Title: Downregulation of the microRNA biogenesis components and its association with poor prognosis in hepatocellular carcinoma

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Running title: Downgulation of miRNA biogenesis pathway in HCC

Keywords: HCC, miRNA, biogenesis, non-viral, histone modification

Abstract

Genetic alterations and deregulation of the miRNAs biogenesis pathway components have been reported in human tumors. Tissue-specific deletion of the Dicer gene, which encodes an essential miRNA processing enzyme, promotes carcinogenesis in animal models. These indicate that aberrant miRNA biogenesis components are directly associated with cancer. Here we conducted quantitative reverse-transcription polymerase chain reaction (PCR) of fourteen genes that are related to the miRNA biogenesis pathway in forty-seven paired samples of primary HCC and matched non-cancerous liver. Expression of seven genes (Dgcr8, p68, p72, Dicer, Ago3, Ago4 and Piwil4) was significantly decreased in primary HCC, especially in non-viral HCC subtypes, compared to the non-cancerous liver. Combinations of decreased expression of the miRNA biogenesis components in non-cancerous liver were related to cigarette smoking, alcohol intake and diabetes that are known to be risk factors for HCC, and were also associated with the occurrence of multicentric tumors. Reduction of two of these genes (Dicer and p68) in HCC was associated with poor prognosis. Trimethylation of histone H3 lysine 27 in the promoters is implicated in the deregulation of these miRNA-biogenesis-related genes in non-HBV genome integrated HCC cell lines. In conclusion, deregulation of the miRNA biogenesis pathway components is frequently observed in non-viral associated HCC and is associated with etiological risk factors and poor prognosis. Our study further showed that epigenetic regulation could be implicated

in the deregulation of these genes during hepatocarcinogenesis. (230 words)

Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; ChIP-PCR, chromatin immunoprecipitation PCR; EED, Embryonic Ectoderm Development; H3K27me3, trimethylation of histone H3 lysine 27; HCC, hepatocellular carcinoma; miRBir gene, miRNA biogenesis pathway-related gene; miRNA, microRNA; NBNC, non-HBV-non-HCV; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse-transcription PCR; siRNA, small interfering RNA

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide [1]. Although previous studies have revealed multiple etiologic factors responsible for HCC occurrence (hepatitis viruses (HBV and HCV), intake of alcohol or aflatoxin B1-contaminated food, cigarette smoking, obesity and diabetes) [1, 2], the overall view of the molecular abnormalities induced by these environmental agents during hepatocarcinogenesis is still incomplete.

Micro-RNAs are a family of small (18~22 nucleotides (nt) in length) endogenous single-stranded RNAs [3]. Their aberrant expression is associated with human diseases, including cancer [4]. Most mammalian miRNA genes are initially transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II (Figure S1). These pri-miRNAs are then cleaved endonucleolytically by the Drosha complex to form ~70-nt hairpin-structured precursor miRNAs (pre-miRNAs). The Drosha complex comprises the ribonuclease III (RNase III) enzyme Drosha, the DiGeoge syndrome critical region gene 8 (DGCR8) and multiple RNA-associated proteins including the RNA helicases p68 (also known as DDX5) and p72 (also known as DDX17) [5]. Pre-miRNAs are transported into the cytoplasm with the help of Exportin-5 (XPO5) and are cleaved into double-stranded ~22-nt duplexes by Dicer, another RNase III enzyme, in the cytoplasm. One of the strands associates with an Argonaute (Ago) protein, functioning as a guide to repress target mRNA [3, 5-7]. Argonaute contains a

RNA-binding PAZ domain that is shared with another family of small-RNA-binding proteins, PIWILs [8].

Previous miRNA profiling studies have revealed global deregulation of mature miRNAs in human cancers [4, 6]. Such a broad change of miRNA gene expression is due primarily to aberrant transcriptional regulation including CpG hypermethylation silencing of the promoter or abnormalities of transcriptional factors. Alternatively, molecular defects of miRNA biogenesis would also severely affect the mature miRNA profiles in cancer, and recent studies have shown that cancer-related signal molecules (such as TP53 and SMAD) regulate this process [9-11]. Downregulation of Drosha and Dicer genes have been reported in ovarian and other cancers [12, 13]. Importantly mutational impairments of the miRNA-processing pathway including the Dicer [14, 15] and Xpo5 [16] genes have been reported in human tumors. Notably, tissue-specific deletion of the Dicer gene, which encodes an essential processing enzyme, promotes lung and liver carcinogenesis in mice, indicating that aberrant miRNA biogenesis is directly associated with cancer [17, 18].

Materials and methods

Primary HCC and liver tissues

Forty-seven paired samples of primary HCC and matched adjacent non-cancerous liver tissues, and ten samples of non-HCC-associated liver tissue were obtained from surgical specimens resected at the National Cancer Research Center Hospital, Tokyo, between December 1998 and March 2010, after obtaining approval from the institutional review board and informed consent from the patients. Among the 47 HCC patients, 16 were immunologically positive for HCV infection, 11 for persistent HBV infection (hepatitis B surface antigen positive), two had a history of previous HBV infection which we excluded in our analysis for the association with virus status) and 18 were negative for both HCV and HBV infection. Ten samples of non-HCC related liver tissues were negative for either HCV or HBV infection. The clinicopathological data for all cases are shown in Table 1.

RNA extraction and reverse-transcription and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using a mirVana miRNA Isolation Kit (Ambion, Austin, TX) and the RNA samples were treated with DNase I (New England Bio Labs, Ipswich, MA). Total RNA (5 μg) was used to generate complementary DNA by reverse transcription with a First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed on a Light Cycler 480 (Roche, Manheim, Germany) using TaqMan probes (Applied Biosystems, Foster City, CA). Expression of each gene was determined with the $2^{-\Delta\Delta Ct}$ method and normalized relative to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and TATA box binding protein (TBP). The sequences of the qRT-PCR primers are listed in Table S1. For miRNA detection, total RNA (10ng) from each samples was used, mature has-let-7a was reverse-transcribed with specific RT primer with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed on a Light Cycler 480 (Roche, Indianapolis, IN), quantified and normalized by U6 small nuclear RNA using TaqMan miRNA assays (Applied Biosystems).

Cell culture, drug treatments and small interfering RNA (siRNA)-mediated gene silencing

HuH-1 cells were obtained from the Human Science Research Resource Bank (Osaka, Japan). HepG2 and Alex (PLC/PRF/5) cells were provided from the Riken Cell Bank (Tsukuba, Japan). KYN2 have been described previously [19]. HepG2 and KYN2 cell lines are validated as negative for HBV genome integration (data not shown). HuH-1 and Alex cells are derived from HBV-positive HCC [20, 21]. Cell lines were treated with 10μM 5-aza-2'-deoxycytidine (5-aza-dC, Sigma-Aldrich, St. Louis, MO)

for 72 hours [22]. siRNAs targeting the Embryonic Ectoderm Development (EED) and GAPDH geneswere purchased from Dharmacon (Lafayette, CO) and were transfected using Lipofectamine RNAiMAX reagent (Invitrogen).

Bisulfite sequencing

Bisulfite treatment of genomic DNA (500 ng) was performed using an EZ DNA methylation kit (Zymo Research, Orange, CA). PCR products encompassing the CpG islands were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and at least 14 independent clones were sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems). We used a CpG island Searcher (http://cpgislands.usc.edu/) to predict the CpG islands and QUMA (http://quma.cdb.riken.jp/top/index.html) for methylation quantification.

Chromatin immunoprecipitation PCR (ChIP-PCR) assay

Approximately $1x10^7$ cells of each cell line were fixed with 1% formaldehyde for 10 min at room temperature. The formaldehyde was then quenched by addition of a 1/20 volume of 2.5 M glycine to the plates, and the cells were harvested. The chromatin was then sonicated to create DNA fragments with a length of 200 to 1000 base pairs. Fragmented chromatin was subjected to immunoprecipitation with/without a polyclonal anti-histone H3 trymethyl Lys 27 antibody (Active Motif, Carlsbad, CA). The

co-immunoprecipitated fragments of DNA extracted using a QIAquick PCR Purification Kit (Qiagen, GmbH, Germany) were amplified by PCR. The PCR primer sequences are listed in Table S1. The assays were performed more than twice.

Immunoblot analysis

Cells were washed once with PBS, and proteins in the nuclear fraction were extracted using ProteoExtract (Merck, Damstadt, Germany) with a protease inhibitor cocktail (Roche). The proteins (20 μg) were electrophoresed, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), and blotted with a polyclonal anti-histone H3 trymethyl Lys 27 antibody (Active Motif, Carlsbad, CA) or a rabbit monoclonal anti-histone H3 antibody (clone 3H1, Cell Signaling Technology, Danvers, MA).

Statistical analyses

Statistical analyses were performed using the Statview 5.0 software package (Abacus Concepts, Berkeley, CA). Statistical analysis of the expression data was performed using Student's *t* test or Welch's *t* test (two-group *t* test: Unpaired); the latter was used after dispersion of the data had been calculated by F-test, and Two-group *t* test: Paired or Wilcoxon signed-ranks test was used. Pearson's correlation and Spearman's rho coefficient test were calculated to examine the correlations among the

relative expressions of the various genes. Chi-square test, Fisher's exact test and Multivariate logistic regression analysis were used for comparing subgroups. Kaplan-Meier plots were used for calculating disease-free survival probabilities, and the log-rank test was employed for testing statistical significance. Data are expressed as mean ± standard deviation. All reported *P* values are two-tailed, except for the F-test (one-tailed), and differences were considered significant at $P < 0.05$.

Results

Decreased expression of miRNA biogenesis pathway-related genes (miRBir genes) in HCC

Using qRT-PCR, we measured the expression of 14 mRNAs related to the miRNA biogenesis pathway (Drosha, Dgcr8, p68, p72, Dicer, Xpo-5, Ago1-4 and Piwil1-4) in 47 paired samples of primary HCC and corresponding non-cancerous liver tissue and 10 samples of non-HCC-associated liver tissue. Seven miRBir genes (Dgcr8, p68, p72, Dicer, Ago3, Ago4 ($P < 0.01$) and Piwil4 ($P < 0.05$), unpaired t test) were significantly down-regulated in tumors relative to the non-cancerous liver (Figure 1A). Among the 14 genes, none of the seven miRBir genes were down-regulated in samples of HCV-positive HCC, and three genes (p68, Dicer and Ago3) ($P < 0.05$) showed decreased expression in HBV-positive HCCs (Figure 1B). In contrast, six miRBir genes (Dgcr8, p68, p72, Dicer, Ago3 and Ago4) were significantly ($P < 0.01$) downregulated in samples of non-HBV-non-HCV (NBNC) HCC (Figure 1B).

