Sequential analysis of multistage hepatocarcinogenesis reveals that miR-100 and PLK1 dysregulation is an early event maintained along tumor progression.

UNIVERSITÀ DEGLI STUDI DI TORINO

Sequential analysis of multistage hepatocarcinogenesis reveals that miR-100 and PLK1 dysregulation is an early event maintained along tumor progression

A Petrelli[1], A Perra[2], K Schernhuber[1], M Cargnelutti[1], A Salvi[3], C Migliore[1], E Ghiso[1], A Benetti[4], S Barlati[3], G M Ledda-Columbano[2], N Portolani[5], G De Petro[3], A Columbano[2,6] and S Giordano[1,6]

[1]IRCC, Institute for Cancer Research and Treatment, University of Torino School of Medicine, Torino, Italy
[2]Department of Toxicology, Unit of Oncology and Molecular Pathology, University of Cagliari, Cagliari, Italy
[3]Department of Biomedical Sciences and Biotechnologies, Division of Biology and Genetics, School of Medicine, University of Brescia, Brescia, Italy
[4]Department of Pathology, School of Medicine, University of Brescia, Brescia, Italy
[5]Department of Medical and Surgical Sciences, Surgical Clinic, School of Medicine, University of Brescia, Brescia, Italy

Correspondence: Dr A Petrelli or Professor S Giordano, Division of Molecular Oncology, Institute for Cancer Research and Treatment, S.P. 142km 3.9, Candiolo TO 10060, Italy. E-mail: annalisa.petrelli@ircc.it orsilvia.giordano@unito.it; Professor A Columbano, Professor A Columbano, Department of Toxicology, Unit of Oncology and Molecular Pathology, Via Porcell 4, 09124 Cagliari, Italy. E-mail: columbano@unica.it

[6]These authors equally contributed to the work.

ABSTRACT

MicroRNAs (miRNAs) have an important role in a wide range of physiological and pathological processes, and their dysregulation has been reported to affect the development and progression of cancers, including hepatocellular carcinoma (HCC). However, in the plethora of dysregulated miRNAs, it is largely unknown which of them have a causative role in the hepatocarcinogenic process. In the present study, we first aimed to determine changes in the expression profile of miRNAs in human HCCs and to compare them with liver tumors generated in a rat model of chemically induced HCC. We found that members of the miR-100 family (miR-100, miR-99a) were downregulated in human HCCs; a similar downregulation was also observed in rat HCCs. Their reduction was paralleled by an increased expression of polo like kinase 1 (PLK1), a target of these miRNAs. The introduction of miR-100 in HCC cells impaired their growth ability and their capability to form colonies in soft agar. Next, we aimed at investigating, in the same animal model, if dysregulation of miR-100 and PLK1 is an early or late event along the multistep process of hepatocarcinogenesis. The obtained results showed that miR-100 downregulation (i) is already evident in very early preneoplastic lesions generated 9 weeks after carcinogenic treatment; (ii) is also observed in adenomas and early HCCs; and (iii) is not simply a marker of proliferating hepatocytes. To our knowledge, this is the first work unveiling the role of a miRNA family along HCC progression.

Keywords: miRNA; HCC; animal model; PLK1; hepatocarcinogenesis

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third cause of cancer-related deaths worldwide, and a major health problem, due to its poor prognosis and few treatment options (Parkin, 2001). Genome-wide gene expression microarray and quantitative real-time reverse transcriptase PCR (RT–PCR) studies have tried to identify the genes abnormally expressed in HCC and to generate molecular signatures able to distinguish among different types of liver tumors and between evolutive and non-evolutive lesions (Lee and Thorgeirsson, 2004). These studies indicated a general aberrant activation of signaling pathways involved in cellular proliferation, survival, differentiation and angiogenesis, which are heterogeneously present in each tumor. However, they did not result in a consensus for a signature or for a single pathway that is prominent and characteristic of HCC.

