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Transcriptome profiling of infectious diseases and cancer in zebrafish

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Chapter 5

Liver tumor-related microRNA expression is conserved between zebrafish and human

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Abstract

The use of zebrafish models for liver cancer has been supported by a strong conservation of gene expression signatures between zebrafish and human liver tumors. The expression levels of genes involved in many cellular processes are modulated by the activity of microRNAs. Aberrant expression of these small non-coding RNAs has been associated with many diseases, including cancer. In this study we compared the miRNA transcriptomes of zebrafish liver tumors and human hepatocellular carcinoma (HCC). Microarray analysis of five individual zebrafish with carcinogen-induced liver tumors indicated a consistent tumor-specific expression signature for a large set of known and predicted miRNAs. The most notable similarities between zebrafish and human liver cancer were the common up-regulation of miR-21, miR-23a, miR-146a/b, miR-221 and miR-222, and the common down-regulation of miR-1 and miR-122. In addition, several miRNAs not previously linked to HCC but linked to several other types of human cancer showed differential expression in zebrafish liver tumors. A subset of the liver tumor-related miRNAs, including members of the miR-21 and miR-146 families, was also responsive to *Mycobacterium marinum* infection in zebrafish, suggesting a common role in cancer and the immune response. Finally, we investigated the possible conservation of miRNA target genes and found that several putative target genes of the miR-1, miR-146 and miR-221/miR-222 families are conserved between human and zebrafish. The conserved expression signatures of these tumor-related miRNAs and the involvement of their predicted target genes in cancer-related processes and signal transduction pathways corroborate the similarity between zebrafish and human liver cancer.

Introduction

Zebrafish is increasingly used as a model organism for cancer research (Amatruda et al., 2002; Berghmans et al., 2005; Feitsma and Cuppen, 2008; Lam and Gong, 2006). Several types of cancers have been studied in zebrafish as they can spontaneously develop almost any type of tumors with notable similarities to human and mammalian tumors. The ease of performing tumor transplantations and visualization of tumor invasiveness stimulated the development of zebrafish cancer models using forward and reverse genetic screens and transgenesis methods.

Recently, zebrafish has been used as a model species to study liver cancer. It has been shown that zebrafish and human liver tumors share molecular, biochemical and cellular similarities (Chu and Sadler, 2009; Lam and Gong, 2006; Lam et al., 2006; Ung et al., 2009). They have largely overlapping expression profiles of genes involved in cell cycle/proliferation, apoptosis, DNA replication and repair, metastasis and cell adhesion, cytoskeletal organization and cell motility, and RNA processing and protein synthesis. Differential expression of genes in these processes is consistent with the characteristics of human liver cancer such as defective apoptosis, uncon-

trolled proliferation, limitless replication and loss of tissue-specific functions (Chu and Sadler, 2009; Lam and Gong, 2006; Lam et al., 2006).

MicroRNAs (miRNA) are highly conserved small non-coding RNAs that control gene expression by regulating mRNA stability and translation. They play crucial roles in essential physiological functions including cell cycle, differentiation, development, and metabolism (Schmidt et al., 2009; Sonkoly and Pivarcsi, 2009). A large number of recent studies support that miRNAs are important regulators of the immune system and that specific alterations of their expression contribute to pathogenesis of cancer and other diseases (Calin et al., 2002; Garofalo et al., 2008; Garzon et al., 2009; Lu et al., 2005; Lu and Liston, 2009; Medina and Slack, 2008). More than 50% of miRNA genes were found to reside in cancer-associated genomic regions (Calin et al., 2004).

Differentially expressed miRNAs in cancer can act either as direct causatives of tumorigenesis (oncogenic and tumor suppressor oncomirs) or can be indirectly involved in genomic, epigenomic or physiological changes associated with cancer (Esquela-Kerscher and Slack, 2006). Recently, a significant group of miRNAs have been found to be expressed in the liver and to modulate several liver functions (Chen, 2009). Their deregulation may play a crucial role in liver diseases including hepatocellular carcinoma (HCC). HCC is the most common malignancy in the liver and one of the most common causes of death from cancer triggered either by viral (hepatitis B and C) or nonviral (alcohol and aflatoxin B1) agents. Revealing alterations in miRNA expression and uncovering their mRNA targets is important to our understanding of carcinogenesis and could lead to novel therapeutic strategies based on the modulation of miRNA activity. Using a murine liver cancer model, it was recently shown that systemic administration of miR-26a, a miRNA showing reduced expression in HCC cells, could suppress tumorigenesis (Kota et al., 2009). In addition to this miRNA replacement strategy, the application of synthetic inhibitors (antagomirs) of miRNAs up-regulated in HCC may prove to be a promising approach to liver cancer treatment (Krutzfeldt et al., 2005; Pineau et al.; Tsai et al., 2009).

While numerous studies have uncovered miRNA profiles of different cancer types, their exact functions are not well understood. MiRNAs that have been linked to the progression of HCC and metastasis among others include miR-10, miR-15a, miR-21, miR-26a, miR-29a, miR-34a, miR-101, miR-122, miR-146, miR-199, miR-221/222, and miR-224 (Budhu et al., 2008; Chen, 2009; Huang et al., 2008; Ladeiro et al., 2008; Murakami et al., 2006; Su et al., 2009; Varnholt et al., 2008). Several miRNAs that are differentially expressed in HCC show similar alterations in other cancers, suggesting that there are common miRNAs regulating basic processes of carcinogenesis. Each miRNA is predicted to regulate several hundreds of target mRNAs. The identification of true *in vivo* targets is crucial for understanding of miRNA function. A number of targets of deregulated miRNAs in HCC and other types of cancer have now been experimentally validated, including several oncogenes and tumor suppressor genes, inhibitors of apoptosis, cell cycle regulators and genes involved in cell

proliferation, migration and invasion (Bueno et al., 2008; Mann et al., 2007; Medina and Slack, 2008; Mott, 2009; Qin and Tang, 2002).

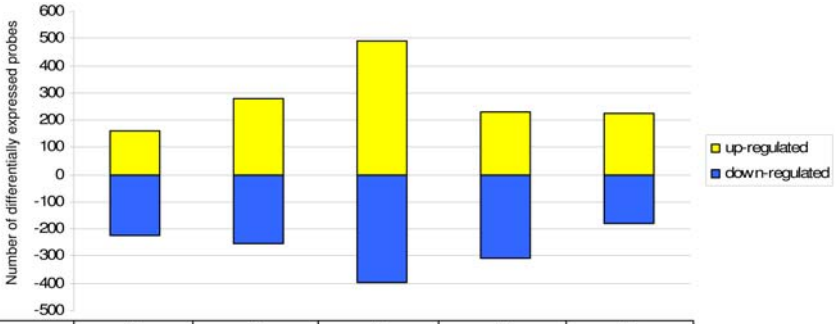
Here, we analyzed the miRNA transcriptome of zebrafish liver tumors and compared our data set with miRNAs previously detected in human liver cancer. Furthermore, we determined the overlap between the liver cancer-related miRNAs and miRNAs identified in previous infection studies of zebrafish (chapter 4). Finally, we compiled experimentally validated targets of differentially expressed tumor-related miRNAs to shed more light on their role in cancer mechanisms. With these studies we have defined tumor and immune-related miRNA marker sets and predicted targets conserved between human and zebrafish.

