components of extracellular matrix (ECM) that results in liver fibrogenesis, cirrhosis, and, subsequently, liver carcinoma. Effectively, these authors found that piR-823 was significantly upregulated in activated HSCs from CCL4-treated C57BL/6 mice compared to those HSCs from control mice. In fact, they proved that high piR-823 levels in LX-2 cells (a human hepatic stellate cell line) induced greater proliferation and higher levels of two pro-fibrotic proteins: α-smooth muscle actin (α-SMA) and collagen I (COL1A1). Conversely, the inhibition of piR-823 by antagomiRs significantly reduced the activity of HSCs. Pull-down assays showed that piR-823 can interact with several types of proteins, such as SFPQ, PLOD1, and EIF3B. In particular, EIF3B is a translation promoter of TGF-β1, which is a well-recognized factor in the pathogenesis of liver fibrosis; therefore, the interaction between piR-823 and EIF3B can induce a higher TGF-β1 expression that leads to greater activation of HSCs to initiate liver fibrosis. In this manner, piR-823 may be also considered a promising target in the treatment of liver fibrosis and, hence, its modulation would help to avoid the development of HCC [49].

2.5. Gallbladder Cancer (GBC)

Gallbladder cancer (GBC) is the most frequent neoplasm of the biliary tract, representing ~80–95% of biliary tract malignancies [50]. Around the world, GBC is the 22nd most incident but 17th most deadly cancer, representing 1.7% of all cancer-related deaths and constituting the fifth most common gastrointestinal neoplasms [27][50]. The incidence of GBC varies depending on the geographic region and ethnicity, being an important cause of mortality in Japan, India, and Chile, among other countries [51]. The most important risk factor described for the GBC is gallstone disease (GSD), but some environmental and lifestyle risk factors can induce the development of gallstones and the subsequent GBC formation such as age, geographical location, female gender, ethnicity, nutritional aspects (high-fat and high-sugar diets), bacterial infections (e.g., Salmonella typhi), chronic diseases (e.g., sclerosing cholangitis, type II diabetes, metabolic syndrome, and/or dyslipidemias), parasitic infections, smoking, and alcohol consumption [52][53][54]. Unfortunately, since GBC is usually asymptomatic in the early stages and thereby its diagnosis is late, this diagnosis occurs in an advanced stage of the disease that shows an invariable course to death because all treatments become ineffective, showing an overall survival rate of only 6 months on average, with a 5-year survival rate of 5% [55]. Thus, new markers or therapeutic targets are needed to overcome these screening issues observed in GBC.

There are scarce studies that have analyzed the expression of piRNAs in GBC. The most important study was the one performed by Gu et al., who aimed to identify the piRNA signature present in blood exosomes of five cholangiocarcinoma (CCA) and four GBC patients and five healthy individuals. The authors focused on selecting those differentially expressed piRNAs with the highest fold-change values between healthy donors and patient groups (CCA + GBC), resulting in the identification of 10 upregulated piRNAs within CCA + GBC cases, including piR-2660989, piR-10506469, piR-20548188, piR-10822895, piR-23209, and piR-18044111. In particular, piR-23209 and piR-17603885 were suggested as promising biomarkers in the diagnosis of both CCA and GBC cases. On the contrary, certain piRNAs such as piR-17802142, piR-12355115, piR-4262304, piR-5114107, piR-9052713, and piR-14022777 were found downregulated in this CCA + GBC group. Interestingly, piR-12355115 showed dramatically low levels in both CCA and GBC cases, suggesting that its downregulation can be used as a common biomarker for these two diseases.

Later, some of these piRNAs were evaluated in a validation cohort composed of blood samples from 50 healthy individuals, 40 CCA patients, and 25 GBC patients. Results showed that piR-10506469 expression was significantly increased in the plasma exosomes of CCA and GBC patients, confirming the results of RNA-seq.

Additionally, piR-20548188 and piR-14090389 were found significantly upregulated only in the exosomes of CCA patients. In particular, the levels of piR-14090389 were increased as the malignant grade increased of GBC cases.