Recently, it became clear that the classification and stratification of tumors can be performed not only on the bases of mRNA expression analysis but also—and likely more accurately—by evaluating the modulation of microRNAs (miRNAs), small non-coding RNAs that negatively control gene expression. The involvement of miRNAs in cancer pathogenesis is now well established and miRNA expression profiles accurately classify tumors at different stages and distinguish among subsets of patients with different molecular pathologies (Lu *et al.*, 2005). Although changes in the expression of miRNAs between tumor specimens and the normal corresponding tissues have been investigated also in HCC, nevertheless the obtained results were often discordant and do not yet allow the identification of miRNAs critical for HCC development and progression (Imbeaud *et al.*, 2010). Furthermore, among the miRNAs whose expression is changed, several are probably quantitatively altered not as a cause but as a consequence of the tumorigenic status. Microarray profiling of mRNAs and miRNAs performed on human HCCs does not help to discriminate the alterations that are indeed driving the tumorigenic process from those which are the result of the process. Unfortunately, knowledge of the molecular events in early stages of human HCC development is hampered by the clinical difficulty of diagnosing early lesions. This problem can be circumvented by the use of animal models of hepatocarcinogenesis, in which discrete lesions at different stages of progression can be obtained and analyzed. These experimental models could thus help to identify molecular alterations already present in early preneoplastic stages. Although assessment of the translational value of animal models of human cancer poses a major challenge, the usefulness of the resistant hepatocyte (R-H) model (Solt *et al.*, 1977) has already been demonstrated; indeed, comparative functional genomics studies on laser-capture microdissected early lesions showed that the gene expression signature associated to the progenitor cell marker cytokeratin-19 (CK19) can successfully predict the clinical outcome of human HCC (Andersen *et al.*, 2010).

Here, we analyzed HCCs obtained from human patients and compared them with chemically-induced rat HCCs. Next, we analyzed the multistep process of liver carcinogenesis in rats over a time period of several months, thus allowing the identification of very early lesions (hyperplastic nodules), adenomas and early and advanced HCCs. The results showed that miR-100 and miR-99a, belonging to miR-100 family, were downregulated in both human and rat HCCs. The downregulation of these miRNAs is a very early event and it is associated with all phases of the hepatocarcinogenic process. The decrease of miR-100 and miR-99a was paralleled by the augmented expression of polo like kinase 1 (PLK1), a target of these miRNAs, whose oncogenic role has already been established (Pellegrino *et al.*, 2010). The biological significance of miR-100 was demonstrated in HCC cell lines where its exogenous expression significantly impaired growth ability.

RESULTS

miRNA expression profiling in human HCCs

To identify miRNAs differentially expressed between HCC and peri-tumoral (PT) tissue, we profiled specimens obtained from six HCC patients with the TaqMan Low Density Array. In order to avoid bias due to samples heterogeneity, we analyzed pooled samples on three different miRNA CARDs: (i) RNAs extracted from the PT tissues of six patients, and (ii) two pools of three HCCs each. In our study, 85 miRNAs showed a dysregulated expression of at least twofolds in tumors versus PT tissues, most of them being downregulated, and only 15 upregulated (see Supplementary Table 1 for the most downregulated miRNAs). The obtained results were in line with those published by other groups (Imbeaud *et al.*, 2010); indeed, the expression of miRNAs, such as miR29c, -130a, -199a, -199a*, -214, (downregulated) and miR-183, -216, -221, -224 (upregulated), was found to be modified. Interestingly, in both the screenings, we found among the first 30 underexpressed miRNAs three microRNAs belonging to the same family—namely miR-100, miR-99a and miR-99b. According to the main bioinformatic tools available for target prediction these miRNAs share the same putative targets; of note, one of them is PLK1, a serine/threonine kinase frequently overexpressed in solid tumors (Eckerdt *et al.*, 2005; Pellegrino *et al.*, 2010; Shi *et al.*, 2010).

Expression of miR-100 family is frequently downregulated in human HCCs

In light of these results, we focused on miR-100, miR-99a and miR-99b. We first validated the data obtained with the miRNA CARDs on matched-pairs of HCC and PT tissues obtained from 34 patients who underwent surgical resection of the tumor. The characteristic of the tissue samples are described in Supplementary Table 2.

To confirm the downregulation of the three members of the miR-100 family, we performed quantitative real time PCR (qRT–PCR) on retro-transcribed RNAs. As shown (Figure 1), miR-100, miR-99a and miR-99b were indeed significantly downregulated in tumors compared with their normal counterparts in 79%, 88%and 73% of the samples, respectively (P-value=2.1×10^{-5} for miR-100; P=2.4×10^{-6} for miR-99a; P=0.00034 for miR-99b).