Results

Differentially expressed miRNAs in liver tumor samples from zebrafish

Previously, it has been shown that gene expression profiles between zebrafish and human liver tumors are strongly overlapping (Lam and Gong, 2006). In order to determine if miRNA expression is also conserved between zebrafish and human liver tumors, we obtained samples from these previous studies and analyzed them using a custom-designed Agilent 8x15k microarray for relative quantification of miRNA expression. We compared liver tissues from 5 fish with liver tumors induced by carcinogen (DMBA) treatment with normal liver tissues from 5 healthy control fish. In the five biological replicates the numbers of differentially expressed probes showing more than 1.5-fold induction or repression ($P < 1.00E{-}5$) ranged between 385 and 888 (Fig. 1). The percentage of up-regulated probes (45%-60%) was similar to that of down-regulated probes in all replicates (Fig.1). The custom-designed zebrafish microarray platform contained probes for both known and predicted miRNAs, the latter classified into three categories: C1 - novel confident miRNAs for which there is compelling experimental evidence, C2 - candidate miRNAs with clear hairpin structure but less experimental evidence, and C3 - additional hairpins identified in the zebrafish genome sequence that are less likely to be miRNAs. Approximately one third of the differentially expressed probes corresponded to known miRNAs, even though these represent less than one tenth of the total number of probes on the microarray (546 out of 7604 probes). The largest group of probes on the microarray belongs to the C3 category (6942 probes) and between 4-9% of these probes showed differential expression in the microarray analysis. In the smaller C1 (62 probes) and C2 (54 probes) categories only a few probes showed altered expression in the replicate liver tumor samples (C1: 4 to 8; C2: 1 to 5). Next, we determined the overlap of differentially expressed probes between the biological replicates (Fig. 2). Taking together the known miRNA probes and the C1/C2 probes for experimentally supported miRNA candidates, we found that 72 probes were consistently changed in at least 4 out of 5 biological replicates, of which 26 probes showed differential expression in all 5 replicates. A good overlap was also observed for the probes in the C3 category of putative

A



B

	#1	#2	#3	#4	#5
known up	59	104	113	106	130
known down	40	32	90	36	17
C1 up	2	2	5	4	4
C1 down	3	2	3	2	2
C2 up	0	1	1	2	1
C2 down	4	3	2	3	0
C3 up	101	174	373	120	90
C3 down	176	214	301	267	161

Figure 1. Differentially expressed miRNA probes in zebrafish liver tumor samples. (A) Total number of up-regulated and down-regulated probes in five biological replicates of healthy liver versus liver tumor. (B) Number of up-regulated and down-regulated probes in four annotation categories: known miRNAs, and predicted miRNAs, C1, C2, and C3, with decreasing level of confidence as further detailed in the text. The total number of probes on the array per category is: 546 probes in known, 62 probes in C1, 54 probes in C2 and 6942 probes in C3 category.

miRNAs without experimental support, where 140 probes were changed in at least 4 out of 5 biological replicates. In conclusion, our microarray analysis of five individual fish with carcinogen-induced liver tumors indicated a consistent tumor-specific expression signature for a large set of known and predicted miRNAs.

Comparison of miRNA regulation between zebrafish and human

To study the miRNA transcriptome similarities between zebrafish and human liver tumors we compiled in Table 1 those miRNAs that were differentially expressed in at least 4 replicates of the zebrafish liver tumor study and investigated which of these miRNAs have previously been related to human hepatocellular carcinoma (HCC) or to other types of human cancer. Furthermore, we also included in Table 1 all other human miRNAs that were previously linked to HCC in the literature and indicated the number of zebrafish liver tumor replicates in which these miRNAs were differentially expressed. The results of the comparative analysis in Table 1 are summarized in Figure 3, showing that several miRNAs were commonly regulated in zebrafish and human liver cancer. MiRNAs previously reported to show increased expression in HCC and that we also found up-regulated in zebrafish liver tumors included miR-

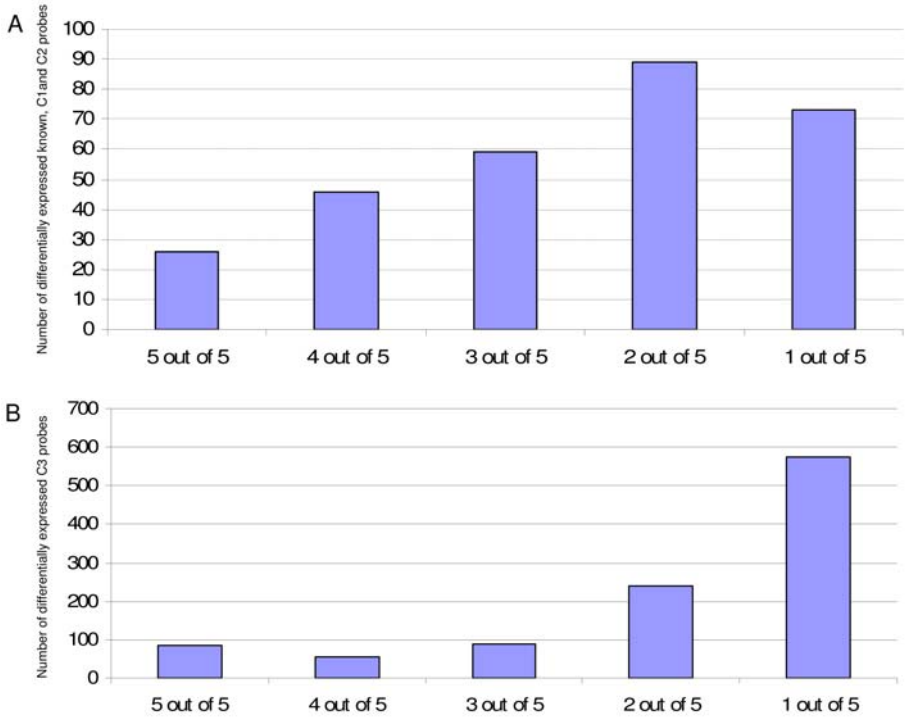


Figure 2. Overlap of differentially expressed miRNA probes between biological replicates of healthy liver versus liver tumor. (A) Overlap of probes in the combined categories of known miRNAs and C1 and C2 miRNAs that include candidate miRNAs supported by experimental evidence. (B) Overlap of probes in the C3 category of putative miRNAs not supported by experimental evidence. Probes were sorted according to the number of replicates in which they were differentially expressed.

21, miR-23a, miR-146a, miR-146b, miR-221 and miR-222, which were consistently changed in at least 4 replicates of our microarray study. Furthermore, miR-1 and miR-122, for which decreased expression was reported in HCC, were consistently down-regulated in zebrafish tumors. Other miRNAs increased (miR-10a/b, miR-18c, miR-93, miR-125b and miR-181a/b, miR-338) or decreased (let-7a, miR-101b, miR-148 and miR-199) in HCC, were also changed in the same direction in zebrafish liver tumors, but only in 2 or 3 out of the 5 replicates. We also found miRNAs (including miR-9, miR-15a, miR-29a/b and miR-34 changed in 4-5 replicates, and miR-125a and miR-223 changed in 2-3 replicates) for which the observed direction of change in zebrafish liver tumors was opposite to changes reported for HCC (Figure 3). In addition, several miRNAs not previously linked to HCC showed differential expression in at least 4 replicates of the zebrafish liver tumor study, including miR-15b, miR-16-a/b, miR-27a/b, miR-132, miR-153a/b/c, miR-193a/b, miR-210, miR-212, miR-217, miR-

Table 1. Liver cancer-related miRNA probes.

Zebrafish miRNA	Expression in zf liver cancer	Human miRNA	Expression in HCC	Other associations to cancer	Functional links	References
dre-let-7a (let-7a-1_l, let-7a-2_l, let-7a-3_l, let-7a-4_l, let-7a-6_l)	down (2)	hsa-let-7a	down	down in lung squamous carcinoma	tumor suppression, proliferation, cell death	Mott JL 2009 Yang J et al 2008 Kutay H et al 2006
dre-miR-1 (miR-1-1_r, miR-1-2_r)	down (5)	hsa-miR-1	down	down in lung cancer, colon cancer	apoptosis	Datta J et al 2008 Nasser MW et al 2008 Melkamu T et al 2009 Tang Y et al 2009 Sarver AL et al 2009
dre-miR-7a* (miR-7a-2_r)	down (4)	hsa-miR-7-1*, hsa-miR-7-2*		hsa-mir-7: down in glioblastoma, breast cancer, urothelial carcinomas, lung cancer	hsa-mir-7: tumor suppression, aggressiveness	Kefas B et al 2008 Foekens JA et al 2008 Veerla S et al 2009 Webster RJ et al 2009 Reddy SD et al 2008
dre-miR-7b (miR-7b_l)	up (3) / down (1)	hsa-miR-7	-			
dre-miR-9 (miR-9-1_l, miR-9-3_l, miR-9-6_l)	down (4)	hsa-miR-9	up	down in gastric carcinoma	metastasis, invasion	Zhao Y et al 2009 Hao-Xiang T et al 2009 Luo H et al 2009 Budhu et al 2008
dre-mir-10a (mir-10a_l)	up (2)	hsa-miR-10a	up	up in pancreatic cancer, urothelial carcinoma, down in CML, CLL, AML,	metastasis	Varnholt H et al 2008 Weiss FU et al 2009 Veerla S et al 2009 Lund AH et al 2009
dre-mir-10b (mir-10b-1_l, mir-10b-2_l)	up (2) / down (1)	hsa-miR-10b	up	up in breast cancer, glioma	cell migration, metastasis, invasion	Ladiero et al 2008 Sasayama T et al 2009 Ma L et al 2007 Lund AH et al 2009
dre-miR-10d (miR-10d-1 _l)	up (3) / down (1)	-	-	-	-	
dre-miR-15a (miR-15a-1_l)	up (5)	hsa-miR-15a	down,deletion	up in APL, down in CLL, prostate cancer, adenomas	dedifferentiation, chromosomal instability, apoptosis, tumor suppression	Budhu et al 2008 Skawran B et al 2008 Aqeilan RI et al 2009
dre-miR-15b (miR-15b_l)	up (4)	hsa-miR-15b		up in melanomas, colorectal cancer, cervical cancer, APL, pancreatic cancer, down in gastric cancer	proliferation, apoptosis	Satzger I etal 2009 Xia L et al 2008 Wang X et al 2008 Zhang Y et al 2009
dre-miR-15c (miR-15c_l)	up (4)	-	-	-	-	
dre-miR-16a (miR-16a_l)	up (5)	hsa-mir-16	-	up in APL, cervical cancer, down in CLL	apoptosis, tumor suppression	Aqeilan RI et al 2009 Calin GA et al 2002 Xia L et al 2008 Wang X et al 2008
dre-miR-16b (miR-16b_l)	up (5)					