The most interesting results of this study were found when researchers compared the expression of certain piRNAs in the blood of patients before surgery and 1 week after the surgery. For instance, the expression levels of piR-10506469 and piR-20548188 were found considerably diminished in plasma of CAA and GBC cases 1 week after surgery compared to individuals before surgery, demonstrating that these piRNAs constitute potential circulating diagnostic markers to differentiate CCA and GBC cases from healthy individuals and to carry out post-treatment follow-up in patients with gallbladder diseases. On the other hand, the expressions of piR-4333713 and piR-14090389 were found significantly repressed in the respective blood samples of GBC and CCA obtained 1 week after surgeries compared to those samples before surgery, suggesting that these two piRNAs can be used to discriminate the progression of a healthy subject to CCA and then to GBC, becoming potential biomarkers for the diagnosis of gallbladder preneoplastic and neoplastic diseases [56].

Undoubtedly, GBC is a disease with a large and unknown field to investigate the expression of piRNAs and their role in the development of this neoplasm.

References

1. Muñoz-López, M.; García-Pérez, J.L. DNA Transposons: Nature and Applications in Genomics. Curr. Genom. 2010, 11, 115.
2. Cordaux, R.; Batzer, M.A. The impact of retrotransposons on human genome evolution. Nat. Rev. Genet. 2009, 10, 691–703.
3. Anwar, S.L.; Wulaningsih, W.; Lehmann, U. Transposable Elements in Human Cancer: Causes and Consequences of Deregulation. Int. J. Mol. Sci. 2017, 18, 974.
4. Lin, X.; Xia, Y.; Hu, D.; Mao, Q.; Yu, Z.; Zhang, H.H.; Li, C.; Chen, G.; Liu, F.; Zhu, W.; et al. Transcriptome-wide piRNA profiling in human gastric cancer. Oncol. Rep. 2019, 41, 3089–3099.
5. Tóth, K.F.; Pezic, D.; Stuwe, E.; Webster, A. The piRNA Pathway Guards the Germline Genome against Transposable Elements. Adv. Exp. Med. Biol. 2016, 886, 51.
6. Wang, C.; Lin, H. Roles of piRNAs in transposon and pseudogene regulation of germline mRNAs and lncRNAs. Genome Biol. 2021, 22, 27.
7. Yu, Y.; Gu, J.; Jin, Y.; Luo, Y.; Preall, J.B.; Ma, J.; Czech, B.; Hannon, G.J. Panoramix enforces piRNA-dependent cotranscriptional silencing. Science 2015, 350, 339–342.
8. Post, C.; Clark, J.P.; Sytnikova, Y.A.; Chirn, G.W.; Lau, N.C. The capacity of target silencing by Drosophila PIWI and piRNAs. RNA 2014, 20, 1977–1986.