Downregulation of miR-100 family correlates with increased expression of PLK1 in human HCCs

Previous works have indicated PLK1, whose oncogenic role has already been reported, as a target of miR-100 (Shi *et al.*, 2010). We thus assessed PLK1 expression by qRT–PCR in tumors and normal surrounding tissues. As shown inFigure 2a, PLK1 was overexpressed in most of the tumors compared with their normal counterparts and a clear inverse correlation with the expression of miR-100 family members was observed. The anti-correlation is also evident in the scatter plots shown in Figure 2b, where we defined a Pearson value of −0.605 and of −0.498 for miR-100 and miR-99a, respectively. It is noteworthy that, as highlighted by the dendrogram (Figure 2a), among the members of this miRNA family, miR-100 and miR-99a showed an almost superimposable expression in human HCCs, while miR-99b was slightly divergent, suggesting a different regulation.

To confirm that the increased level of PLK1 mRNA indeed corresponded to an increase of PLK1 protein, we performed immunohistochemistry on slices derived from the same human HCCs. As seen in Figure 2c and in Supplementary Figure 1, while the expression of PLK1 was almost completely absent in the PT tissue, a clear positivity was observed in the tumor area. Immunohistochemical evaluation showed that PLK1 was more expressed in HCC compared with the PT in 27 out of 28 samples examined. In particular, we observed a weak PLK1 increase (one-

to threefold increase) in 18%, a moderate (four- to eightfold increase) in 35% and a strong increase (>eightfold increase) in 42% of the samples.

MiR-100 overexpression impairs proliferation of HCC cell lines

To define the biological link between the miR-100 family and PLK1, we moved to *in vitro* studies. We first evaluated the endogenous expression of miR-100, miR-99a and PLK1 in nine human HCC cell lines. As shown in Figure 3a and in the scatter plots (Figure 3b), in cells where the miRNAs were expressed at high levels, PLK1 was present in low amount and vice versa, confirming the inverse correlation observed *in vivo* (Pearson value −0.836 for miR-100/PLK1 and −0.866 for miR-99a/PLK1).

To establish the biological effect of the modulation of miR-100 levels in HCC cells, we performed growth curve assays and soft agar assays (measuring, respectively, the ability of the cells to grow in an anchorage-dependent or -independent manner). To prove the physiological role of miR-100, we transfected different liver cancer cell lines with a specific miR-100 inhibitor (antagomir). As shown in Figure 4a, inhibition of the endogenous miRNA resulted in a significative increase of growth ability. On the other hand, miR-100 overexpression significantly impaired the ability of HCC cells to grow in an anchorage-independent manner (Figure 4b). It has to be noted that miR-100 inhibition was accompanied by an increase of PLK1 levels, whereas miRNA overexpression induced downregulation of PLK1 (Figure 4c).

All together these results suggest that restoration of miR-100 can hamper the growth of HCC cells.

MiR-100 and miR-99a are downregulated and PLK1 is increased in rat HCCs

As reported above, we found downregulation of the three members of the miR-100 family in human liver tumors. As we were interested in evaluating the exact stage of neoplastic progression where this alteration occurs, we investigated this problem in a model of rat carcinogenesis, the so called R-H model (Solt *et al.*, 1977), where the different steps of tumorigenesis can be dissected. In this model, liver cells are initiated by a single dose of a chemical carcinogen (for example, diethylnitrosamine) and promoted by a brief treatment with 2-acetylaminofluorene combined with partial hepatectomy (PH). A total of 100% of the animals subjected to this protocol develop HCCs in 14 months after diethylnitrosamine initiation. We thus killed the rats at this time and explanted their livers. Macroscopically, several tumors were clearly identifiable in the liver of all animals examined; by microscopic analysis these tumors showed the characteristic pattern of HCC (Figure 5a, left). To establish whether rat HCCs also had an altered expression of miR-100 and miR-99a, we performed qRT–PCR on RNA extracted from eight tumors. As shown (Figure 5b), neoplastic lesions showed a significant decrease of both miR-100 and miR-99a, compared with non-neoplastic liver (P=4.3 × 10^{-5} for miR-100; P=3.4 × 10^{-5} for miR-99a). Western blot analysis performed on proteins extracted from the same samples (Figure 5b, lower panels) showed that miRNA downregulation was paralleled by increase of PLK1 in all the HCCs examined, confirming the inverse correlation observed in human tumors (Pearson value=−0.522 for miR-100/PLK1 and −0.449 for miR-99a/PLK1; Figure 5c). Accordingly, immunohistochemical analysis showed a clear PLK1 positivity of the neoplastic cells, whereas the surrounding PT tissue was essentially negative (Figure 5a, right).