Zebrafish miRNA	Expression in zf liver cancer	Human miRNA	Expression in HCC	Other associations to cancer	Functional links	References
-	-	hsa-miR-17-92 cluster	up	up in lung cancer, colorectal adenoma, ALL	proliferation, angiogenesis	Li Y et al 2009 Liu WH 2009 Connolly E et al 2008 Kutay H et al 2006 Ebi H et al 2009 Zanette DL et al 2007
dre-miR-18c (miR-18c_l)	up (2) / down (3)	hss-mir-18b	up	-	tumor suppression	Li Q et al 2009 Li Y et al 2009
dre-miR-21 (miR-21-1_l, miR-21-2_l)	up (5)	hsa-miR-21				
dre-miR-21* (miR-21-1_r)	up (5)	hsa-miR-21*	hsa-miR-21: up	hsa-mir-21: up in glioma, colon cancer, breast cancer, gastric cancer	hsa-mir-21: tumor growth, invasion, metastasis, cell cycle progression, cell death, angiogenesis	Gramantieri L et al 2008 Connolly E et al 2008 Ladeiro et al 2008 Meng F et al 2007 Kutay H et al 2006 Zhang et al 2008
dre-miR-23a (miR-23a-1_r, miR-23a-3_r)	up (4)	hsa-miR-23a	up	up in bladder cancer	cell growth	Kutay H et al 2006 Kulshreshtha R et al 2007 Huang S et al 2008 Gottardo F et al 2007
dre-miR-24* (miR-24-3_l)	up (4)	hsa-miR-24	-	up in kaposi sarcoma, hodgkin lymphoma, deregulated in colorectal cancer	erythropoiesis, cellular transformation, cellular proliferation	O'Hara AJ et al 2009 Gibcus JH et al 2009 Mishra PJ et al 2009
-	-	hsa-mir-25	up	up in gastric cancer, esophageal adenocarcinoma, prostate cancer	proliferation, tumorigenicity	Li Y et al 2009 Kim YK et al 2009 Kan T et al 2009 Ambs S et al 2009
dre-mir-26a (mir-26a-1_l, mir-26a-2_l, mir-26a-3_l	up (1) / down (1)	hsa-miR-26a	down/up	up in glioma, colorectal cancer, down in thyroid anaplastic carcinomas, Burkitt lymphoma	cell cycle regulation, tumor suppression	Ji J et al 2009 Kota J et al 2009 Huse JT et al 2009 Xi Y et al 2006
dre-miR-27a (miR-27a_r)	up (5)	hsa-miR-27a				Gottardo F et al 2007 Feng J et al 2009 Tsuchiya Y et al 2006 Lin Q et al 2009
dre-miR-27b (miR-27b_r)	up (5)	hsa-miR-27b				
dre-miR-27d (miR-27d_r)	up (4)	-	-	up in renal cell carcinoma, down in breast cancer	differentiation	
dre-miR-27e (miR-27e_r)	up (5)	-				
dre-miR-29a (miR-29a-1_r, miR-29a-2_r)	up (4)	hsa-miR-29a				Su H et al 2009 Ura S et al 2009 Xiong Y et al 2009 Mott JL et al 2007 Park SY et al 2009 Mraz M et al 2009 Calin GA et al 2007 Fabbri M et al 2007 Garzon R et al 2008 Li W et al 2008
dre-miR-29b (miR-29b-1_r, miR-29b-2_r, miR-29b-3_r)	up (4)	hsa-miR-29b	down	up in mesenchymal, metastatic mouse breast cells, AML, down in CLL, lung cancer,	apoptosis	
-	-	hsa-miR-29c				

Zebrafish miRNA	Expression in zf liver cancer	Human miRNA	Expression in HCC	Other associations to cancer	Functional links	References
dre-miR-34 (miR-34_l)	up (4)	hsa-mir-34a	down	down in melanomas, lung cancer, osteosarcoma	maintenance and survival of cancer stem cells, tumour suppression, apoptosis, cell-cycle arrest, senescence	Satzger I etal 2009 Ji Q et al 2009 Christoffersen NR et al 2009 Hermeking H. et al 2009 Yamakuchi M, Lowenstein CJ 2009 Pogribny IP et al 2007 Gallardo E et al 2009 Li N et al 2009 Pineau P et al 2009
dre-mir-93 (mir-93_l)	up (2)	hsa-miR-93	up	up in gastric cancer, dult T-cell leukemia, ovarian carcinoma, esophageal adenocarcinoma, prostate cancer	tumor suppression	Li W et al 2008 Kim YK et al 2009 Yeung ML et al 2008 Nam EJ et al 2008 Kan T et al 2009 Ambs S et al 2008
dre-mir-101a (mir-101a_r)	up (1) / down (1)	hsa-miR-101	down	down in endometrial serous adenocarcinomas, colon cancer	apoptosis, tumorigenicity	Mott JL 2009 Su H et al 2009 Li W et al 2008 Hiroki E et al 2009 Strillacci A et al 2009
dre-miR-101b (miR-101b_r)	down (2)					
dre-miR-103 (miR-103_r)	up (3) / down (1)	hsa-miR-103		up in esophageal squamous cell carcinoma	-	Guo Y et al 2008
-	-	hsa-miR-106b	up	up in esophageal adenocarcinoma, gastric cancer, prostate cancer	progression, proliferation	Li W et al 2008 Kan T et al 2009 Kim YK et al 2009 Ambs S et al 2008
dre-miR-107 (miR-107_r)	up (3) / down (1)	hsa-miR-107		up in esophageal squamous cell carcinoma, pancreatic cancer	-	Guo Y et al 2008 Lee KH et al 2009
dre-miR-122 (miR-122_l)	down (4)	hsa-miR-122	down	-	metastasis, tumor suppression	Bai S et al 2009 Coulouarn C et al 2009 Tsai WC et al 2009 Lin CJ et al 2008 Gramantieri L et al 2007 Kutay H et al 2006
dre-mir-125a (mir-125a-1_l, mir-125a-2_l)	up (2)	hsa-miR-125a	down	breast cancer, down in ovarian cancer	tumor suppression, apoptosis	Mott JL 2009 Meng F et al 2007 Guo X et al 2009 Cowden Dahl KD et al 2009
dre-mir-125b (mir-125b-1_l, mir-125b-2_l, mir-125b-3_l)	up (2)	hsa-miR-125b	hsa-miR-125b: up	hsa-miR-125b: up in leukemia, down in oral squamous cell carcinoma, bladder cancer, breast cancer	hsa-miR-125b: proliferation, apoptosis	Varnholt H et al 2008 Meng F et al 2007 Xia HF et al 2009 Henson BJ et al 2009 Ichimi T et al 2009 Hui AB et al 2009 Gefen N et al 2010
dre-mir-125b* (mir-125b-2_r, mir-125b-3_r)	up (3)	hsa-miR-125b-2*				