We also examined expression of 14 miRBir genes in HCC cell lines. Eight genes (Drosha, Dgcr8, p72, Ago3, Ago4, Piwil1, Piwil2 and Piwil4) were significantly reduced in HCC cell lines which are negative for HBV and HCV infection compared to the non-cancerous liver tissues (Figure S2A). Six genes (p72, Ago1, Ago3, Ago4, Piwil1 and Piwil2) also significantly decreased in HBV-associated two HCC cell lines (Alex and HuH-1) (Figure S2B).

We then examined correlations among the relative expression ratios (tumor versus non-cancerous liver) of these seven genes. Correlations among the relative expressions of the Dgcr8, p68 and p72 genes, all of which encode the Drosha complex in the nucleus, and among those of the Dicer, Ago3 and Ago4 genes, were notable (Pearson's correlation coefficient > 0.70, except between the Dicer and Ago4 genes (0.679), $P < 0.05$) (Figure 1C, Table S2). We found that reduced Dicer expression was significantly correlated with downregulation of let-7a in HCC (Figure 1D), which has been reported to be decreased in HCC and negatively regulates cell proliferation [23].

HCC risk factors and multi-centric tumor occurrence are associated with reduced expression of miRBir genes in non-cancerous liver

Frequent decrease of the miRBir genes in NBNC HCC prompted us to examine whether any etiological factors other than virus infection may be associated with this aberrant expression. We first examined whether there was any correlation between expression change of the miRBir genes in HCC and non-viral etiological factors (alcohol intake, smoking and HbA1c). However, reduction of all genes in tumor was not associated with these etiological factors. (Figure S3).

We then examined the relationship between miRBir gene expressions in non-cancerous liver tissues and etiological factors (Figure 2). Firstly, we examined the relationship between background liver histology and expression of microRNA

biogenesis components. Significant difference in the Ago3 gene expression was observed between normal liver and chronic hepatitis (CH). Significantly decreased expression of the Ago3, Ago4 and Piwil4 genes was detected in precirrhosis (PC) status compared to the normal liver (Figure S4). There was no significant association between background liver histology and other etiological factors (smoking, alcohol and diabetes) (Table S3). Lastly we examined relationship between expression of miRBir gene and etiological factors. Notably expression of five miRBir genes (Ago1, Ago2, Ago3 Exportin-5, Dgcr8) was already reduced in the non-cancerous liver of patients who smoked. Decreased expression of Piwil 1 was found to be associated with habitual alcohol intake. Reduced expression of the Ago1 gene was also associated with an elevated level of HbA1c, a serum marker of diabetes. Multivariate analysis revealed that reduced expression of Ago1 gene was independently associated with smoking and diabetes (Tables 2, 3 and S4). Therefore aberrant expression of most miRBir genes in non-cancerous liver is associated with non-viral etiological factors.

Previous studies showed that etiological high risk factors of HCC are associated with the occurrence of multi-centric tumor [1, 2]. Interestingly expression of seven miRBir genes (Ago1, Ago3, Ago4, Dicer, Piwil1, Piwil2 and p72) was significantly reduced in non-cancerous tissues with multicentric tumors (Figure 2). There was no significant relationship between the presence of intra-hepatic metastasis and expression of the miRBir genes in non-cancerous liver (Figure S5).

Prognostic significance of miRBir gene reduction in HCC

Expression of the p68 and p72 genes in tumors relative to that in the paired samples of non-cancerous liver was significantly (P<0.01) decreased in more poorly differentiated cases (Figure 3A). Decreased expression of the p68 and Dicer genes but not others was significantly associated with shorter recurrence-free survival time (p68: P = 0.0217, Dicer: P = 0.0273 by log-rank test) (Figure 3B).

H3K27me3 in promoter regions partly regulates the silencing of miRBir genes

Because reduced expression of the miRBir genes is already evident in non-cancerous liver tissues with characteristic etiological backgrounds such as smoking, we hypothesized that epigenetic regulation may play a role in this aberrant gene expression.

To examine whether methylation of CpG islands is involved in deregulation of the miRBir genes, we chose two non-HBV genome integrated HCC cell lines (HepG2 and KYN2 cells). Bisulfite sequencing of CpG islands in the promoter regions revealed that fully methylation in the p72 gene, partial methylation in the Ago4 gene and unmethylation in the Dgcr8 gene (Figure S6) in these cells. We treated these cell lines with 5-aza-dC, a DNA methyltransferase inhibitor and measured the expressions of these genes by qRT-PCR. We validated the effect of 5-aza-dC treatment on the

expression of the SFRP1 gene, which has been previously confirmed to be silenced by CpG island methylation in HCC (Figure S7) [24]. Even in this condition, expression of the seven genes was little affected or even decreased after treatment in HepG2 and KYN2 cells (Figure 4A).

We next focused on trimethylated H3K27 (H3K27me3), a repressive histone modification that plays a major role in epigenetic silencing in stem cells and cancer, and is independent of, or coexists with, DNA methylation [25]. We investigated the presence of H3K27me3 in the promoter regions of seven miRBir genes using ChIP-PCR in these HCC cell lines (Figure S8). We detected the presence of H3K27me3 modification in the promoter regions of the p72, Dicer, Ago3 and Piwil4 genes in two cell lines, while H3K27me3 in the promoter regions of Dgcr8, p68 and Ago4 genes was detected only in HepG2 cells (Figure 4B).

To determine whether this histone modification is actively implicated in the epigenetic silencing of target genes, we then attempted to decrease H3K27me3 modification in these cells. H3K27 methylation is catalyzed by two highly-related histone methyltransferases (HMTs) in the Polycomb Repressive Complex 2 (PRC2) [26]. PRC2 contains multiple proteins, among which EED protein plays a pivotal role in the propagation of H3K27me3 marking [27]. We knocked down EED expression using siRNA, which decreased H3K27me3 in the two cell lines (Figure 4C). In HepG2 cells, five genes out of seven increased when EED was knocked down, and four genes

increased by EED siRNA treatment in KYN2 cells (Figure 4D).

Discussion

Our expression analysis of the miRNA biogenesis pathway revealed that seven key molecules, including the Dicer gene, were frequently and simultaneously downregulated in HCC, indicating that decreased miRNA biogenesis pathway is associated with human hepatocarcinogenesis, as has been proposed in a mouse model [17]. Furthermore, we found that reduced expression of the miRNA biogenesis components occurred more frequently in HCC without hepatitis viral infection. Downregulation of five genes (Ago1, Ago2, Ago3 Exportin-5 and Dgcr8) in non-cancerous liver was associated with a history of smoking. Other genes (Piwi4 and Ago1) were reduced in background liver of patients with habitual alcohol intake and diabetes. Especially reduction of Ago1 gene was independently associated with smoking and diabetes. Izzotti *et al.* [28] reported that cigarette smoke exposure caused downregulation of miRNA in the lungs of rats, which suggests that miRNA deregulation resulting from chronic exposure to non-viral carcinogens or metabolic stress could be a more general phenomenon leading to malignancy. In addition to inducing downregulation of tumor-suppressive miRNAs, deregulation of miRNA biogenesis may have other biological significance in hepatocarcinogenesis. Recently Dicer and Drosha are reported to be indispensable for DNA repair and DNA damage response in normal cells [29, 30]. It is possible that aberrant regulation of miRNA synthesis pathway also plays an important role in genomic instability of HCC.

Reduced expression of the Dicer or Drosha gene is reportedly associated with clinical aggressiveness or poorer prognosis of tumors arising from various organs, including the lung and ovary [12, 31]. Decreased Dicer expression in cancer conferred increased proliferative ability and an invasive phenotype [32, 33]. Our analysis has revealed that downregulation of seven components of the miRNA-biogenesis pathway is associated with the presence of multicentric tumors. Downregulation of the p68 and Dicer genes was also significantly associated with shorter recurrence-free survival time. These results suggest that deregulation of miRNA biogenesis components is tightly associated with a higher risk of both liver carcinogenesis and tumor recurrence.

The molecular mechanisms underlying deregulation of the miRNA biogenesis pathway in tumorigenesis are only now becoming clearer [9-11], and recent studies have demonstrated downregulation of the Dicer gene by either induction of Dicer-targeting miRNAs (miR-103/107) [32] or suppression of its direct trans-activator (TAp63) [33], as well as recurrent mutations of the Xpo5, TARBP2 and Dicer genes in mismatch repair-deficient cancers or other tumors [13-15, 34]. A discrepancy of the down regulated genes between in primary HBV-positive HCC and in cell lines derived from HBV-positive HCC indicated the status of viral protein activity may be associated with expression of miRBir genes. Based on our findings in clinical samples, we

hypothesized that epigenetic silencing might be responsible for downregulation of the miRNA pathway in hepatocarcinogenesis. ChIP-PCR analysis revealed the frequent presence of H3K27me3 in the promoters of downregulated miRNA biogenesis components and a global decrease of trimethylation of lysine 27 in histone H3 (H3K27me3) by EED knockdown induced partial recovery of the expression of the target genes and let-7a in HCC cells (Figure S9). However, there was also a discrepancy between the expression of Dicer gene and let-7a in HepG2 cells, suggesting that complicated molecular mechanisms exist to regulate tumor suppressive miRNA expression [35].

H3K27me3 is one of the major epigenetic histone modifications in silenced chromatin, and is catalyzed by HMTs (EZH1 and EZH2) [26, 27]. Increased expression and oncogenic activity of HMTs, especially EZH2, has been reported in HCC [36] and H3K27me3 modification silences tumor suppressor genes in other cancer types [37]. Our results suggest that aberrant H3K27me3 modification modulates the miRNA biogenesis pathway in HCC.

In conclusion, our study has for the first time demonstrated that reduced expression of genes related to the miRNA biogenesis pathway is frequent, and especially associated with specific etiological backgrounds and poor prognosis in HCC. Additional analyses of HCC cell lines have shown that histone modification could be at

least partly implicated with this deregulation. The identification of this novel molecular alteration should be of clinical significance for designing diagnostic or preventive modalities and evaluating the prognosis for liver cancers, especially the non-viral subtype.