9. Kuramochi-Miyagawa, S.; Watanabe, T.; Gotoh, K.; Totoki, Y.; Toyoda, A.; Ikawa, M.; Asada, N.; Kojima, K.; Yamaguchi, Y.; Ijiri, T.W.; et al. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev. 2008, 22, 908–917.
10. Liu, Y.; Dou, M.; Song, X.; Dong, Y.; Liu, S.; Liu, H.; Tao, J.; Li, W.; Yin, X.; Xu, W. The emerging role of the piRNA/piwi complex in cancer. Mol. Cancer 2019, 18, 123.
11. Goh, W.S.S.; Falciatori, I.; Tam, O.H.; Burgess, R.; Meikar, O.; Kotaja, N.; Hammell, M.; Hannon, G.J. piRNA-directed cleavage of meiotic transcripts regulates spermatogenesis. Genes Dev. 2015, 29, 1032–1044.
12. Ng, K.W.; Anderson, C.; Marshall, E.A.; Minatel, B.C.; Enfield, K.S.S.; Saprunoff, H.L.; Lam, W.L.; Martinez, V.D. Piwi-interacting RNAs in cancer: Emerging functions and clinical utility Kevin. Mol. Cancer 2016, 15, 5.
13. Rouget, C.; Papin, C.; Boureux, A.; Meunier, A.C.; Franco, B.; Robine, N.; Lai, E.C.; Pelisson, A.; Simonelig, M. Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo. Nature 2010, 467, 1128–1132.
14. Balmeh, N.; Mahmoudi, S.; Karabedianhajiabadi, A. piRNAs and PIWI proteins: From biogenesis to their role in cancer. Gene Rep. 2021, 22, 101013.
15. Czech, B.; Hannon, G.J. One Loop to Rule Them All: The Ping-Pong Cycle and piRNA-Guided Silencing. Trends Biochem. Sci. 2016, 41, 324–337.
16. Guo, B.; Li, D.; Du, L.; Zhu, X. piRNAs: Biogenesis and their potential roles in cancer. Cancer Metastasis Rev. 2020, 39, 567–575.
17. Sellitto, A.; Geles, K.; D’Agostino, Y.; Conte, M.; Alexandrova, E.; Rocco, D.; Nassa, G.; Giurato, G.; Tarallo, R.; Weisz, A.; et al. Molecular and Functional Characterization of the Somatic PIWIL1/piRNA Pathway in Colorectal Cancer Cells. Cells 2019, 8, 1390.
18. Han, Y.N.; Li, Y.; Xia, S.Q.; Zhang, Y.Y.; Zheng, J.H.; Li, W. PIWI Proteins and PIWI-Interacting RNA: Emerging Roles in Cancer. Cell. Physiol. Biochem. 2017, 44, 1–20.
19. Jeong, H.; Park, K.H.; Lee, Y.; Jeong, A.; Choi, S.; Kim, K.W. The Regulation and Role of piRNAs and PIWI Proteins in Cancer. Processes 2021, 9, 1208.
20. Wild, C.P.; Weiderpass, E.; Stewart, B.W. (Eds.) World Cancer Report 2020: Cancer Research for Cancer Prevention; IARC Press: Lyon, France, 2020.
21. Zhang, X.Y.; Zhang, P.Y. Gastric cancer: Somatic genetics as a guide to therapy. J. Med. Genet. 2017, 54, 305–312.
22. Cheng, J.; Guo, J.M.; Xiao, B.X.; Miao, Y.; Jiang, Z.; Zhou, H.; Li, Q.N. PiRNA, the new non-coding RNA, is aberrantly expressed in human cancer cells. Clin. Chim. Acta 2011, 412, 1621–1625.

23. Cheng, J.; Deng, H.; Xiao, B.; Zhou, H.; Zhou, F.; Shen, Z.; Guo, J. piR-823, a novel non-coding small RNA, demonstrates in vitro and in vivo tumor suppressive activity in human gastric cancer cells. Cancer Lett. 2012, 315, 12–17.
24. Martinez, V.D.; Enfield, K.S.S.; Rowbotham, D.A.; Lam, W.L. An atlas of gastric PIWI-interacting RNA transcriptomes and their utility for identifying signatures of gastric cancer recurrence. Gastric Cancer 2016, 19, 660–665.
25. Cui, L.; Lou, Y.; Zhang, X.; Zhou, H.; Deng, H.; Song, H.; Yu, X.; Xiao, B.; Wang, W.; Guo, J. Detection of circulating tumor cells in peripheral blood from patients with gastric cancer using piRNAs as markers. Clin. Biochem. 2011, 44, 1050–1057.
26. Ge, L.; Zhang, N.; Li, D.; Wu, Y.; Wang, H.; Wang, J. Circulating exosomal small RNAs are promising non-invasive diagnostic biomarkers for gastric cancer. J. Cell. Mol. Med. 2020, 24, 14502–14513.
27. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. 2018, 68, 394–424.
28. Herzig, D.O.; Tsikitis, V.L. Molecular markers for colon diagnosis, prognosis and targeted therapy. J. Surg. Oncol. 2015, 111, 96–102.
29. Chen, H.; Xu, Z.; Liu, D. Small non-coding RNA and colorectal cancer. J. Cell. Mol. Med. 2019, 23, 3050.
30. Yin, J.; Jiang, X.Y.; Qi, W.; Ji, C.G.; Xie, X.L.; Zhang, D.X.; Cui, Z.J.; Wang, C.K.; Bai, Y.; Wang, J.; et al. piR-823 contributes to colorectal tumorigenesis by enhancing the transcriptional activity of HSF1. Cancer Sci. 2017, 108, 1746–1756.
31. Sabbah, N.A.; Abdalla, W.M.; Mawla, W.A.; Abdalmonem, N.; Gharib, A.F.; Abdul-Saboor, A.; Abdelazem, A.S.; Raafat, N. PiRNA-823 is a unique potential diagnostic non-invasive biomarker in colorectal cancer patients. Genes 2021, 12, 598.
32. Weng, W.; Liu, N.; Toiyama, Y.; Kusunoki, M.; Nagasaka, T.; Fujiwara, T.; Wei, Q.; Qin, H.; Lin, H.; Ma, Y.; et al. Novel evidence for a PIWI-interacting RNA (piRNA) as an oncogenic mediator of disease progression, and a potential prognostic biomarker in colorectal cancer. Mol. Cancer 2018, 17, 16.
33. Yin, J.; Qi, W.; Ji, C.G.; Zhang, D.X.; Xie, X.L.; Ding, Q.; Jiang, X.Y.; Han, J.; Jiang, H.Q. Small RNA sequencing revealed aberrant piRNA expression profiles in colorectal cancer. Oncol. Rep. 2019, 42, 263–272.
34. Iyer, D.N.; Wan, T.M.H.; Man, J.H.W.; Sin, R.W.Y.; Li, X.; Lo, O.S.H.; Foo, D.C.C.; Pang, R.W.C.; Law, W.L.; Ng, L. Small RNA Profiling of piRNAs in Colorectal Cancer Identifies Consistent