Zebrafish miRNA	Expression in zf liver cancer	Human miRNA	Expression in HCC	Other associations to cancer	Functional links	References
dre-mir-126* (mir-126_l)	up (1)	hsa-miR-126	down	down in breast cancer, colon cancer, B-lineage ALL, lung cancer	invasion, cell cycle	Wong QW et al 2008 Liu B et al 2009 Fulci V et al 2009 Zhang J et al 2008 Guo C et al 2008 Crawford M et al 2008
dre-mir-126 (mir-126_r)	up (1)	hsa-miR-126*	down	down in lung cancer	invasion	Ladiero et al 2008
dre-miR-128* (miR-128-2_l)	up (4)	-	-	hsa-mir-128: prostate cancer, up in acute lymphoblastic leukemia, down in glioma	hsa-mir-128: invasion	Khan AP et al 2009 Fulci V et al 2009 Godlewski J et al 2009, Zhang Y et al 2009, Ciafrè SA et al 2005 Mi S, et al 2007
dre-miR-132 (miR-132-1_r, miR--132-2_r)	up (5)	hsa-miR-132	-	up in squamous cell carcinoma of tongue, down in adult T-cell leukemia	innate immunity	Bellon et al 2009 Wong et al 2008
dre-miR-135c (miR-135c-1_l, miR-135c-3_l)	up (3) / down (1)	hsa-135a	-	up in colorectal adenomas, megakaryoblastic leukemia		Nagel R et al 2008 Garzon R et al 2006
dre-miR-143 (miR-143-1_l)	up (3) / down (1)	hsa-miR-143*	hsa-mir-143: up	hsa-miR-143: colorectal cancer, T-cell leukemia, down in B-cell malignancies, gastric cancers	hsa-miR-143: metastasis, apoptosis	Zhang X et al 2009 Ng EK et al 2009 Akao Y et al 2009, 2007 Takagi T et al 2009 Clapé C et al 2009
dre-miR-146a (miR-146a_l)	up (5)	hsa-miR-146a	hsa-miR-146a: up, SNP association	hsa-miR-146a: up in breast cancer, SNP in thyroid carcinoma, breast/ovarian cancer, down in pancreatic cancer	innate immunity, invasiveness	Xu T et al 2008 Belair C et al 2009 Shen J et al 2008 Huang YS et al 2008 Li Y et al 2010 Pogribny IP et al 2010 Jazdzewski K et al 2008
dre-miR-146a* (miR-146a_r)	up (4)	hsa-miR-146a*				
dre-miR-146b (miR-146b-1_l)	up (5)	hsa-miR-146b-5p	up	up in lung cancer, leukemia, down in adult T-cell leukemia, breast cancer		Bellon et al 2009 Hurst DR et al 2009 Huang YS et al 2008 Patnaik SK et al 2010 Fulci V et al 2009
dre-mir-148* (mir-148_l)	down (1)	hsa-miR-148a*/b*	hsa-miR-148a, b: down	hsa-miR-148a, b: CLL	hsa-miR-148a, b: metastasis	Budhu et al 2008 Visone R et al 2009 Lujambio A et al 2008 Zhao Y et al 2009
dre-mir-148 (mir-148_r)	down (2)	hsa-miR-148a, b				
dre-miR-153a (miR-153a_r)	up (4)	hsa-miR-153	-	down in glioblastoma	-	Xu J et al 2009
dre-miR-153b (miR-153b_r)	up (4)	hsa-miR-153				
dre-miR-153c (miR-153c_r)	up (4)	hsa-miR-153				

Zebrafish miRNA	Expression in zf liver cancer	Human miRNA	Expression in HCC	Other associations to cancer	Functional links	References
dre-miR-181a (miR-181a_l, mir-181a-1_l, mir-181a-2_l,	up (2) / down (1)	hsa-miR-181a	up	up in oral squamous cell carcinoma, down in AML, CLL, glioma		Ji J et al 2009 Larson RA et al 2009 Visone R et al 2009
dre-mir-181b (mir-181b-1_l, mir-181b-2_l)	up (2) / down (1)	hsa-miR-181b	up	down in AML, CLL	aggressiveness	Shi L et al 2008 Cervigne NK et al 2009 Schaefer A et al 2010
dre-mir-181c (mir-181c_l)	up (1) / down (1)	hsa-miR-181c	up	down in AML, CLL		
dre-miR-192 (miR-192_l)	up (1) / down (3)	hsa-miR-192	polymorphism	colon cancer	tumor suppression	Yang J et al 2008 Song B et al 2008 Georges SA et al 2008 Braun CJ et al 2008 Yantiss RK et al 2009
dre-miR-193a (miR-193a_r, miR-193a-1_r)	up (4)	hsa-miR-193a-3p	-	epigenetically silenced during oral carcinogenesis	tumor suppression	Kozaki K et al 2008
dre-miR-193b (miR-193b_r)	up (5)	hsa-miR-193b	-	down in breast cancer, adenocarcinoma	metastasis, tumor progression, invasion	Li XF et al 2009 Leivonen SK et al 2009 Wu W et al 2009
-	-	hsa-miR-195	down	up in breast cancer, down in adrenocortical carcinoma, bladder cancer, prostate carcinoma	cell cycle control	Xu T et al 2009 Soon PS et al 2009 Zhang H et al 2009 Ichimi T et al 2009 Zanette DL et al 2007
dre-miR-199 (miR-199_l, mir-199-1_l, mir-199-3_l)	down (2)	hsa-miR-199a-5p	down	up in cervical carcinomas, AML, CLL, down in bladder cancer,ovarian cancer	metastasis, apoptosis	Chen XM 2009 Mott JL 2009 Li W et al 2008 Ichimi T et al 2009 Kim S et al 2008 Lee JW et al 2008 Garzon R et al 2008 Iorio MV et al 2007
dre-miR-199* (miR-199_r, mir-199-1_r, mir-199-3_r)	up (1) / down (1)	hsa-miR-199a-3p				
dre-miR-210 (miR-210_r)	up (4)	hsa-miR-210	-	up in renal cell carcinoma, pancreatic tumor, breast cancer, B-cell lymphoma	metastasis, cell cycle regulation, differentiation, DNA repair, regulation of angiogenesis, neuronal cell survival	Juan D et al 2009 Greither T et al 2010 Foekens JA et al 2008 Crosby ME et al 2009 Fasanaro P et al 2009
dre-miR-212 (miR-212_r)	up (5)	hsa-miR-212	-	down in gastric cancer	-	Wada R et al 2009
dre-miR-217 (miR-217-1_l)	down (4)	hsa-miR-217	-	down in pancreatic cancer	-	Szafranska AE et al 2007
dre-miR-221 (miR-221_r)	up (4)	hsa-miR-221	up	-	apoptosis, proliferation, aggressiveness	Pineau P et al 2009 Gramantieri L et al 2009 Fornari F et al 2008
dre-miR-222* (miR-222_l)	up (4)	hsa-miR-222*				Wong QW et al 2008 Ladeiro Y et al 2008 Sun T et al 2009 Greither T et al 2010 Ueda R et al 2009 Liu X et al 2009
dre-miR-222 (miR-222_r)	up (4)	hsa-miR-222	hsa-mir-222: up	hsa-mir-222: up in pancreatic tumor, prostate cancer	hsa-mir-222: invasion	

Zebrafish miRNA	Expression in zf liver cancer	Human miRNA	Expression in HCC	Other associations to cancer	Functional links	References
dre-mir-223* (mir-223_l)	up (1)	hsa-miR-223*	hsa-miR-223: down	hsa-miR-223: gastric cancer, small cell lung cancer, down in CLL, ovarian cancer	-	Wong QW et al 2008 L X et al 2009 Miko E et al 2009 Visone R et al 2009 Laios A et al 2008
dre-mir-223 (mir-223_r)	up (2)	hsa-miR-223				
-	-	hsa-miR-224	up	up in pancreatic ductal adenocarcinoma, AML, prostate cancer	proliferation, migration, invasion	Chen XM 2009 Su H et al 2009 Li W et al 2008 Wang Y et al 2008 Li Z et al 2008 Mees ST et al 2009 Prueitt RL et al 2008
dre-miR-338 (miR-338_r, mir-338-1_r, mir-338-3_r)	up (2) / down (1)	hsa-miR-338-3p	down	gastric cancer, up in squamous cell carcinoma of tongue	aggressiveness	Huang XH et al 2009 Zhao Y et al 2009 L X et al 2009 Wong TS et al 2008
dre-miR-363 (miR-363_r)	up (2) / down (2)	hsa-miR-363	-	up in T-cell lymphoma, down in malignant bronchial epithelial cells	-	Lum AM et al 2007 Shen YL et al 2009
dre-miR-365 (miR-365-1_r, miR-365-2_r, miR-365-3_r)	up (5)	hsa-miR-365	-	up-regulated in breast cancer	-	Yan LX et al 2008
dre-miR-455 (miR-455_l)	up (5)	hsa-miR-455-5p	-	down in endometrial serous adenocarcinoma	differentiation, invasion	Walden TB et al 2009 Hiroki E et al 2009
dre-miR-455* (miR-455_r)	up (5)	hsa-miR-455-3p				
dre-miR-457a (miR-457a_l)	up (4)	-	-	-	-	
dre-miR-457b (miR-457b_l)	up (4)	-	-	-	-	
dre-miR-489* (miR-489_l)	up (1) / down (4)	-	-	hsa-miR-489: down in breast cancer	-	Miller TE et al 2008
dre-miR-499 (miR-499_l)	up (1) / down (3)	hsa-miR-499-5p	-	breast cancer	senescence	Catucci I et al 2009 Hu Z et al 2009 Lafferty-Whyte K et al 2009
dre-miR-722 (miR-722_r)	up (2) / down (3)	-	-	-	-	
dre-miR-733 (miR-733_l)	up (2) / down (2)	-	-	-	-	
dre-miR-735 (miR-735_r)	up (5)	-	-	-	-	
(block361064_C1_r)	up (4)	-	-	-	-	
(sblock169393_C1_r)	down (5)	-	-	-	-	