MiR-100 downregulation is an early event in the process of hepatocarcinogenesis and is associated with all the phases of the process

The R-H model allows dissecting the different steps of the carcinogenic process, as it is possible to isolate phenotypically distinct lesions at well-defined timings. Histologically, the livers sequentially

develop hyperplastic nodules (1–2 months after initiation), adenomas and early HCCs (9 months), and by 14 months fully advanced HCCs, with possible lung metastases. To determine whether miR-100 downregulation is an early event during liver carcinogenesis, we laser micro-dissected preneoplastic lesions developed 9 weeks after initiation and identified by their positivity for placental form of glutathione-*S*-transferase (GSTP) (Satoh *et al.*, 1985) (Figure 6b). As shown in Figure 6a, miR-100 was significantly downregulated in the nodules examined, compared with the non-nodular liver tissue (P=4.4 × 10^{-9}). Accordingly, PLK1 expression was increased (Figure 6c), as observed in human and rat HCCs. These results thus demonstrate that the decrease of miR-100 and the upregulation of PLK1 are very early events during the carcinogenic process. Due to the low amount of RNA extracted from the nodules, the expression of miR-99a could not be evaluated.

Next, we analyzed miR-100 expression in adenomas and early HCCs that developed 9 months after diethylnitrosamine initiation. Adenomas were composed of cells closely resembling normal hepatocytes, although not organized in plates and lacking a lobular architecture (Supplementary Figure 2A). Early HCCs had cell plates thicker than three cells, no discernible normal lobular architecture and signs of nuclear atypia (large and small hepatocytes were seen in the same lesion) (Supplementary Figure 2B). The absence of a glandular appearance indicates that these lesions were still in an early stage of cancer progression. Microdissection of adenomas and early HCCs and determination of miR-100 expression revealed that this miRNA was downregulated in 8/9 lesions, with values ranging from 2 to 10 folds, compared with non-tumoral liver (P=0.017 for adenomas, P=0.023 for early carcinomas) (Supplementary Figure 2C). We conclude that downregulation of miR-100 coincided spatially and temporally with the onset of preneoplastic lesions in the liver, and that continued downregulation of miR-100 expression is probably necessary for the maintenance and progression of these lesions.

MiR-100 and PLK1 dysregulation are not simply markers of hepatocyte proliferation

Preneoplastic and neoplastic hepatocytes are characterized by a proliferative rate higher than that of normal hepatocytes. Therefore, it is possible that the observed downregulation of miR-100 can be simply a marker of cell proliferation. To establish whether miR-100 downregulation is simply a reflection of increased proliferative activity of preneoplastic and neoplastic cells, we performed experiments in regenerating livers upon 2/3 PH. As shown in Figure 7, expression of miR-100 and miR-99a was not significantly modified 24 h after PH (time of maximal DNA synthesis), compared with livers from the same animals excised at the time of surgery (P=0.339 for miR-100 and P=0.511 for miR-99a). It should be noted that the labeling index of hepatocytes 24 h after PH (25%±0.6 s.e.m.) was higher than that of preneoplastic lesions (12%±1 s.e.m.) (Supplementary Figure 3). No significant change in PLK1 protein expression was observed during liver regeneration (Figures 6d and 7 lower panels).

We conclude that miR-100 downregulation and PLK1 upregulation are intimately linked to the carcinogenic process and not merely to the reentry of hepatocytes into the cell cycle.