Overexpression of piR-24000 That Correlates Clinically with an Aggressive Disease Phenotype. Cancers 2020, 12, 188.

35. Chu, H.; Xia, L.; Qiu, X.; Gu, D.; Zhu, L.; Jin, J.; Hui, G.; Hua, Q.; Du, M.; Tong, N.; et al. Genetic variants in noncoding PIWI-interacting RNA and colorectal cancer risk. Cancer 2015, 121, 2044–2052.
36. Vychytilova-Faltejskova, P.; Stitkovcova, K.; Radova, L.; Sachlova, M.; Kosarova, Z.; Slaba, K.; Kala, Z.; Svoboda, M.; Kiss, I.; Vyzula, R.; et al. Circulating PIWI-interacting RNAs piR-5937 and piR-28876 are promising diagnostic biomarkers of colon cancer. Cancer Epidemiol. Biomark. Prev. 2018, 27, 1019–1028.
37. Mai, D.; Ding, P.; Tan, L.; Zhang, J.; Pan, Z.; Bai, R.; Li, C.; Li, M.; Zhou, Y.; Tan, W.; et al. PIWI-interacting RNA-54265 is oncogenic and a potential therapeutic target in colorectal adenocarcinoma. Theranostics 2018, 8, 5213–5230.
38. Mai, D.; Zheng, Y.; Guo, H.; Ding, P.; Bai, R.; Li, M.; Ye, Y.; Zhang, J.; Huang, X.; Liu, D.; et al. Serum piRNA-54265 is a new biomarker for early detection and clinical surveillance of Human Colorectal Cancer. Theranostics 2020, 10, 8468–8478.
39. Tosar, J.P.; García-Silva, M.R.; Cayota, A. Circulating SNORD57 rather than piR-54265 is a promising biomarker for colorectal cancer: Common pitfalls in the study of somatic piRNAs in cancer. RNA 2021, 27, 403–410.
40. Qu, A.; Wang, W.; Yang, Y.; Zhang, X.; Dong, Y.; Zheng, G.; Wu, Q.; Zou, M.; Du, L.; Wang, Y.; et al. A serum piRNA signature as promising non-invasive diagnostic and prognostic biomarkers for colorectal cancer. Cancer Manag. Res. 2019, 11, 3703–3720.
41. Adamska, A.; Domenichini, A.; Falasca, M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. Int. J. Mol. Sci. 2017, 18, 1338.
42. Rawla, P.; Sunkara, T.; Gaduputi, V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. World J. Oncol. 2019, 10, 10–27.
43. Müller, S.; Raulefs, S.; Bruns, P.; Afonso-Grunz, F.; Plötner, A.; Thermann, R.; Jäger, C.; Schlitter, A.M.; Kong, B.; Regel, I.; et al. Next-generation sequencing reveals novel differentially regulated mRNAs, lncRNAs, miRNAs, sdRNAs and a piRNA in pancreatic cancer. Mol. Cancer 2015, 14, 94.
44. Xie, J.; Xing, S.; Shen, B.Y.; Chen, H.T.; Sun, B.; Wang, Z.T.; Wang, J.W.; Lu, X.X. PIWIL1 interacting RNA piR-017061 inhibits pancreatic cancer growth via regulating EFNA5. Hum. Cell 2021, 34, 550–563.
45. El-Serag, H.B. Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. Gastroenterology 2012, 142, 1264.