The miRNAs that showed more than 1.5-fold differential expression (P<10-5) in four or more zebrafish liver tumor experiments and a set of miRNAs previously linked to human hepatocellular carcinoma (HCC) in the literature are listed in the table. MiRNA names were derived from miRBase and star sequences (not in all cases present in miRBase) were named accordingly. Probe names on the custom microarray are indicated in brackets behind the zebrafish (dre) miRNA names, and the corresponding human (hsa) miRNA names are given. For some human miRNAs there are no probes for corresponding zebrafish miRNA on the microarray, which is due to absence of the miRNA genes in zebrafish (miR-106, miR-195, miR-224) or an omission/error in the array design (miR-25, miR-29c, miR-92a). The direction of change of miRNA expression is indicated both for the zebrafish liver tumor samples and for the data on HCC derived from the literature. Numbers in brackets indicate the number of biological replicates of zebrafish healthy liver versus liver tumor where the miRNA probes were differentially expressed. Data on association to other types of cancer in human and on biological processes affected by miRNA function were collected from the literature. In some cases we observed differential expression of the miRNA star sequences in addition to or instead of expression of the mature miRNAs reported in miRBase. As indicated in the table, the literature data are for primary miRNAs. Abbreviations: ALL - acute lymphocytic leukemias, AML - acute myeloid leukemia, APL - acute promyelocytic leukemia, CLL - chronic lymphocytic leukemia, CML - chronic myeloid leukemia, HCC-hepatocellular carcinoma.

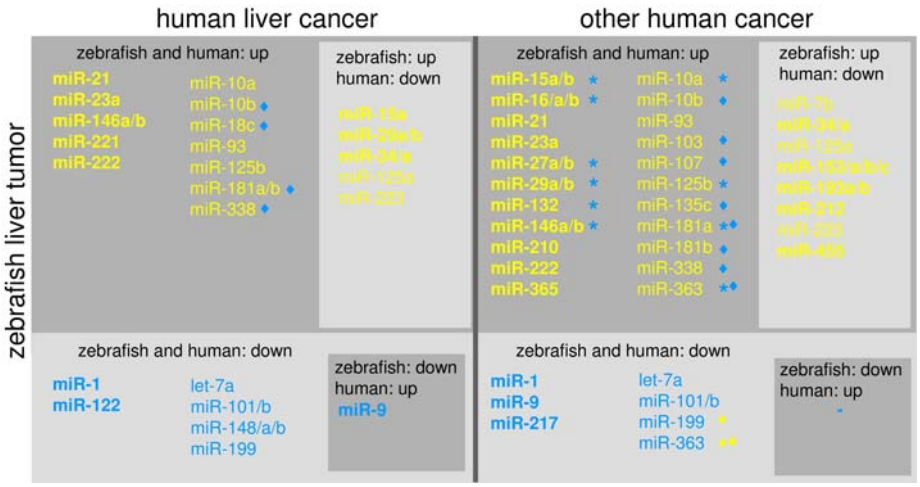


Figure 3. Common regulation of miRNA expression in zebrafish liver tumors and human cancer. Differential expression of miRNAs in zebrafish liver tumors is compared with literature data on the expression of the homologous human miRNAs in human liver cancer (hepatocellular carcinoma) and in other types of cancer as further detailed in Table 1. Yellow indicates miRNAs up-regulated in zebrafish liver tumor tissue compared to normal liver and blue indicates down-regulated miRNAs in zebrafish liver tumors. MiRNAs showing differential expression in 4 out of 5 or 5 out of 5 biological replicates are in bold and miRNAs differentially expressed in 2 or 3 biological replicates are in normal script. MiRNAs for which both up- and down-regulation has been reported in human cancer are indicated with asterisks. MiRNAs for which both up- and down-regulation was observed in zebrafish liver tumors are indicated with diamonds.

365 and miR-455. These miRNAs have been linked to other types of cancer in human and like those linked to HCC, these miRNAs have also been suggested to play roles in tumorigenicity, tumor suppression, apoptosis, cell proliferation, invasion, and metastasis (Table 1, Figure 3).

Expression of miRNA star sequences

Besides regulation of the described mature miRNAs, in several cases we also detected differential expression of probes matching miRNA star sequences (Table 1). Star sequences (miRNA*) are derived from the other arm of the pre-miRNA hairpin structure during miRNA biogenesis, but are not loaded into the RISC complex and are degraded. However, sometimes miRNA* can have similar 5' end stability as the mature miRNA strands and similar incorporation efficiency into the RISC complex, and therefore can also be functional (Soares AR et al 2009). For several miRNAs (miR-21, miR-125b, miR-126, miR-146a, miR-148, miR-199, miR-222, miR-223, miR-455) we found both the primary miRNA and the miRNA* to be differentially expressed in liver tumors, while in other cases (miR-7a*, miR-24*, miR-128*, miR-

Table 2. Common tumor- and immune-related miRNAs.

miRNA	Liver cancer	Mycobacterium infection			
		chronic strain (E11)		acute strain (Mma20)	
		1 dpi	6 dpi	1 dpi	6 dpi
dre-miR-15c (miR-15c_l)	up (4)				up
dre-miR-16a (miR-16a_l)	up (5)			up	
dre-miR-21 (**miR-21-1_l, miR-21-2_l**)	up (5)				up
dre-miR-21* (**miR-21-1_r**)	up (5)	down			up
dre-miR-34 (miR-34_l)	up (4)		up	down	
dre-miR-128* (miR-128-2_l)	up (4)	down	down	down	down
dre-miR-132 (**miR-132-1_r**, miR-132-2_r)	up (5)			down	
dre-miR-146a (miR-146a_l)	up (5)				up
dre-miR-146a* (miR-146a_r)	up (4)				up
dre-miR-146b (miR-146b-1_l)	up (5)				up
dre-miR-212 (miR-212_r)	up (5)			down	
dre-miR-217 (miR-217-1_l)	down (4)	down		down	down
dre-miR-455 (miR-455_l)	up (5)			down	
dre-miR-455* (miR-455_r)	up (5)			down	

Differentially regulated miRNAs in the zebrafish liver tumor study were compared with data from our previous infection study where we used *Mycobacterium marinum* strains Mma20 and E11 as pathogens causing acute disease or chronic granulomatous tuberculosis in zebrafish adults, respectively (chapter 4). Microarray analysis was carried out using RNA samples collected from two time points, 1 and 6 days post infection (dpi). Probe names on the custom microarray are indicated in brackets behind the zebrafish (dre) miRNA names. If there are more probes for one miRNA, those that were expressed in the infection study are indicated in bold. The direction of change of miRNA expressions is indicated both for the zebrafish liver tumor samples and the samples from the infection study. Only the probes that showed consistent up- or down-regulation in at least four biological replicates of the liver tumor study were considered for comparison with the infection data. Significance cut-offs were set at $P<10\text{-}5$ and more 1.5-fold change for analysis of both the liver tumor and infection data sets.

489*) altered expression of only the miRNA* was detected. Most of the zebrafish miRNA* that we found to be differentially expressed in liver tumors have not been previously reported in miRBase. However, miRBase provides evidence for expression of several of these miRNA* in human, such as miR-7-1/2*, miR-21*, miR-24*, miR-125b*, miR-126*, miR-143*, miR-146a*, miR-148a*/b*, miR-222*, and miR-223*. Our microarray data support that these miRNAs* are also expressed in zebrafish and suggest that they play a role in liver cancer similar as the primary miRNAs.