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Figure legends

Figure 1. Decreased expression of miRNA-biogenesis-related (miRBir) genes in primary HCC. (A) Relative expression of each gene related to miRNA biogenesis was measured by quantitative RT-PCR in tumor (T), non-cancerous liver (N). The adjusted expression data to that of non-cancerous liver tissue in each gene is shown. Significant difference was indicated by asterisk (* P<0.05, ** P<0.01). (B) Relative expression of the miRBir genes in virus-positive (HCV or HBV) and virus-negative (NBNC) HCC. Significant difference was indicated by asterisk (* P<0.05, ** P<0.01). (C) Correlations among the relative expressions ratio (T/N) of the Dgcr8, p68 and p72 genes (left), and those of the Dicer, Ago3 and Ago4 genes in HCC. Pearson's correlation coefficient > 0.70, except between the Dicer and Ago4 genes (0.679) (P < 0.05). (D) Significant correlation between Dicer and let-7a expression in HCC. Pearson's correlation coefficient was 0.422 (P < 0.01).

Figure 2. The association between relative miRBir gene expression in non-cancerous liver and etiological factors. Relative expressions of miRBir genes in non-cancerous liver of patients with (closed column) or without (open column) alcohol intake (top), smoking (upper middle), increased HbA1C (lower middle) and Multiplicity (bottom) were shown. Significant difference was indicated by asterisk (* P<0.05, ** P<0.01).

Figure 3. Reduction of the miRBir genes is associated with prognosis in HCC. (A) Association between expression of the p68 and p72 genes and tumor histology.

Expression of the miRBir genes in tumors relative to that in the paired samples of non-cancerous liver (T/N) in different histological category was shown. Expression of the p68 and p72 genes was significantly ($P < 0.01$) decreased in poorly differentiated cases (indicated by asterisk). (B) Decreased expression of the p68 and Dicer genes was associated with shorter recurrence-free survival time. Kaplan-Meier plots of the patients segregated according to the relative (tumor versus non-cancerous liver (T/N)) expression of the two genes are shown. Significant difference was indicated by asterisk (* P<0.05, ** P<0.01).

Figure 4. Epigenetic regulation of the miRBir genes by histone H3 lysine 27 trimethylation in HCC cells. (A) Expression of the miRBir-related genes in HepG2 and KYN2 cells upon treatment with 5-Aza-dC. Expression of miRBir genes in drug-treated HCC cells relative to the untreated ones is shown. (B) Detection of H3K27me3 in the promoter regions of the p72, Dicer, Ago3 and Piwil4 genes in all cell lines, while H3K27me3 in the promoter regions of Dgcr8, p68 and Ago4 genes was detected in three cell lines. Chromatin immunoprecipitates obtained with anti-H3K27me3 antibody (H3K27me3) or control IgG (NC) were amplified with primers covering the promoters of the miRBir genes and electrophoresed. (C) Decrease of H3K27me3 in HCC cells with EED knockdown. Immunoblot analysis of H3K27me3 and total histone H3 in the nuclear fraction of siRNA-treated HepG2 and KYN2 cells. (D) Recovery of the downregulated miRBir genes in EED-knockdown HCC cells. Relative expressions of

1 the seven miRBir genes in the EED-knockdown cells are shown. Significant difference
2 was indicated by asterisk (* $P<0.05$, ** $P<0.01$).
3

Supporting information

Figure S1. The biogenesis of miRNAs in mammalian cells.

Figure S2. The relative expressions of miRNA biogenesis pathway-related (miRBir) genes in HCC cell lines.

Figure S3. The association between relative miRBir gene expression in HCC and etiological factors.

Figure S4. The relationship between background liver histology and expression of miRNA biogenesis components in non-cancerous liver.

Figure S5. The relationship between intra-liver metastasis and expression of the miRBir genes in non-cancerous liver.

Figure S6. Methylation status of CpG islands in the promoters of the three miRBir genes.

Figure S7. Methylation specific PCR revealed intense CpG island methylation of the SFRP1 gene promoter in these cell lines.

Figure S8. Positions of CpG island and ChIP-PCR primer range in seven miRBir genes are presented.

Figure S9. Decreased of let-7a miRNA in HepG2 cell line and Increased in KYN2 with EED siRNA knockdown.

Table S1 PCR primers.

Table S2 Pearson's correlation among the relative expression ratios (tumor vs. non-cancerous liver) of the 7 miRBir genes.

Table S3 Statistical analysis between subgroups of patients background (Alcohol, Tobaccl and Diabetes).

Table S4 Mutivriate logistic regression analysis for Alcohol.

Fig. 1

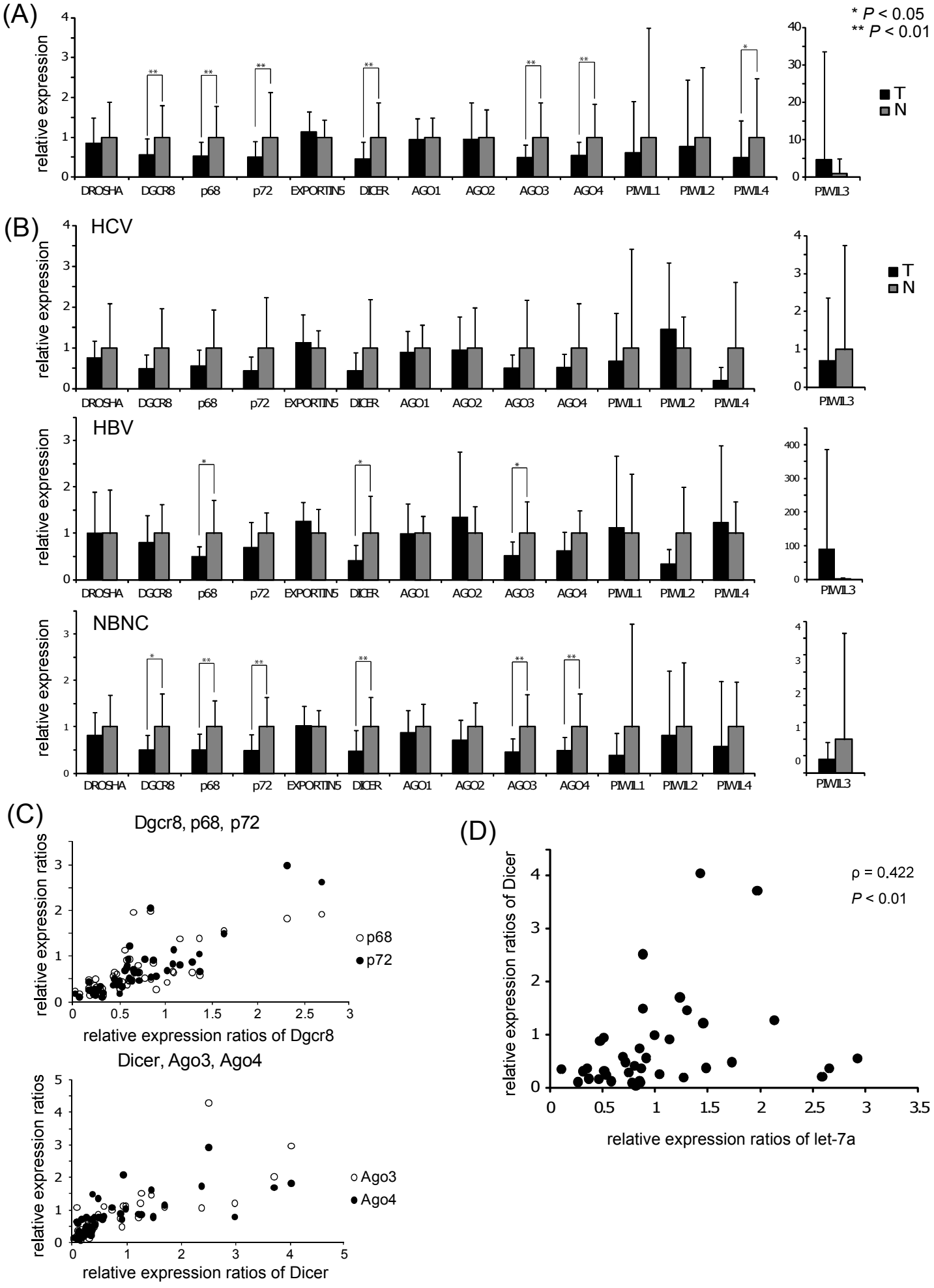

(A)
* P < 0.05
** P < 0.01
T
N
relative expression
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL4
PIWIL3
(B)
HCV
HBV
NBNC
(C)
Dgcr8, p68, p72
relative expression ratios
relative expression ratios of Dgcr8
p68
p72
Dicer, Ago3, Ago4
relative expression ratios of Dicer
Ago3
Ago4
(D)
relative expression ratios of Dicer
relative expression ratios of let-7a
ρ = 0.422
P < 0.01