DISCUSSION

The miR-100 family of miRNAs comprises three members, namely miR-100, miR-99a and miR-99b. Comparative studies indicate that miR-100 is the oldest known animal miRNA (Christodoulou *et al.*, 2010) and that it is widely expressed in vertebrates (Wienholds *et al.*, 2005). Moreover, it has been shown that this miRNA modulates differentiation of mouse embryonic stem

cells (Tarantino *et al.*, 2010). Its role in cancer is quite contradictory as it can behave either as an oncogene or a tumor suppressor gene, depending on the tumor type examined. Decreased expression of miR-100 has been found, in fact, in nasopharyngeal cancer (Shi *et al.*, 2010), oral cancer (Henson *et al.*, 2009), squamous cell carcinomas of tongue (Wong *et al.*, 2008), hepatoblastoma (Cairo *et al.*, 2010) and serous ovarian carcinomas (Nam *et al.*, 2008), whereas overexpression has been described in medulloblastomas (Liu *et al.*, 2009), gastric (Ueda *et al.*, 2010), pancreatic (Lee *et al.*, 2007) and prostate (Leite *et al.*, 2009) cancers. Also in HCC, the role of miR-100 is controversial, as a decreased expression of miR-100 has been reported by Su *et al.* (2009) and Hou *et al.* (2011), while Varnholt *et al.* found miR-100 overexpression in HCV-associated HCCs (Varnholt *et al.*, 2008). Very recently, Cairo *et al.* (2010) have shown a Myc-dependent downregulation of the miR-100/let-7a-2/miR125b-1 cluster in poorly differentiated hepatoblastomas. However, as underlined by Thorgeirsson in a commentary to this paper, although these results give an important contribution to the understanding of hepatoblastoma, the role of Myc and miRNAs in HCC pathogenesis still remains to be elucidated (Thorgeirsson, 2011).

Our present study clearly demonstrates that the miR-100 family is downregulated in human HCCs of different etiology. Moreover, it shows that reduced expression of these miRNAs is also species independent, as a strong decrease takes place also in rat HCCs.

More important, a novel and relevant finding of this study is the discovery of miR-100 downregulation from very early phases (9 weeks after initiation) of a process that culminates in advanced HCCs, 12–14 months later. Moreover, the finding of miR-100 downregulation also in adenomas and early HCCs, further supports a critical role of this miRNA along all HCC development.

Although assessment of the translational value of animal models of human cancer poses a major challenge, the usefulness of the R-H model has been clearly demonstrated (Andersen *et al.*, 2010). Early rat lesions positive for the progenitor cell marker CK19+, in fact, display a gene expression profile that robustly cosegregates with the human CK19+HCC subtype, characterized by the worst clinical prognosis. Thus, the downregulation of miR-100 observed in the several phases of the multistep process of hepatocarcinogenesis (early preneoplastic lesions, adenomas, early HCCs and advanced HCCs) is likely to recapitulate the natural history of human HCC.

Another critical finding stemming from this study is that downregulation of miR-100 is not simply a marker of proliferating hepatocytes. Since the early lesions developed in the R-H model are characterized by a proliferation rate higher than that of the surrounding liver, we indeed considered the possibility that the decreased miR-100/miR-99a expression could simply be linked to cell division—either as a trigger or as a consequence—and not necessarily to events related to the carcinogenic process. However, this possibility was ruled out by the finding that no significant variations of miR-100/miR-99a levels took place during liver regeneration post-PH, a condition characterized by a proliferative response considerably higher than that found in preneoplastic lesions. These findings, together with the observation that miR-100/miR-99a are downregulated in HCC of different etiology and in two diverse species, strongly support the role of these miRNAs in the tumorigenic process.

Nevertheless, although downregulation of these miRNAs appears to be critical in the cancer process, the loss of the oncosuppressive ability of miR-100 may not be sufficient to allow the evolution of HCC. Indeed, miR-100 was found to be downregulated in almost 100% of the rat preneoplastic lesions; however, it is well established that during the carcinogenic process, a slow but continuous regression of the vast majority of the preneoplastic lesions occurs, that is

characterized by a progressive loss of the less-differentiated phenotype and by the return to a normal one (remodeling) (Enomoto and Farber, 1982). MiR-100 downregulation is likely not to discriminate between lesions that undergo spontaneous regression and those that will progress to HCC. If this is the case, one possible interpretation is that, as in the case of many other genes endowed with an oncosuppressive function, the loss of miR-100 represents a permissive condition for the development of preneoplastic lesions but not sufficient for progression to HCC. We presume that additional lesions have to take place in a cellular context where miR-100 is poorly expressed, to allow progression. In the absence of this further event, the preneoplastic lesions possibly undergo regression.