46. Llovet, J.M.; Kelley, R.K.; Villanueva, A.; Singal, A.G.; Pikarsky, E.; Roayaie, S.; Lencioni, R.; Koike, K.; Zucman-Rossi, J.; Finn, R.S. Hepatocellular carcinoma. Nat. Rev. Dis. Prim. 2021, 7, 6.
47. Law, P.T.Y.; Qin, H.; Ching, A.K.K.; Lai, K.P.; Co, N.N.; He, M.; Lung, R.W.M.; Chan, A.W.H.; Chan, T.F.; Wong, N. Deep sequencing of small RNA transcriptome reveals novel non-coding RNAs in hepatocellular carcinoma. J. Hepatol. 2013, 58, 1165–1173.
48. Rizzo, F.; Rinaldi, A.; Marchese, G.; Coviello, E.; Sellitto, A.; Cordella, A.; Giurato, G.; Nassa, G.; Ravo, M.; Tarallo, R.; et al. Specific patterns of PIWI-interacting small noncoding RNA expression in dysplastic liver nodules and hepatocellular carcinoma. Oncotarget 2016, 7, 54650–54661.
49. Tang, X.; Xie, X.; Wang, X.; Wang, Y.; Jiang, X.; Jiang, H. The combination of piR-823 and eukaryotic initiation factor 3 B (EIF3B) activates hepatic stellate cells via upregulating TGF-β1 in liver fibrogenesis. Med. Sci. Monit. 2018, 24, 9151–9165.
50. Rakić, M.; Patrlj, L.; Kopljar, M.; Kliček, R.; Kolovrat, M.; Loncar, B.; Busic, Z. Gallbladder cancer. Hepatobiliary Surg. Nutr. 2014, 3, 221.
51. Hickman, L.; Contreras, C. Gallbladder Cancer: Diagnosis, Surgical Management, and Adjuvant Therapies. Surg. Clin. North. Am. 2019, 99, 337–355.
52. Espinoza, J.A.; Bizama, C.; García, P.; Ferreccio, C.; Javle, M.; Miquel, J.F.; Koshiol, J.; Roa, J.C. The inflammatory inception of gallbladder cancer. Biochim. Biophys. Acta 2016, 1865, 245–254.
53. Bertran, E.; Heise, K.; Andia, M.E.; Ferreccio, C. Gallbladder cancer: Incidence and survival in a high-risk area of Chile. Int. J. Cancer 2010, 127, 2446–2454.
54. Hundal, R.; Shaffer, E.A. Gallbladder cancer: Epidemiology and outcome. Clin. Epidemiol. 2014, 6, 99–109.
55. Buchegger, K.; Silva, R.; López, J.; Ili, C.; Araya, J.C.; Leal, P.; Brebi, P.; Riquelme, I.; Roa, J.C. The ERK/MAPK pathway is overexpressed and activated in gallbladder cancer. Pathol. Res. Pract. 2017, 213, 476–482.
56. Gu, X.; Wang, C.; Deng, H.; Qing, C.; Liu, R.; Liu, S.; Xue, X. Exosomal piRNA profiling revealed unique circulating piRNA signatures of cholangiocarcinoma and gallbladder carcinoma. Acta Biochim. Biophys. Sin. 2020, 52, 475–484.