Comparison of miRNA expression in liver cancer and tuberculosis infection

Besides being involved in the pathogenesis of cancer, miRNAs are also known to play important regulatory roles in the development and function of the immune system (Lu and Liston, 2009). Previously we used the same microarray platform to investigate miRNA expression during the immune response of adult zebrafish to infection with *Mycobacterium marinum*, the causative agent of fish tuberculosis (chapter 4). Here we compared the data from this infection study with the liver cancer study in order to define common tumor and immune-related miRNAs (Table 2).

In our infection study we used *Mycobacterium marinum* strains Mma20 and E11, which respectively cause acute disease or chronic granulomatous tuberculosis in adult zebrafish (van der Sar et al., 2004; van der Sar et al., 2009), and determined miRNA profiles at 1 and 6 days post infection (dpi). Comparing the datasets of liver tumor-related miRNAs to *M. marinum* infection-related miRNAs we found that 8 miRNAs, miR-15c, miR-16a, miR-21, miR-21*, miR-34, miR-146a, miR-146a* and miR-146b, were commonly up-regulated in liver tumors and at either the 1 or 6 dpi time point of infection with the Mma20 or E11 strains (Table 2). Two of these miRNAs (miR-21* and miR-34) were up-regulated by only one of the *M. marinum* strains, but down-regulated by the other. Expression of miR-217 was down-regulated in liver tumors and at different time points of *M. marinum* infection with both strains. Five miRNAs (miR-128*, miR-132, miR-212, miR-455, miR-455*) were up-regulated in liver tumors, but down-regulated at one or more time points of infection with the *M. marinum* strains. Other miRNAs that were up-regulated in liver tumors (miR-15a, miR-15b, miR-16b, miR-23a, miR-24*, miR-27a/b/c/d, miR-29a/b, miR-153a/b/c, miR-193a/b, miR-210, miR-221, miR-222, miR-222*, miR-365, miR-457a/b and miR-735) or down-regulated in liver tumors (miR-1, miR-7a*, miR-9, miR-122) were not differentially expressed in the samples of the *M. marinum* infection study. Among the miRNAs commonly regulated in the liver cancer and tuberculosis studies, in particular the members of the miR-21 and miR-146 family can be strongly linked to the immune response, as they were also found to be induced by different bacterial infections in zebrafish embryos (chapter 4).

Experimentally validated targets of liver tumor-related miRNAs

To shed further light on the role of differentially expressed liver tumor-related miRNAs in the mechanism of cancer we searched the TarBase database for putative

Table 3. Pathways and processes in which the experimentally validated target genes of liver tumor-related miRNAs are involved.

Pathway/process	Gene symbol	miRNA	Additional pathway
Apoptosis signaling	*ACVR1B*	miR-24	cell cycle, immune system process, MAPK signaling pathway
	API5	miR-224	
	BCL2	miR-15A, miR-15B	cell proliferation, cell cycle, immune system process
	CDKN1A	miR-106B	cell proliferation, cell cycle, immune system process, ErbB signaling pathway, p53 signaling pathway
	CDKN1B	miR-221, miR-222	cell proliferation, cell cycle, cell motility, immune system process, cytoskeleton organization and biogenesis, ErbB signaling pathway
	CDKN2A	miR-24	cell proliferation, cell cycle, cell adhesion, p53 signaling
	CXCR4	miR-146A	cell proliferation, cell motility, immune system process,
	FADD	miR-146A	I-kappaB kinase/NF-kappaB cascade,
	GCH1	miR-1	
	GJA1	miR-1	cell proliferation, I-kappaB kinase/NF-kappaB cascade
	IHPK2	miR-1	
	KRAS	miR-146B-5P	cell proliferation, cell cycle, cytoskeleton organization and biogenesis, small GTPase mediated signal transduction, ErbB signaling pathway, MAPK signaling pathway, VEGF signaling pathway, Fc epsilon RI signaling pathway
	MCL1	miR-29B	
	PDCD4	miR-1, miR-21	cell cycle
	POGK	miR-1	
	PTEN	miR-21	cell proliferation, cell cycle, cell motility, cell adhesion, p53 signaling pathway
	ROCK1	miR-146A	cytoskeleton organization and biogenesis, small GTPase mediated signal transduction, Wnt signaling
	TIMP3	miR-1	
Cell proliferation	***CDKN1C***	miR-221, miR-222	cell cycle
	ERBB2	miR-125A	immune system process, ErbB signaling pathway
	HDAC4	miR-1	cell cycle, immune system process
	KIT	miR-221, miR-222	immune system process
	IGF1	miR-1	cell motility, small GTPase mediated signal transduction, p53 signaling pathway
	LAMC1	miR-29C	cell motility, cell adhesion
	MXD4	miR-1	
	MYCN	miR-101	
	NF2	LET-7A	cell cycle, cell motility, cell adhesion, cytoskeleton organization and biogenesis
	NOTCH1	miR-24, miR-27B	immune system process
	SMAD1	miR-26A	
	CDK6	miR-34a	cell cycle, cell adhesion, immune system process, p53 signaling
Cell cycle	*AXL*	miR-1	
	DMTF1	miR-15A	
	EML4	miR-1	cytoskeleton organization and biogenesis
	MAPK7	miR-143*	MAPK signaling pathway
	E2F3	miR-34a	
	CCND1	miR-34a	p53 signaling, wnt signaling

Pathway/process	Gene symbol	miRNA	Additional pathway
Cell motility	*CAP1*	miR-1	cytoskeleton organization and biogenesis
	MAPK14	miR-24	MAPK signaling pathway, VEGF signaling pathway, Fc epsilon RI signaling pathway
	SERPINB5	miR-1, miR-21	p53 signaling pathway
	TPM1	miR-21	
	TPM4	miR-1	
Cell adhesion	*COL15A1*	miR-29C	
	CXCL12	miR-23A	cytoskeleton organization and biogenesis, immune system process
	VCAM1	miR-126	
Cytoskeleton organization and biogenesis	*ARHGEF18*	miR-1	small GTPase mediated signal transduction
	HCN4	miR-1	
	LASP1	miR-1	p53 signaling pathway
	PREX1	miR-1	small GTPase mediated signal transduction, immune system process
	TMSB4X	miR-1	
I-kappaB kinase/NF-kappaB cascade	***IRAK1***	miR-146A, miR-146B-5P	
	IRAK2	miR-146A	
	TRAF6	miR-146A, miR-146B-5P	apoptosis, immune system process, MAPK signaling pathway
	STAT1	miR-146A	apoptosis, cell cycle, immune system processs,
	IRF5	miR-146A, miR-146B-5P	
ErbB signaling pathway	*AKT2*	miR-146B-5P	MAPK signaling pathway, VEGF signaling pathway, Fc epsilon RI signaling pathway
	ERBB3	miR-125A	
Small GTPase mediated signal transduction	*ARF3*	miR-1	
	ARF4	miR-1	
	RABGAP1L	miR-1	
	RABL2A	miR-1	
	RABL2B	miR-1	

Experimentally validated targets were collected from the TarBase v5 database. Gene ontology analysis using DAVID tools classified these targets into various cancer-related pathways and processes listed in the table. Target genes predicted to be conserved in zebrafish are indicated in bold.