Fig. 2

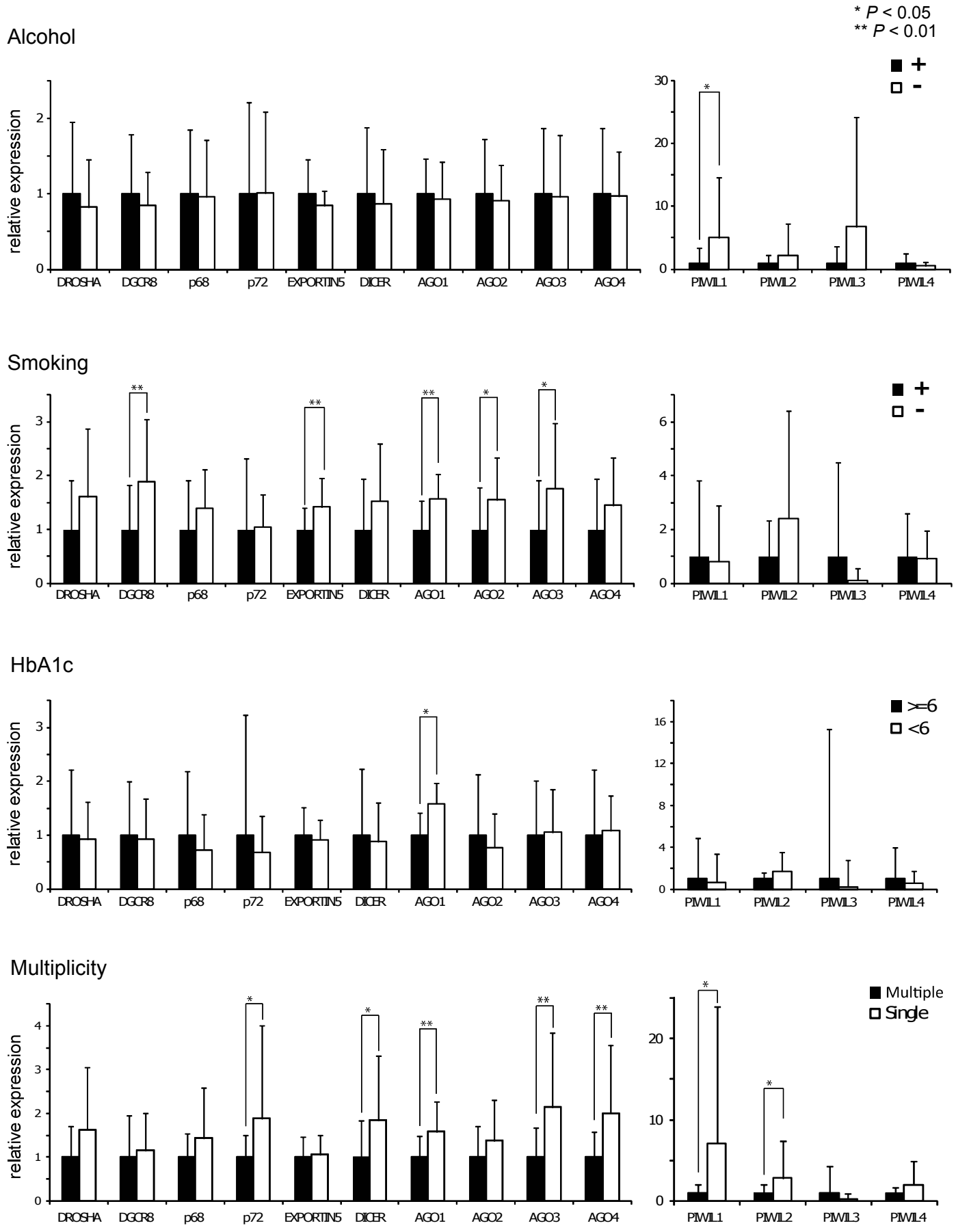

* P < 0.05
** P < 0.01
Alcohol
Smoking
HbA1c
Multiplicity
relative expression
+
-
>=6
<6
Multiple
Single
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL3
PIWIL4

Fig. 3

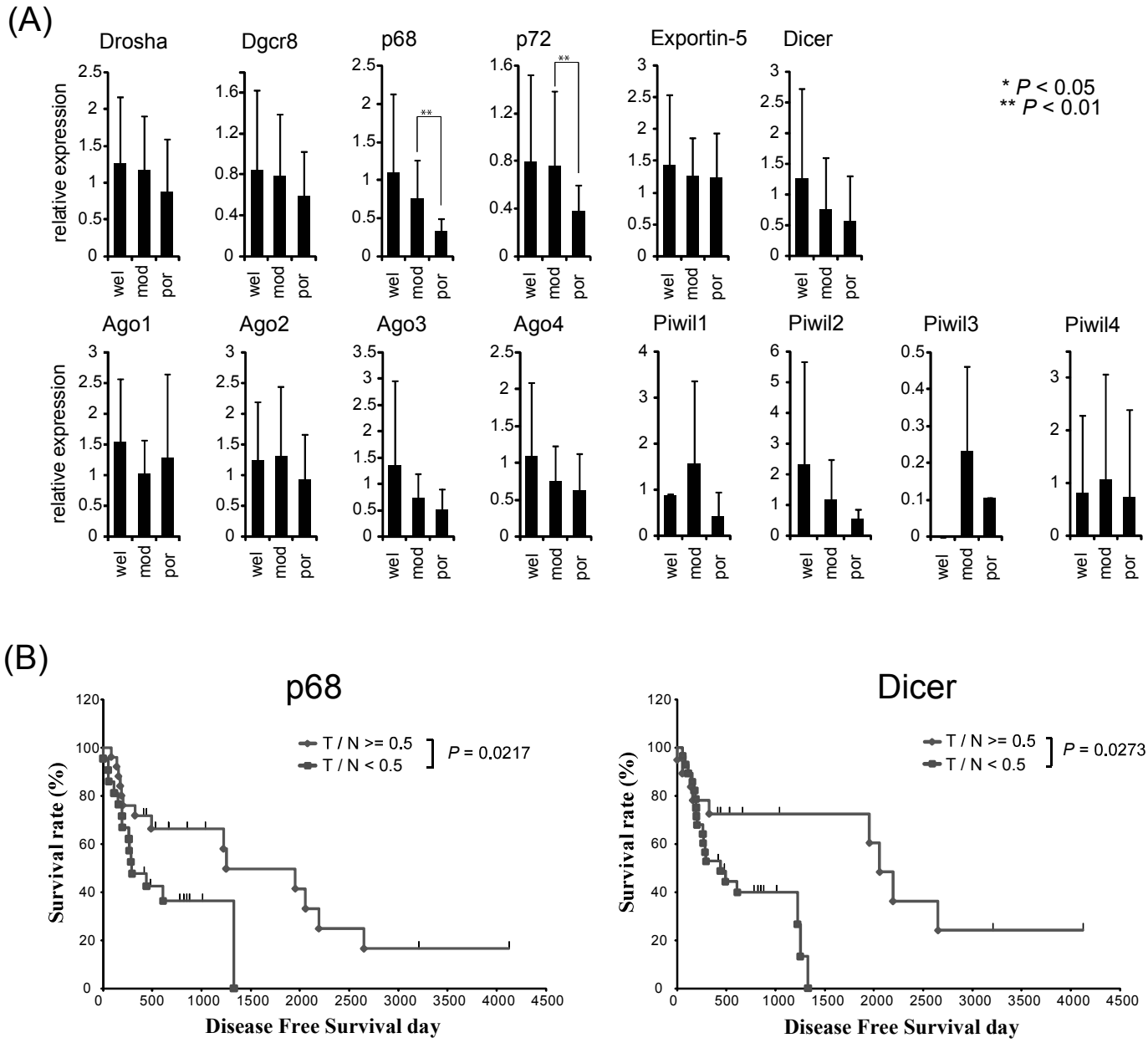

(A)
Drosha
Dgcr8
p68
p72
Exportin-5
Dicer
relative expression
wel
mod
por
* P < 0.05
** P < 0.01
Ago1
Ago2
Ago3
Ago4
Piwil1
Piwil2
Piwil3
Piwil4
(B)
p68
T / N >= 0.5
T / N < 0.5
P = 0.0217
Survival rate (%)
Disease Free Survival day
Dicer
T / N >= 0.5
T / N < 0.5
P = 0.0273
Survival rate (%)
Disease Free Survival day

Fig. 4

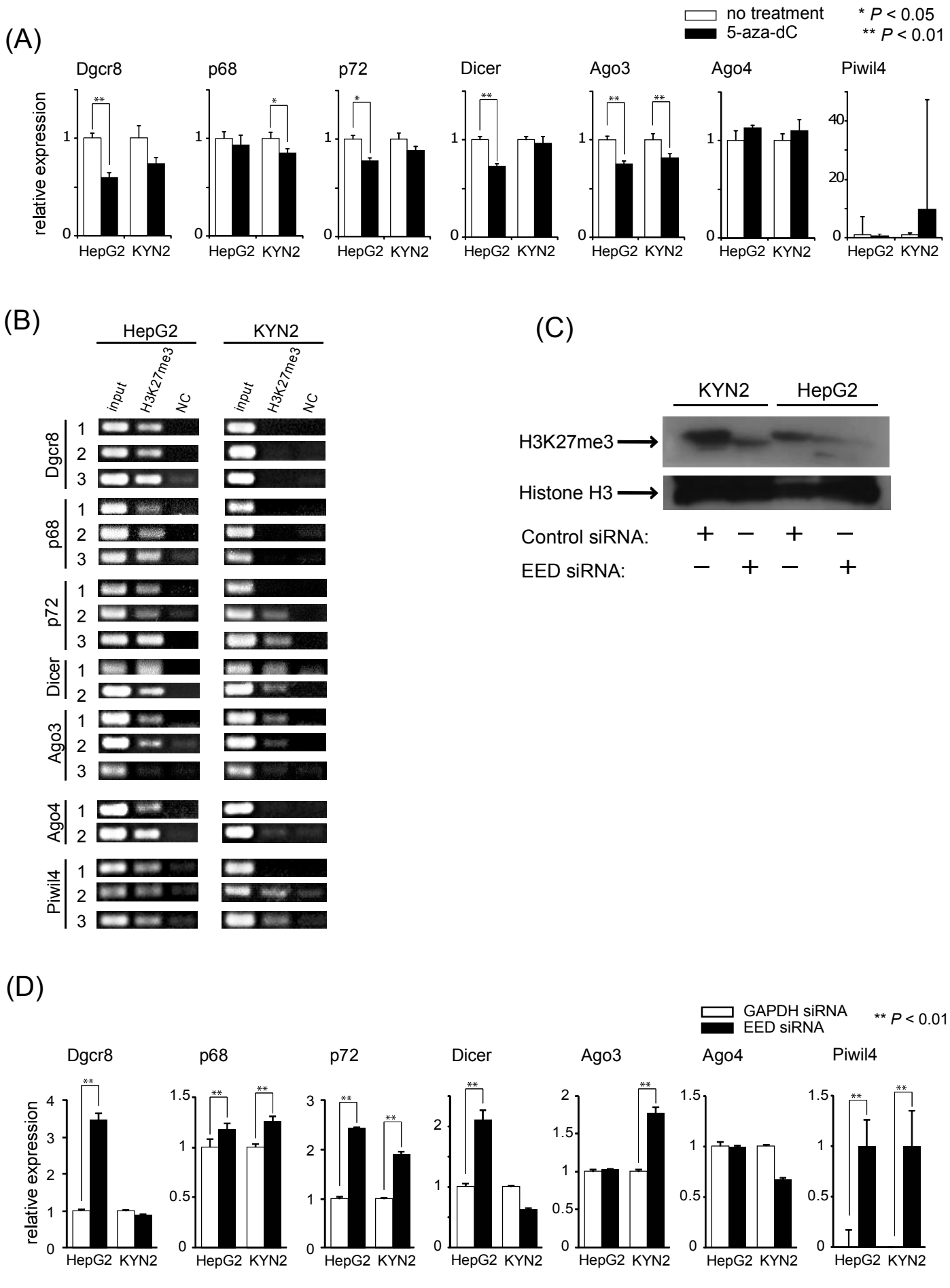

(A)
no treatment
5-aza-dC
* P < 0.05
** P < 0.01
Dgcr8
p68
p72
Dicer
Ago3
Ago4
Piwil4
relative expression
HepG2
KYN2
(B)
HepG2
KYN2
input
H3K27me3
NC
Dgcr8
p68
p72
Dicer
Ago3
Ago4
Piwil4
(C)
KYN2
HepG2
H3K27me3
Histone H3
Control siRNA:
EED siRNA:
(D)
GAPDH siRNA
EED siRNA
** P < 0.01
Dgcr8
p68
p72
Dicer
Ago3
Ago4
Piwil4
relative expression
HepG2
KYN2

Table 1. The Clinicopathological data of the 47 cases.