In *in vitro* experiments, we show that reintroduction of miR-100 in HCC cells was able to impair cell growth; on the other hand, inhibition of the endogenous miRNA resulted in increased growth ability, further supporting the oncosuppressive role of this miRNA. Several targets have been proposed for miR-100; among them are PLK1, mTOR (Wang *et al.*, 2008) and FGFR3 (Oneyama *et al.*, 2011). Although we did not find any correlation between mTOR or FGFR3 and miR-100 expression in HCC samples (data not shown), we did find an inverse correlation between miR-100 and PLK1. PLK1 is a protein involved in the control of the G2/M phase and whose oncogenic role has been demonstrated *in vitro* (Smith *et al.*, 1997). Moreover, several works have shown that this protein is frequently overexpressed in human tumors and that its expression correlates with tumor genetic instability (Eckerdt *et al.*, 2005). As we have not been able to revert the inhibitory growth effect or miR-100 by reexpressing PLK1 in miR-100-transfected cells (data not shown), it is plausible that, since miRNAs have several targets, the reexpression of PLK1 alone might not be sufficient to recover the inhibitory effect on cell growth due to miR-100 overexpression. Alternatively, the main role of PLK1 in HCC cells may not be linked to cell proliferation but rather to other properties of this molecule. The present work unveils an important contribution for miR-100 and its family in the early phases of hepatocarcinogenesis and suggests a post-transcriptional regulation mechanism that can explain the increase of PLK1 expression, frequently observed in human HCCs, in the absence of gene amplification. It has to be noted that, although PLK1 was increased in most of the human HCCs and in all rat HCCs, there was not a strict mathematic anti-correlation between the levels of miR-100 and PLK1. It is likely that the level of expression of PLK1 does not depend only on the presence of the miRNAs but also on other regulators, such as transcription factors or proteins that modulate its degradation. As a consequence, the amplitude of PLK1 increase due to miR-100 downregulation is likely to be context dependent.

In conclusion, the findings reported in this work have potential translational outcomes. As miR-100 downregulation and PLK1 overexpression are early events during hepatocarcinogenesis, they can represent new biomarkers of the neoplastic evolution of a pathological liver and could help in the identification of progressive lesions that are not clearly identified at the pathological analysis. Moreover, as we showed that reintroduction of miR-100 in HCC cell lines resulted in impairment of their growth, it is likely that the restoration of this miRNA in the neoplastic liver could affect tumor progression. A recent work demonstrates that in a mouse model of liver cancer, ectopic expression of a single miRNA (miR-26) could reverse disease progression (Kota *et al.*, 2009). Restoring miRNA levels in hepatic cancer cells is therefore a new therapeutic option for the treatment of liver cancer. In this context, miR-100 could be a promising tool, being lost in cancer cells but expressed at considerable levels in normal hepatocytes and being able to interfere with cancer cell growth upon reexpression.

MATERIAL AND METHODS

Cell lines and animals

For cell lines and *in vitro* experiments see Supplementary Information.

Guidelines for the Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari and the Italian Ministry of Health. Male Fischer F-344 rats (100–125 g) were purchased from Charles River Italia (Lecco, Italy). Preneoplastic lesions and HCCs were induced according to the R-H model (Solt *et al.*, 1977). For detailed experimental procedures see Supplementary Information.

Quantitative analysis of miRNAs and mRNAs in human and rat samples

Human HCC samples (n=34) and the corresponding PT tissues (resected 1–2 cm from the tumor) were from specimens surgically resected for pathological examination. The clinicopathological features of HCCs are summarized in Supplementary Table 2. Each specimen was obtained with the patient's informed consent. A sample of each HCC and corresponding PT was processed for total RNA extraction (De Petro *et al.*, 1998), whereas the remaining tissue was fixed in 10%buffered formalin and embedded in paraffin for histological analysis.

Analysis of miRNAs and PLK1 expression was performed as described in Supplementary Information.