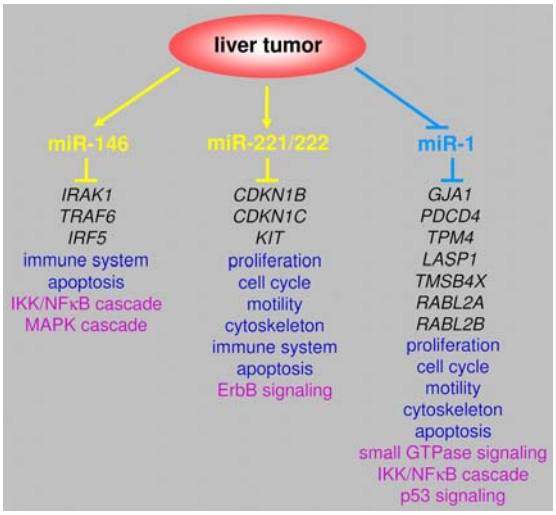


Figure 4. Common predicted target genes of liver tumor-related miRNAs in zebrafish and human. Up-regulation (yellow) of members of the miR-146 and miR-221/miR-222 families and down-regulation (blue) of miR-1 was observed in at least 4 out of 5 biological replicates of zebrafish liver tumor tissue compared to normal liver and changes in the same direction have been reported in the literature on human liver cancer (hepatocellular carcinoma) as detailed in Table 1. Members of the miR-146, miR-221/miR-222 and miR-1 families are predicted to target several genes whose human homologs have been reported as experimentally validated target genes in the TarBase database. The figure shows the common predicted target genes and the biological pathways and signaling pathways that can be associated with these genes by gene ontology analysis.

target genes that have been experimentally validated targets in human cell-based assays (Table 3). Gene Ontology analysis using DAVID tools linked these target genes to biological processes such as apoptosis signaling, cell proliferation, cell cycle, cell motility, cell adhesion, cytoskeleton organization and biogenesis, and immune system process, as well as to signaling pathways, including the I-kappaB kinase/NF-kappaB cascade, small GTPase mediated signal transduction, ErbB signaling, MAPK signaling, VEGF signaling, p53 signaling, and several KEGG cancer pathways (Table 3). These processes and pathways are often deregulated in HCC suggesting that these miRNAs can be part of signaling pathways that significantly contribute to cancer (Chen et al., 2002; Kim et al., 2004; Neo et al., 2004; Okabe et al., 2001; Xu et al., 2001). Next we used different miRNA target prediction programs to investigate if the homologous zebrafish genes might contain miRNA target sites for the same miRNAs as those involved in HCC. This analysis showed that several target genes of the miR-146 and the miR-221/miR-222 families, which we found to be commonly up-regulated in zebrafish liver tumors and in HCC, might be conserved between zebrafish and human (Table 3, Figure 4). For the miR-146 family these included two pivotal signaling intermediates, *IRAK1* and *TRAF6*, of the NF-kappaB pathway and the *IRF5* transcription factor gene that is a target of this pathway. Predicted common targets of the human and zebrafish miR-221/miR-222 family included cyclin-dependent kinase inhibitor genes (*CDKN1B* and *CDKN1C*), linked with epidermal growth factor receptor (ErbB) signaling, and the proto-oncogene tyrosine-protein kinase

KIT. Furthermore, we could predict 7 conserved target genes for miR-1, which we identified as a commonly down-regulated miRNA in zebrafish liver tumors and in reports on HCC. One of these was a member of the connexin family (*GJA1*, gap junction protein, alpha 1, 43kDa) related to cell-cell signaling and positive regulation of the NF-kappaB pathway. Another predicted conserved miR-1 target was the programmed cell death 4 (*PDC4*) gene, also known as neoplastic transformation inhibitor, which has a still unknown function in apoptosis regulation. Three other genes encoded cytoskeleton-related proteins, including the stress-responsive, actin-binding tropomyosin 4 (TPM4) protein, the actin-binding protein LASP1 (LIM and SH3 protein 1), which has been shown to play an essential role in tumor cell growth and migration and was reported as p53 transcriptional target involved in HCC (Wang et al., 2009a), and the actin-sequestering protein TMSB4X (thymosin beta 4, X-linked), which has been reported to positively regulate the expression of hepatocyte growth factor (Barnaeva et al., 2007) and has been implicated in cell proliferation, migration, and differentiation as well as tumor metastasis and angiogenesis (Wang et al., 2004; Zhang et al., 2008a). Finally, two members of the RAS oncogene family-like 2 (RABL2A and RABL2B) were also predicted to be conserved targets of miR-1 in human and zebrafish. Taken together, our results show that several miRNAs are commonly regulated in human and zebrafish liver cancer and predict conservation of a number of target genes strongly linked to cancer-related biological processes and signaling pathways.

Discussion

Recently, it has been shown that there are remarkable molecular similarities between zebrafish and human liver cancer not only with respect to general molecular hallmarks (Lam et al., 2006) but also at the level of biological modules related to tumorigenesis and transcription factor target modules (Ung et al., 2009). In this study we show that there is conservation at the level of miRNA expression as well. Using a custom microarray platform we observed highly reproducible induction and repression of miRNAs in zebrafish liver tumors, many of which were previously linked to human hepatocellular carcinoma (HCC) or to other types of human cancer. We also show that a subset of these liver tumor-related miRNAs overlap with miRNAs responsive to *Mycobacterium marinum* infection, suggesting a common role in cancer and the immune response. Finally, based on target predictions for zebrafish miRNAs and experimental target validation data reported for human miRNAs, we suggest that several target genes of the miR-1, miR-146 and miR-221/miR-222 families, which are commonly deregulated in liver tumors, are conserved between human and zebrafish.

To compare miRNA expression signatures between human and zebrafish liver tumors we took two approaches. First, using a custom Agilent microarray platform we analyzed differential expression in carcinogen-induced zebrafish liver tumors as

compared to healthy liver tissue. Second, by PubMed abstract searching we compiled human miRNAs previously reported to show altered expression in HCC or associated to HCC by polymorphisms. Together this resulted in the identification of 83 liver tumor-related miRNAs, including 70 primary miRNAs reported in miRBase and 13 miRNA star sequences. The majority of the miRNAs previously linked to HCC showed altered expression in zebrafish liver tumors. The most notable similarities were the common up-regulation of miR-21, miR-23a, miR-146a/b, miR-221 and miR-222, and the common down-regulation of miR-1 and miR-122. These miRNAs showed consistent up- or down-regulation in four or five out of five biological replicates of zebrafish liver tumors and have been reported to show the same direction of expression changes in HCC. A similar number of HCC-linked miRNAs was also changed in the same direction in zebrafish liver tumors, but in less than four replicates. Some other miRNAs, most notably miR-9, miR-15a, miR-29a/b and miR-34, showed opposite changes in zebrafish liver tumors compared to literature reports on HCC. Approximately half of the zebrafish miRNAs with consistent differential expression in liver tumors were not previously linked to HCC. Some of these miRNAs lacked human counterparts, but in most cases the human counterparts of these miRNAs were already associated with various other types of cancer and therefore it is conceivable that associations with human liver cancer may also be found in future studies. Most of the miRNAs associated with HCC or other human tumors, including those commonly regulated in zebrafish liver tumors, have been found to play roles in different mechanisms of cancer such as tumorigenicity, tumor suppression, apoptosis, cell proliferation, differentiation, invasion, and metastasis. These data further support that zebrafish liver tumors are appropriate for modeling human liver tumors and that the molecular similarities between the species extend from conserved tumor-associated gene expression signatures to shared expression alterations of a set of tumor-associated miRNAs.

The custom microarray platform used in this study not only contained probes for almost all known miRNAs reported in miRBase but also for the putative star sequences of all primary miRNAs and for predicted hairpin structures with or without experimental support for miRNA identity. Our microarray data support the expression of several miRNA star sequences that were not yet reported in miRBase and show that in most cases altered expression of these miRNA star sequences in liver tumors occurs concomitantly with expression of the corresponding primary miRNAs. We also noted that 86 probes for predicted hairpin structures without experimental support for miRNA identity were differentially expressed in all five biological replicates of the liver tumor study. Similarly, we previously observed differential expression of many probes in this category during bacterial infection of zebrafish (chapter 4). The possibility that these probes represent an additional group of non-coding small RNAs with putative regulatory functions during cancer and infection is of great interest for further investigation.