Characteristic		n (%)
Sex	Male	40 (85.1)
	Female	7 (14.9)
Median age, y (Range)		66.0 (40-78)
Viral infection	HBV positive[a]	11 (23.4)
	HCV positive[b]	16 (34.0)
	Both HBV and HCV Negative	18 (38.3)
	Past of HBV positive[c]	2 (4.3)
Tobacco	Yes	33 (70.2)
	No	14 (29.8)
Alcohol	Yes	34 (72.3)
	No	8 (17.0)
	Unknown	5 (10.6)
HbA1c	>= 6.0 %	12 (25.5)
	< 6.0 %	28 (59.6)
	Unknown	7 (14.9)
Grade of tumor differentiation	Well differentiated HCC	7 (14.9)
	Moderately differentiated HCC	31 (66.0)
	Poorly differentiated HCC	9 (19.1)
Maximum tumor diameter	< 2.0 cm	4 (8.5)
	> 2.0 cm	43(91.5)
Number of Tumors	Single	32(68.1)
	Multiple	15(31.9)
Non tumor liver	Normal	7 (14.9)
	Chronic hepatitis	10 (21.3)
	Precirrhosis	19 (40.4)
	Cirrhosis	11 (23.4)
intra-hepatic metastasis	positive	8 (17.0)
	negative	39 (83.0)

[a]HBV positive represents positivity for hepatitis B surface antigen.
[b]HCV positive represents positivity for serum HCV antibody.
[c]History of past HBV infection represents negativity for hepatitis B surface antigen, positivity for hepatitis B surface antibody and positivity for hepatitis B core antibody.

Table 2. Mutivriate logistic regression analysis for smoking

Gender	Hazard ratio	95% CI	P value
Non tumor liver			
Normal			
Chronic	0.199	0.154-9.888	0.842
Precirrhosis	0.642	0.170-32.96	0.521
Cirrhosis	0.709	0.189-35.20	0.478
DGCR8	-0.617	0.701-1.203	0.537
EXPORTIN5	-1.089	0.013-3.478	0.276
AGO1	-1.987	0.121-0.986	0.047
AGO2	0.235	0.230-6.504	0.814
AGO3	0.278	0.841-1.259	0.781

Table 3. Mutivriate logistic regression analysis for diabate

Gender	Hazard ratio	95% CI	P value
Non tumor liver			
Normal			
Chronic hepatitis	0.534	0.230-13.05	0.593
Precirrhosis	1.104	0.344-45.80	0.2695
Cirrhosis	0.919	0.183-110.3	0.358
AGO1	2.377	1.287-13.818	0.0175

Fig. S1

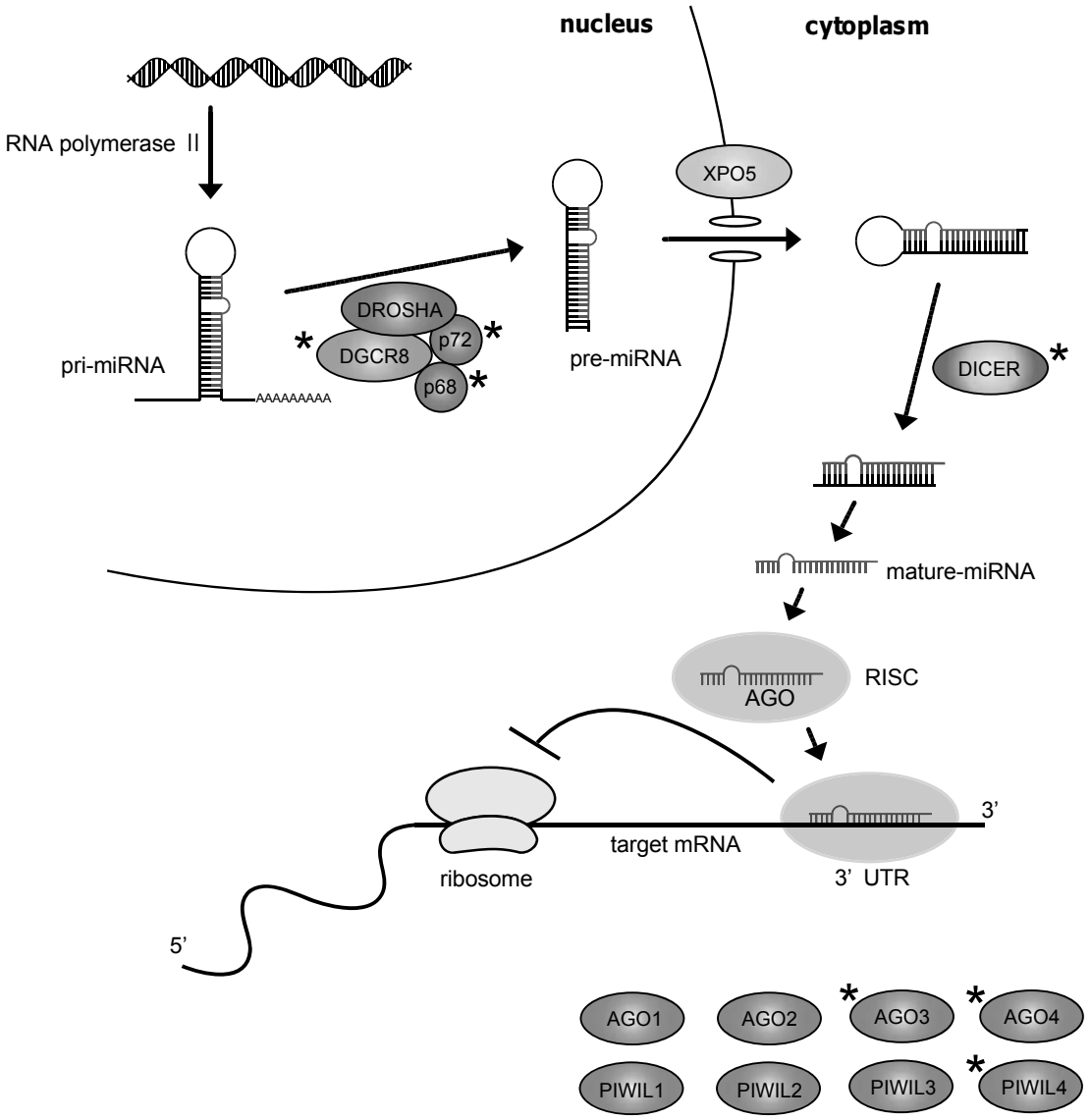

nucleus
cytoplasm
RNA polymerase II
XPO5
pri-miRNA
AAAAAAAAA
DROSHA
DGCR8
p72
p68
pre-miRNA
DICER
mature-miRNA
RISC
AGO
target mRNA
3'
3' UTR
ribosome
5'
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL3
PIWIL4

Fig. S2

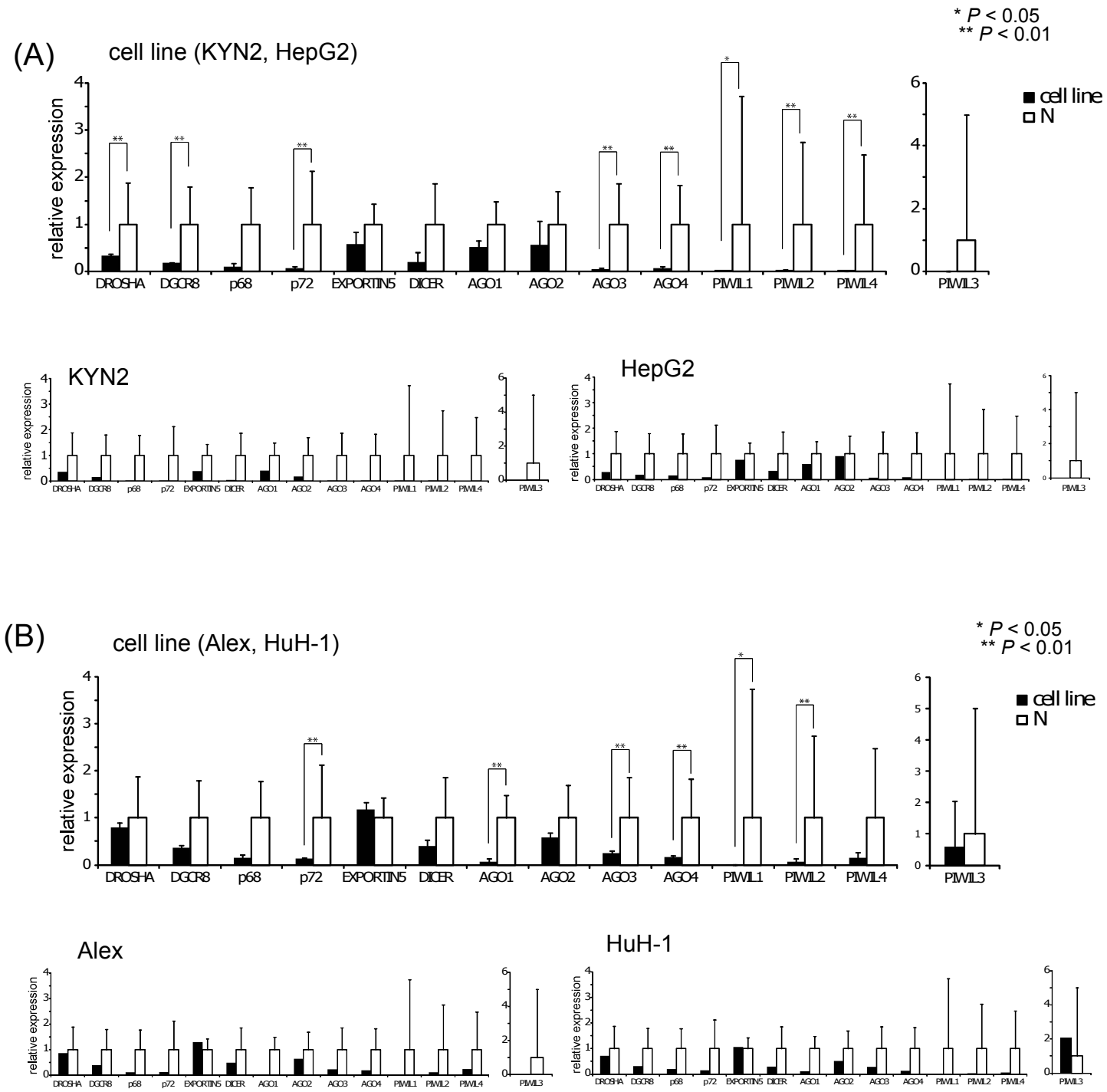

(A)
cell line (KYN2, HepG2)
* P < 0.05
** P < 0.01
relative expression
cell line
N
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL4
PIWIL3
KYN2
HepG2
(B)
cell line (Alex, HuH-1)
Alex
HuH-1

Fig. S3

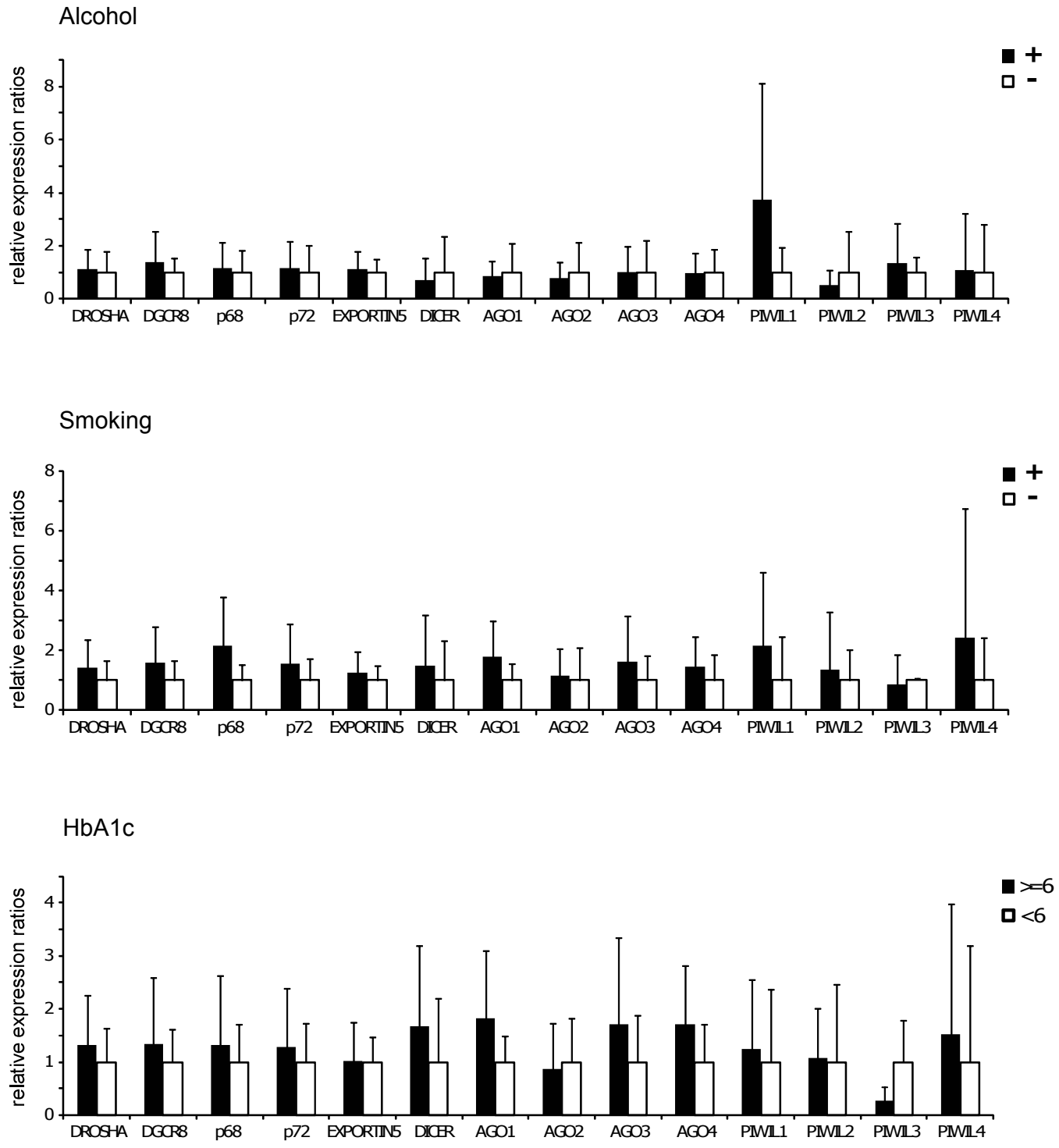

Alcohol
relative expression ratios
0
2
4
6
8
+
-
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL3
PIWIL4
Smoking
relative expression ratios
0
2
4
6
8
+
-
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL3
PIWIL4
HbA1c
relative expression ratios
0
1
2
3
4
>=6
<6
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL3
PIWIL4

Fig. S4

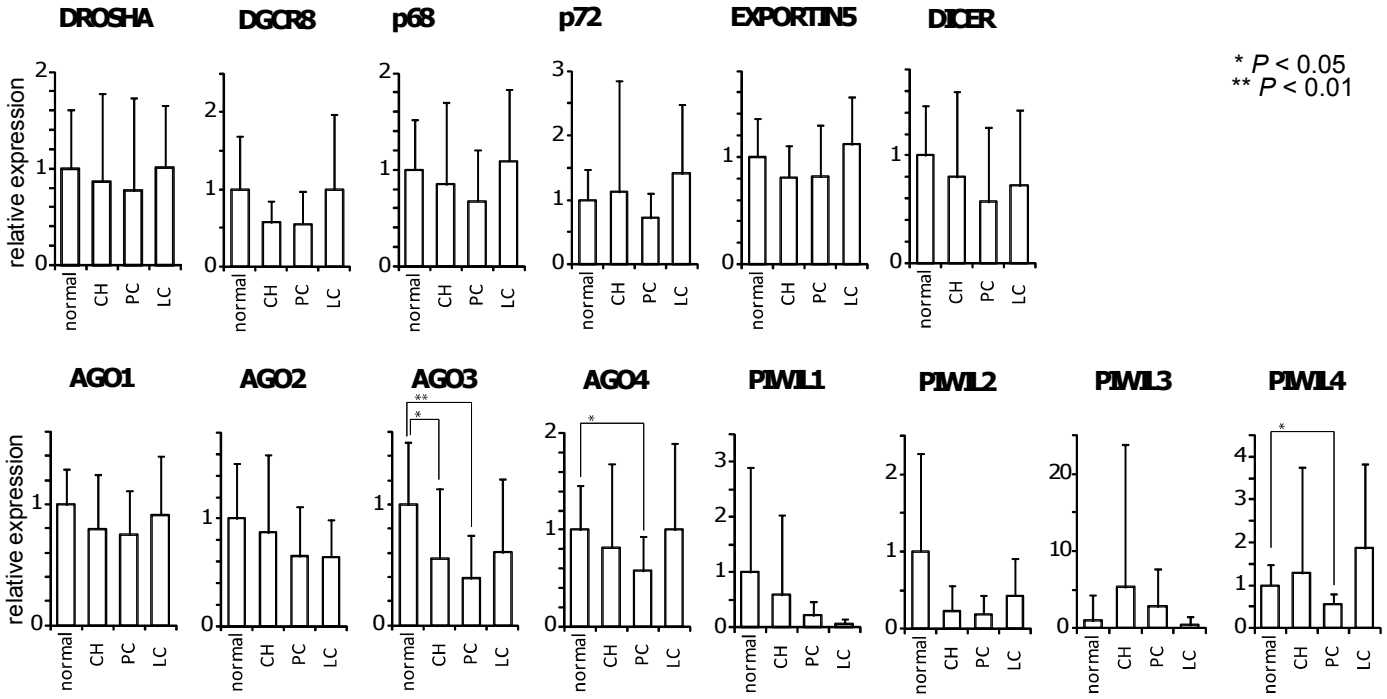

DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
PIWIL1
PIWIL2
PIWIL3
PIWIL4
relative expression
normal
CH
PC
LC
* P < 0.05
** P < 0.01