Differential expression of miRNAs has been implicated not only in different

mechanisms of many types of cancer but also in the immune response. In agreement, our previous work suggests that many miRNAs implicated in cancer processes are also connected to regulation of the immune response in zebrafish (chapter 4). In the previous study we used the same microarray platform to analyze miRNAs induced by *Mycobacterium marinum* infection in zebrafish (chapter 4). *M. marinum* is closely related to *Mycobacterium tuberculosis* and the pathology of *M. marinum* infection in zebrafish strongly resembles that of human tuberculosis. In particular, the formation of granulomas, a hallmark of tuberculosis, is well conserved between zebrafish and human. Granuloma formation involves the aggregation of infected and non-infected macrophages and other immune cells into tight structures. In this respect granuloma formation shares interesting similarities with tumor formation, which generally is also associated with the attraction of macrophages and other immune cells. Therefore, it was of interest to determine the miRNAs commonly regulated in liver tumors and in response to *M. marinum* infection. We identified several miRNAs that were commonly up-regulated in liver tumors and at one or more stages of infection with two *M. marinum* strains, including miRNAs of the miR-15, miR-16, miR-21, miR-34, and miR-146 families. Three of the most interesting commonly regulated miRNAs (miR-146a, miR-146a* and miR-146b) are members of the miR-146 family, which has previously been implicated in regulation of innate immunity signaling pathways and recently suggested also to modulate adaptive immunity (Hou et al., 2009; Perry et al., 2009; Schmidt et al., 2009; Taganov et al., 2006; Williams et al., 2008). Altered expression of human miR-146 miRNAs was observed in viral and bacterial infections, under inflammatory conditions as well as in different types of cancer, including HCC, leukemia and breast cancer (Bellon et al., 2009; Nakasa et al., 2008; Perry et al., 2009; Shen et al., 2008; Williams et al., 2008; Xia et al., 2009; Xu et al., 2008). Similarly, elevated expressed members of the human miR-21 family, which were also commonly up-regulated in zebrafish liver tumors and mycobacterium infection, was previously reported in studies of infection, allergic inflammation and cancer, including HCC, gastric cancer, glioma, colon cancer and breast cancer (Cameron et al., 2008; Connolly et al., 2008; Gramantieri et al., 2007; Lu et al., 2009; Wang et al., 2009b; Zhang et al., 2008b; Zhou et al., 2009).). It has been suggested that the connection between processes of cancer and the immune response might be due to changes in expression of miRNAs regulating shared signaling pathways (Williams et al., 2008). The common and conserved regulation of several miRNAs in tumors and during infection that we observed in this study further supports this connection.

Finally, we investigated the possible conservation of miRNA target genes. Although there are numerous predicted miRNA targets detected by bioinformatics approaches only a few have been validated. In this study we compiled experimentally validated targets of human liver tumor-related miRNAs. Functional classification of these targets by gene ontology analysis revealed their involvement in signaling pathways and biological processes that formerly have been identified as common

molecular hallmarks of zebrafish and human liver cancers (Lam and Gong, 2006; Lam et al., 2006; Ung et al., 2009). Next we checked the 3'UTR regions of the homologous zebrafish genes for the presence of target sites for the same miRNAs. For several miRNAs no conserved targets were predicted by MiRanda, TargetScan and RNA-hybrid algorithms; however, we cannot exclude the possible presence of target sites in introns or the presence of target sites not recognized due to limitations of the target prediction algorithms or incomplete annotation of the 3'UTR regions in the Ensembl database. Interestingly, target predictions indicated several conserved target genes for miRNAs of three families, including the miR-146 and miR-221/miR-222 families that were commonly up-regulated in human and zebrafish liver tumors, and miR-1, a commonly down-regulated miRNA family in human and zebrafish liver tumors. Shared target genes of the miR-146 family encoded signaling intermediates (IRAK1, TRAF6) and a transcription factor (IRF5) involved in the immune response. As discussed above, the miR-146 family is also induced by infection in human and zebrafish; therefore, the predicted conservation of these target genes further supports that this miRNA family functions at the crossroads of cancer and the immune response. Proteins encoded by the predicted conserved target genes of the miR-221/222 family (CDKN1B, CDKN1C, KIT) and the miR-1 family (GJA1, PDCD4, TPM4, LASP1, TMSB4X, RABL2A, RABL2B) are involved in several cancer-related biological processes such as proliferation, cell cycle regulation, apoptosis, and cytoskeletal organization and motility. Furthermore, these genes have been implicated in cancer-related signaling pathways, including ErbB, NF-κB and p53 signaling, and small GTPase-mediated signal transduction. The ErbB signaling pathway has been implicated in the development and malignancy of numerous types of human cancers, including liver cancer (Altimari et al., 2003; Ito et al., 2001; Olayioye et al., 2000). NF-κB signaling is involved in the immune response, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis and imbalanced regulation of this pathway has been associated with development of multiple types of tumors (Steele and Lane, 2005; Sun and Zhang, 2007), similar as for deregulation of the p53 tumor-suppression pathway (Steele and Lane, 2005). Finally, predicted target genes involved in small GTPase-mediated signal transduction are potential markers for tissue invasiveness of liver tumor cells (Lam and Gong, 2006). The predicted conservation of target genes in these pathways and processes further supports the similarity between zebrafish and human liver cancer and that miRNAs might play a pivotal role in regulating these cancer-related mechanisms in both species.

Taken together, our data suggest that there is high conservation between zebrafish and human liver tumors not only at the level of gene expression signatures and transcriptional targets, but also at the level of the regulation by miRNAs. These results further support the usefulness of zebrafish as a useful model for studying liver cancer and functions of miRNAs in the mechanisms of liver cancer.

Materials and methods

Generation of zebrafish liver tumors

Liver tumor samples were obtained from the group of Zhiyuan Gong (National University of Singapore) and generated by carcinogen treatment as described in Lam et al., 2006. In brief, three-week-old juvenile zebrafish were treated with 0.75 ppm 7,12-dimethylbenz[α]anthracene (DMBA) or dimethyl sulfoxide (DMSO, vehicle) for 24 h and the treatment was repeated once at five weeks of age for another 24 hours with 1.25 ppm DMBA or DMSO. Treated fish were rinsed three times in fresh water and transferred into new tanks for maintenance. Fish were sampled during 6-10 months after the onset of DMBA exposure. Tumor samples used for the microarray study were all bigger than 3 mm in diameter. Partial liver tumors were used for RNA extraction and the rest of the tissues were used for histopathological diagnosis. The normal livers were also sampled at 6 months to 10 months after the control treatment (DMSO, vehicle). Zebrafish husbandry was as described (Lam et al., 2006).

Microarray design

Custom-designed 8x15k microarray slides were ordered from Agilent Technologies. The 15k custom design was obtained from Edwin Cuppen and Eugene Berezikov, Hubrecht Institute, Utrecht, The Netherlands and will be submitted into the Gene Expression Omnibus database. The 15k design contained a duplicate of 7604 probes of 60-oligonucleotide length. The probes consisted of 2x22 nucleotide sequences antisense to mature miRNAs separated by a spacer of 8 nucleotides (CGATCTTT) and with a second spacer with the same sequence at the end. From 7604 probes 546 were designed for left (5') and right (3') arms of the hairpins of zebrafish miRNAs that are currently known in miRBase. The remainder 7058 probes were classified into 3 categories: 62 probes into C1 category (novel confident miRNAs for which there is compelling experimental evidence), 54 probes into C2 category (candidate miRNAs with clear hairpin structure but less experimental evidence), and 6942 into C3 category (additional hairpins identified in the zebrafish genome sequence that are less likely to be miRNAs).

RNA isolation, labeling and hybridization

Total RNA from five liver tumors and five normal livers was isolated using the miRNeasy Mini kit to preserve small RNA species. Three of the liver tumor samples were from male zebrafish and two from female zebrafish and each was compared with normal liver samples from fish with the same sex. RNA labeling was carried out with miRCURY™ LNA microRNA, Hy3™/Hy5™ Power Labeling kit (Exiqon) using 1 μg of total RNA according to the manufacturer's instructions. RNA samples from liver tumors were labeled with Hy3 and hybridized against Hy5-labeled RNA samples from normal livers. The dual colour hybridization of the microarray chips was performed according to Agilent protocol GE2-v5_95_Feb07 and GE2_105_Jan09

(www.Agilent.com) for two-color microarray-based gene expression analysis except that hybridization and washing was performed at 37 °C.

Data analysis

Microarray data were processed from raw data image files with Feature Extraction Software 10.5.1 (Agilent Technologies). Processed data were subsequently imported into Rosetta Resolver 7.2 (Rosetta Biosoftware) and subjected to default ratio error modeling. Significance cut-offs for the ratios of infected versus control were set at 1.5-fold change at $P \leq 10-5$. Experimentally validated target genes of human miRNAs were obtained from TarBase V.5 (http://diana.cslab.ece.ntua.gr/tarbase/) (Papadopoulos et al., 2009). Gene ontology and pathway analysis was carried out using Database for Annotation, Visualization and Integrated Discovery (DAVID) software tools (http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009). MiRNA target site predictions for the homologous zebrafish genes were derived from the MicroCosm database and were performed by locally running miRanda, TargetScan and RNAhybrid prediction algorithms using the 3'UTR regions derived from the Ensembl Zv8 zebrafish database. For the zebrafish homolog of *IRAK1* the 3'UTR region experimentally determined in chapter 4 was included in the predictions.

Supplementary data

Supplementary data associated with this chapter can be found at http://apo.szbk.u-szeged.hu/transfer/A_ORDAS/.

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