Fig. S5

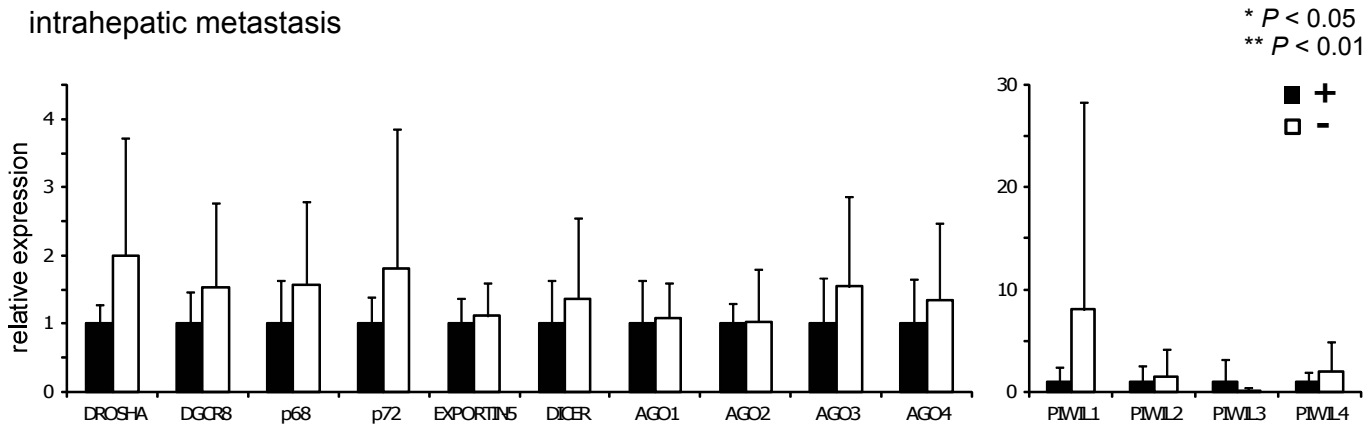

intrahepatic metastasis
* P < 0.05
** P < 0.01
relative expression
0
1
2
3
4
DROSHA
DGCR8
p68
p72
EXPORTIN5
DICER
AGO1
AGO2
AGO3
AGO4
0
10
20
30
PIWIL1
PIWIL2
PIWIL3
PIWIL4
+
-

Fig. S6

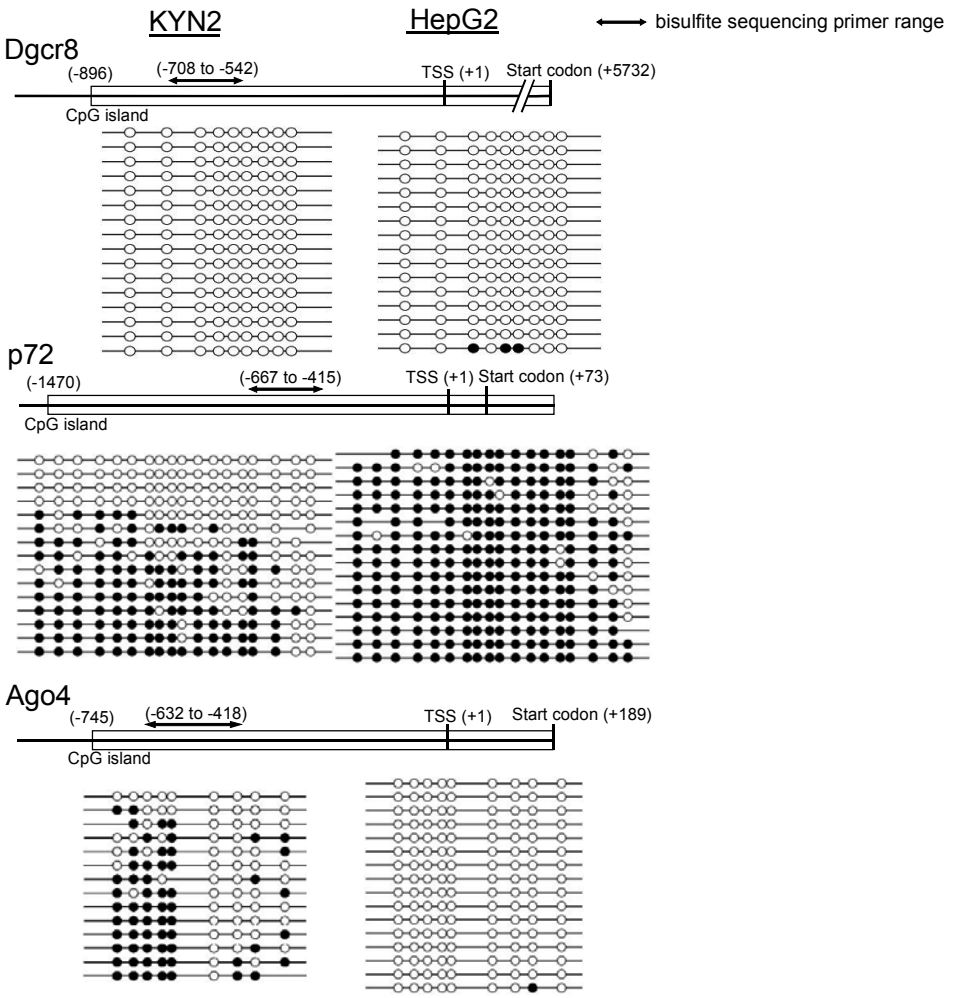
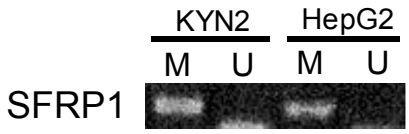

KYN2
HepG2
bisulfite sequencing primer range
Dgcr8
(-896)
(-708 to -542)
TSS (+1)
Start codon (+5732)
CpG island
p72
(-1470)
(-667 to -415)
TSS (+1)
Start codon (+73)
CpG island
Ago4
(-745)
(-632 to -418)
TSS (+1)
Start codon (+189)
CpG island

Fig. S7


KYN2
HepG2
M
U
M
U
SFRP1

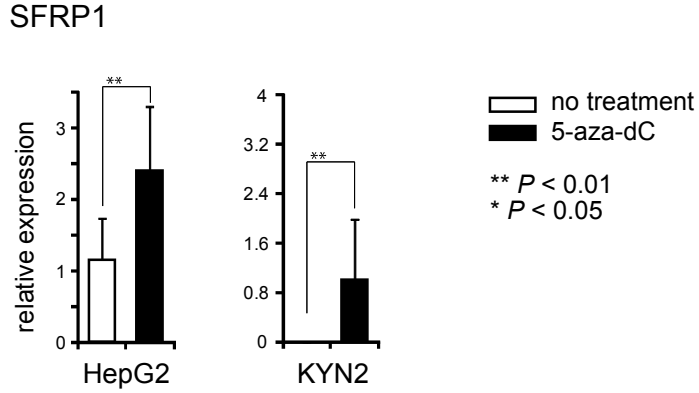
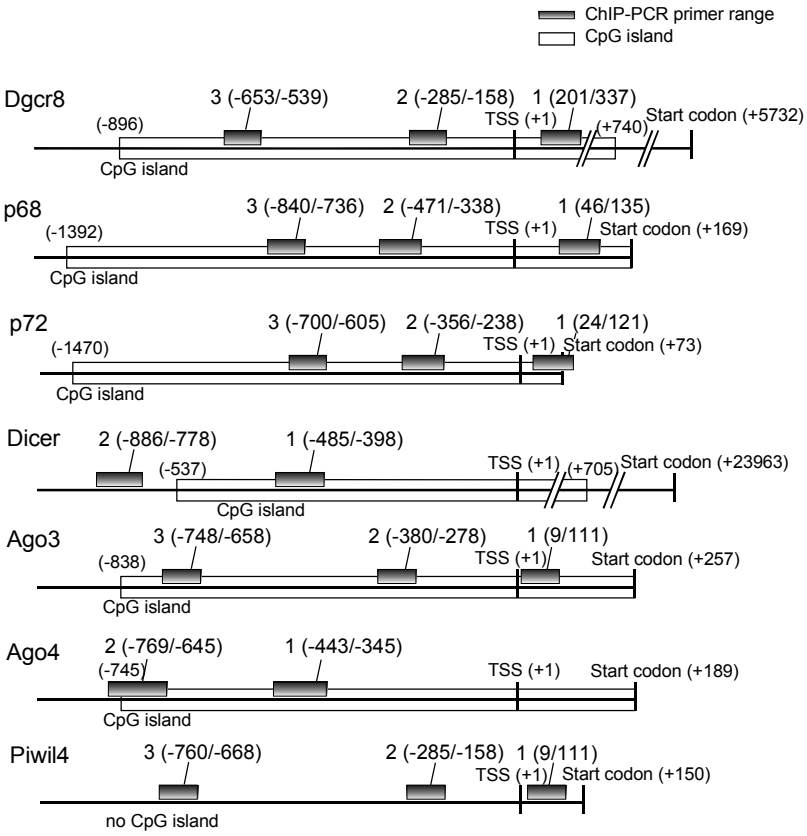

SFRP1
relative expression
**
**
0
1
2
3
0
0.8
1.6
2.4
3.2
4
HepG2
KYN2
no treatment
5-aza-dC
** P < 0.01
* P < 0.05

Fig. S8


ChIP-PCR primer range
CpG island
Dgcr8
3 (-653/-539)
2 (-285/-158)
1 (201/337)
(-896)
TSS (+1)
(+740)
Start codon (+5732)
CpG island
p68
3 (-840/-736)
2 (-471/-338)
1 (46/135)
(-1392)
TSS (+1)
Start codon (+169)
CpG island
p72
3 (-700/-605)
2 (-356/-238)
1 (24/121)
(-1470)
TSS (+1)
Start codon (+73)
CpG island
Dicer
2 (-886/-778)
1 (-485/-398)
(-537)
TSS (+1)
(+705)
Start codon (+23963)
CpG island
Ago3
3 (-748/-658)
2 (-380/-278)
1 (9/111)
(-838)
TSS (+1)
Start codon (+257)
CpG island
Ago4
2 (-769/-645)
1 (-443/-345)
(-745)
TSS (+1)
Start codon (+189)
CpG island
Piwil4
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2 (-285/-158)
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TSS (+1)
Start codon (+150)
no CpG island

Fig. S9

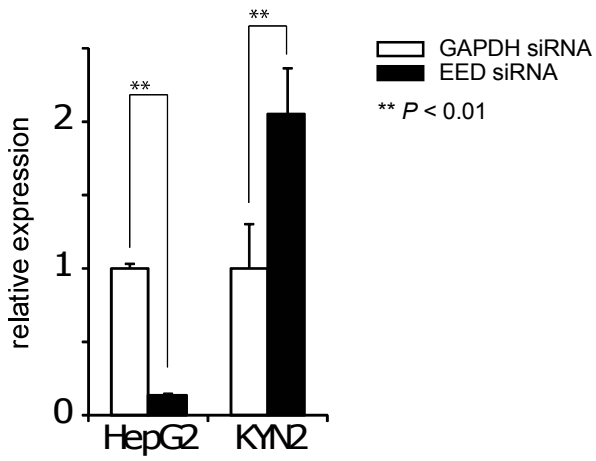
**
**
relative expression
2
1
0
HepG2
KYN2
GAPDH siRNA
EED siRNA
** P < 0.01

Table S1. PCR primers.

Primer name	Sequence		Product size (bp)	UPL probe Number	Annealing temp. (°	Number of cycle
	Forward	Reverse				
qRT-PCR						
Drosha	TCTCTGGAAAGGTCCTACAAAAA	CAGGTTCAGGAACAACCGATA	76	#12		
Dgcr8	AAAACTTGCGAAGAATAAAGCTG	TCTGTTTAACAAAGTCAGGGATGA	68	#47		
p68 (DDX5)	TATGGTTGGAGTGGCACAGA	CCAGCACCAAACAAATAGGC	122	#4		
p72 (DDX17)	AACCACCAGCCATACTTGGA	ATCGGCCACCTGCTGTACT	96	#61		
Exportin-5	TATATACTCTCCGCCGCACA	GCCTCTTCAGAAAGACGTAGTGT	73	#25		
Dicer	AGCAACACAGAGATCTCAAACATT	GCAAAGCAGGGCTTTTCAT	94	#47		
Ago1	CACGGGTATATGGGATGGAA	GTGCCTGGAACACCTGCT	84	#32		
Ago2	GTCTCTGAAGGCCAGTTCCA	ATACAGGCCTCACGGATGG	65	#22		
Ago3	TGTGGGCTATCGCTTGTTTT	TACGCAGCTGGTCTGTGAAA	76	#34		
Ago4	TTCTGTGCAGATAAAACAGAAAGG	ACTCAGATGGATGTGTGATGGTA	89	#40		
Piwil1	AGAGGTTACCAGACCAGAATGG	GTGTGGGAGAAACACTACCACTT	77	#64		
Piwil2	TGTCTCCCATAAGGTCATTCG	CCTGGAAGTGTTCTTTATTCTGC	83	#42		
Piwil3	TGAAAAACGCAAACATCGTG	ACCAGTCTCCTTGTGAATTGG	63	#29		
Piwil4	GCAGGATCTCAACCAACGAT	CACACCTCTTTCCATGAAGGT	74	#9		
SFRP1	ATCCGCAATGTGGAGCAG	TTGCTGTTGGAAATGGTTTG	62	#63		
EED	AATCCGGTTGTTGCAATCTT	CAGAGGATGGCTCGTATTGC	94	#89		
Bisulfite sequencing				touch down method		
Dgcr8 (-708 to 542)	TTTTATTATGTTAGTTAGGTTGGTTT	ATCCCCTTTACTAATACAAAATTTCC	167			
p68 (-1183 to -972)	GATTTTAGGGTTTATAGTGTAAGGG	AAATCTCTCAACTTCACCACCTCT	212		62°C →	
p72 (-667 to -415)	GGGTATTTTAATTTGGAAGGGATAT	ATTATCAAATTTCAAAATCCTCTAAC	253		50°C	
Dicer (-489 to -218)	GGAGGTGTTTAGAGGGAAGTTAAGT	AACCACTCAAAAACAAAAAAACAAC	272		(0.5°	35
Ago3 (-622 to -434)	TTTGTGTAGTGTTTTGTTTGTTAGTTATTT	TAAAACCACCCTCCTTCCTAAAC	189		C/cycle)	
Ago4 (-632 to -418)	GGGATTAGGGAGGGAATGTATTAT	TACTAAAATTCACAAAAACCACCTC	215			
MSP						
SFRP1-M	TGTAGTTTTCGGAGTTAGTGTCGCGC	CCTACGATCGAAAACGACGCGAACG	126		60	35
SFRP1-U	GTTTTGTAGTTTTTGGAGTTAGTGTTGTG	CTCAACCTACAATCAAAAACAACACAAA(	118		60	35
ChIP-PCR						
Dgcr8-1 (201 to 337)	GGCCTTTGTGAGGCAACAT	CGGGAGAGGAGCCTCTTTAC	137		65	43
Dgcr8-2 (-473 to -376)	GGTTGTGTAACGCCTGTGTG	AACAGGGAGCGCGGAGTAC	98		63	40
Dgcr8-3 (-653 to -539)	CTCGACCTCCCAAAACTCTG	CGCTGTCCCCTTTGCTAATA	115		63	40
p68-1 (46 to 135)	GTGTCATCGGTGTCCTTCCT	ATAGAAAAGCGTGCGACAAG	90		63	37
p68-2 (-471 to -338)	CTTCCGGTGAGCTATTTCG	TAACCAAAAGAGGGGGAAGG	134		63	37
p68-3 (-840 to -736)	GGGAGACACTCACCAGCATC	GTGGATCGGTCCTTCCAG	105		63	37
p72-1 (24 to 121)	AGGAAGGAGACGCCTAAACC	GACGGGAGCAAAACACAGAG	98		63	40
p72-2 (-356 to -238)	GTACCGAGATCGGAATCAGG	TCTGGTGCGACGTAACATTC	119		63	37
p72-3 (-700 to -605)	AATGCACCTCAGTGTGAACC	GAGCCGTGTCCCTTTCTTTC	96		63	40
Dicer-1 (-485 to -398)	GTGCTCAGAGGGAAGCTAAG	GACTGCCTCCATTGTTGCTC	89		67	45
Dicer-2 (-886 to -778)	GGGCGCATAGTAGGTTCTGC	TTCAGTGGACCCCCTGATAG	109		65	47
Ago3-1 (9 to 111)	ACGGGACTCCCCTCTGTC	CGCACTCCGAAAGCTCTG	103		63	40
Ago3-2 (-380 to -278)	GGCTCCAGGTAGGCTACTCC	GAGGGGCAGGACTACTCG	103		63	40

Ago3-3 (-748 to -658)	GAGAATTGGAAGCCAACCAG	TTAATAGCGCCTCGTCACAC	111	63	33
Ago4-1 (-443 to -345)	AGGTGGCTCCTGTGAATCTC	CGTCCTGCAAAAGATCTGG	98	63	37
Ago4-2 (-769 to -645)	CAAACTCCTGACCCCAGGT	CTCTGGTCAGCAAATCACC	125	63	37
Piwil4-1 (18 to 130)	GTTGGTTGTGGATGCTGGAC	GCCACAAGAGCTGCAAAGTC	113	63	37
Piwil4-2 (-285 to -158)	GGATACGACAGGCAATTTGC	TGTATTGGGTGGCATCAAAC	129	63	37
Piwil4-3 (-760 to -668)	CGTGGGAATCTGTCATTTGC	GTGAGCCATTCTTCCTTGAC	93	63	37

*M: Methylated, U: Unmethylated

Table S2. Pearson's correlation among the relative expression ratios (tumor vs. non-cancerous liver) of the 7 miRBir genes.

	Dgcr8	p68	p72	Dicer	Ago3	Ago4	Piwil4
Dgcr8	1.000	0.700	0.858	0.507	0.522	0.509	0.367
p68	0.700	1.000	0.804	0.528	0.600	0.415	0.512
p72	0.858	0.804	1.000	0.701	0.702	0.538	0.371
Dicer	0.507	0.528	0.701	1.000	0.854	0.679	0.344
Ago3	0.522	0.600	0.702	0.854	1.000	0.773	0.195
Ago4	0.509	0.415	0.538	0.679	0.773	1.000	0.257
Piwil4	0.367	0.512	0.371	0.344	0.195	0.257	1.000

black letters: > 0.6 and $p < 0.05$

Ago3, Ago4 and Dicer or Dgcr8, p68 and p72 are correlated with each other.

Table S3. Statistical analysis between subgroups of patients background (Alcohol, Tobacco and Diabetes)

Gender	Alcohol			Tobacco			HbA1c		
	Yes N = 34	**No N = 8**	**P value***	**Yes N = 33**	**No N = 14**	**P value***	**>= 6.0 N = 12**	**< 6.0 N = 28**	**P value***
Sex									
Male	32	3	0.0012	30	10	0.1732	12	23	0.298
Female	2	5		3	4		0	5	
Age									
>= 65	18	6	0.4307	18	10	0.3435	9	18	0.7159
< 65	16	2		15	4		3	10	
HBV infection									
HBV positive	10	1	0.6569	7	4	0.7098	0	7	0.081
HBV negative	24	7		26	10		12	21	
HCV infection									
HCV positive	9	5	0.0924	12	4	0.7422	3	10	0.4096
HCV negative	25	3		21	10		9	18	
Tobacco									
Yes	26	4	0.1954	—	—	—	—	—	—
No	8	4		—	—		—	—	
Alcohol									
Yes	—	—	—	26	8	0.2939*	10	18	0.4437
No	—	—		4	4		1	6	
Unknown	—	—		3	2		1	4	
HbA1c									
>= 6.0	10	1	0.3916	11	1	0.0627	—	—	—
< 6.0	18	6		16	12		—	—	
Grade of tumor									
Well differentiated	5	2	0.1939*	5	2	0.5751*	3	4	0.5012*
Moderately	24	3		23	8		8	18	
Poorly defferentiated	5	3		5	4		1	6	
Maximum tumor									
<2.0 cm	17	4	>0.9999	16	8	0.7516	7	14	0.7365
>2.0 cm	17	4		17	6		5	14	

Number of Tumors									
Single	23	5	>0.9999	20	12	0.1698	5	21	0.0705
Multiple	11	3		13	2		7	7	
Non tumor liver									
Normal	8	1	0.5867*	6	5	0.6132*	3	8	0.8493*
Chronic hepatitis	13	4		14	5		6	10	
Precirrhosis	9	1		8	2		2	6	
Cirrhosis	4	2		5	2		1	4	
im									
Positive	5	2	0.6012	7	1	0.4048	3	4	0.4096
Negative	29	6		26	13		9	24	

*Fisher's exact test
**Chi-square test

Table S4. Mutivriate logistic regression analysis for Alcohol

Gender	Hazard ratio	95% CI	P value
Non tumor liver			
Normal			
Chronic hepatitis	-0.92	0.038-4.311	0.4547
Precirrhosis	-0.203	0.060-21.09	0.9372
Cirrhosis	-0.839	0.017-3.661	0.3113
PIWIL1	-1.514	0.485-1.097	0.13