Protein extraction and western blot

For protein analysis, cells and rat liver samples were lysed in 2% SDS, 0.5 M Tris–HCl. Western blots were performed according to standard methods. The following antibodies were used: anti-PLK1 (Cell Signalling Technologies, Beverly, MA, USA), anti-vinculin (Sigma, St Louis, MO, USA) and anti–β-actin (I-9, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Final detection was done with the ECL system (Amersham, Uppsala, Sweden).

Histology, histochemistry and immunohistochemistry

Human HCC serial sections were immunostained for PLK1 with the Abcam (Cambridge, MA, USA) monoclonal antibody (ab17056); Bond Polymer Refine Detection kit allowed to reveal the presence of PLK1 protein. Rat liver sections were fixed in 10% formalin, embedded in paraffin and stained with H&E or processed for immunohistochemical detection. BrdU incorporation into nuclei was stained with mouse antibodies from Becton Dickinson (Franklin Lakes, NJ, USA) (Ledda-Columbano *et al.*, 1998). Placental form of GSTP in the liver was determined by using an anti-rat GSTP polyclonal antibody (MBL, Nagoya, Japan); horseradish peroxidase (for BrdU) or alkaline phosphatase (for GSTP) secondary conjugates were used in double staining. Anti-PLK1 antibody (Abcam, ab47867) was visualized with 3,3′-diaminobenzidine (DAB) in formalin fixed sections or with mouse Alexafluor488 secondary antibody (Molecular Probes, Carlsbad, CA, USA) in cryostat sections.

Laser-capture microdissection

Fifteen-μm-thick serial frozen sections of rat livers were attached to 2 μm RNase-free PEN-membrane slides (Leica, Wetzlar, Germany). Microdissection (Leica, LMD6000) was followed by acetone fixation and a 3½-min H&E staining. Handling time per slide did not exceed 20 min. RNA was extracted from micro-dissected samples using the mirVana miRNA Isolation Kit (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

Statistical analysis was performed with Student's *t*-test and using GraphPad Prism version 5.00 for Windows. To visualize the expression data of miR-100, miR-99a, miR-99b and PLK1 shown in Figure 2a, we exploited the Gedas software (Fu and Medico, 2007).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Andersen JB, Loi R, Perra A, Factor VM, Ledda-Columbano GM, Columbano A*et al*. (2010). Progenitor-derived hepatocellular carcinoma model in the rat.*Hepatology* **51**: 1401–1409.
2. Cairo S, Wang Y, de RA, Duroure K, Dahan J, Redon MJ *et al*. (2010). Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer. *Proc Natl Acad Sci USA* **107**: 20471–20476.
3. Christodoulou F, Raible F, Tomer R, Simakov O, Trachana K, Klaus S *et al*. (2010). Ancient animal microRNAs and the evolution of tissue identity. *Nature***463**: 1084–1088.
4. De Petro G, Tavian D, Copeta A, Portolani N, Giulini SM, Barlati S. (1998). Expression of urokinase-type plasminogen activator (u-PA), u-PA receptor, and tissue-type PA messenger RNAs in human hepatocellular carcinoma.*Cancer Res* **58**: 2234–2239
5. Eckerdt F, Yuan J, Strebhardt K. (2005). Polo-like kinases and oncogenesis.*Oncogene* **24**: 267–276.
6. Enomoto K, Farber E. (1982). Kinetics of phenotypic maturation of remodeling of hyperplastic nodules during liver carcinogenesis. *Cancer Res***42**: 2330–2335.
7. Fu L, Medico E. (2007). FLAME, a novel fuzzy clustering method for the analysis of DNA microarray data. *BMC Bioinformatics* **8**: 3.
8. Henson BJ, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM. (2009). Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. *Genes Chromosomes Cancer* **48**: 569–582.
9. Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q *et al*. (2011). Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. *Cancer Cell* **19**: 232–243
10. Imbeaud S, Ladeiro Y, Zucman-Rossi J. (2010). Identification of novel oncogenes and tumor suppressors in hepatocellular carcinoma. *Semin Liver Dis* **30**: 75–86.
11. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW*et al*. (2009). Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* **137**: 1005–1017.
12. Ledda-Columbano GM, Curto M, Piga R, Zedda AI, Menegazzi M, Sartori C *et al*. (1998). *In vivo* hepatocyte proliferation is inducible through a TNF and IL-6-independent pathway. *Oncogene* **17**: 1039–1044.

13. Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL *et al*. (2007). Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer* **120**: 1046–1054.

14. Lee JS, Thorgeirsson SS. (2004). Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets. *Gastroenterology* **127**: S51–S55.

15. Leite KR, Sousa-Canavez JM, Reis ST, Tomiyama AH, Camara-Lopes LH, Sanudo A *et al*. (2009). Change in expression of miR-let7c, miR-100, and miR-218 from high grade localized prostate cancer to metastasis. *Urol Oncol***29**: 265–269.

16. Liu W, Gong YH, Chao TF, Peng XZ, Yuan JG, Ma ZY *et al*. (2009). Identification of differentially expressed microRNAs by microarray: a possible role for microRNAs gene in medulloblastomas. *Chin Med J (Engl)* **122**: 2405–2411.

17. Lu J, Getz G, Miska EA, varez-Saavedra E, Lamb J, Peck D *et al*. (2005). MicroRNA expression profiles classify human cancers. *Nature* **435**: 834–838.

18. Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH *et al*. (2008). MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* **14**: 2690–2695.

19. Oneyama C, Ikeda J, Okuzaki D, Suzuki K, Kanou T, Shintani Y *et al*. (2011). MicroRNA-mediated downregulation of mTOR/FGFR3 controls tumor growth induced by Src-related oncogenic pathways. *Oncogene* **30**: 3489–3501.

20. Parkin DM. (2001). Global cancer statistics in the year 2000. *Lancet Oncol* **2**: 533–543.

21. Pellegrino R, Calvisi DF, Ladu S, Ehemann V, Staniscia T, Evert M *et al*. (2010). Oncogenic and tumor suppressive roles of polo-like kinases in human hepatocellular carcinoma. *Hepatology* **51**: 857–868.

22. Satoh K, Kitahara A, Soma Y, Inaba Y, Hatayama I, Sato K. (1985). Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. *Proc Natl Acad Sci USA* **82**: 3964–3968.

23. Shi W, Alajez NM, Bastianutto C, Hui AB, Mocanu JD, Ito E *et al*. (2010). Significance of Plk1 regulation by miR-100 in human nasopharyngeal cancer.*Int J Cancer* **126**: 2036–2048.

24. Smith MR, Wilson ML, Hamanaka R, Chase D, Kung H, Longo DL *et al*. (1997). Malignant transformation of mammalian cells initiated by constitutive expression of the polo-like kinase. *Biochem Biophys Res Commun* **234**: 397–405.

25. Solt DB, Medline A, Farber E. (1977). Rapid emergence of carcinogen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. *Am J Pathol* **88**: 595–618.

26. Su H, Yang JR, Xu T, Huang J, Xu L, Yuan Y *et al*. (2009). MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. *Cancer Res* **69**: 1135–1142.

27. Tarantino C, Paolella G, Cozzuto L, Minopoli G, Pastore L, Parisi S *et al*. (2010). miRNA 34a, 100, and 137 modulate differentiation of mouse embryonic stem cells. *FASEB J* **24**: 3255–3263.

28. Thorgeirsson SS. (2011). The almighty MYC: orchestrating the micro-RNA universe to generate aggressive liver cancer. *J Hepatol* **55**: 486–487.

29. Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S *et al*. (2010). Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol* **11**: 136–146.
30. Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP *et al*. (2008). MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. *Hepatology* **47**: 1223–1232.
31. Wang FZ, Weber F, Croce C, Liu CG, Liao X, Pellett PE. (2008). Human cytomegalovirus infection alters expression of cellular microRNA species that affect its replication. *J Virol* **82**: 9065–9074.
32. Wienholds E, Kloosterman WP, Miska E, varez-Saavedra E, Berezikov E, de BE *et al*. (2005). MicroRNA expression in zebrafish embryonic development.*Science* **309**: 310–311.
33. Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI. (2008). Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue.*Clin Cancer Res* **14**: 2588–2592.

ACKNOWLEDGMENTS

We thank L Tamagnone, S Corso, MM Angioni and our colleagues for helpful discussions; C Manca for the preparation of rat samples and C Isella for statistical analysis; L Palmas for technical assistance. Work was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC grants to SG, AC), Regione Piemonte (grant to SG) and MIUR (grants to SG, AC, GMLC, GDP).

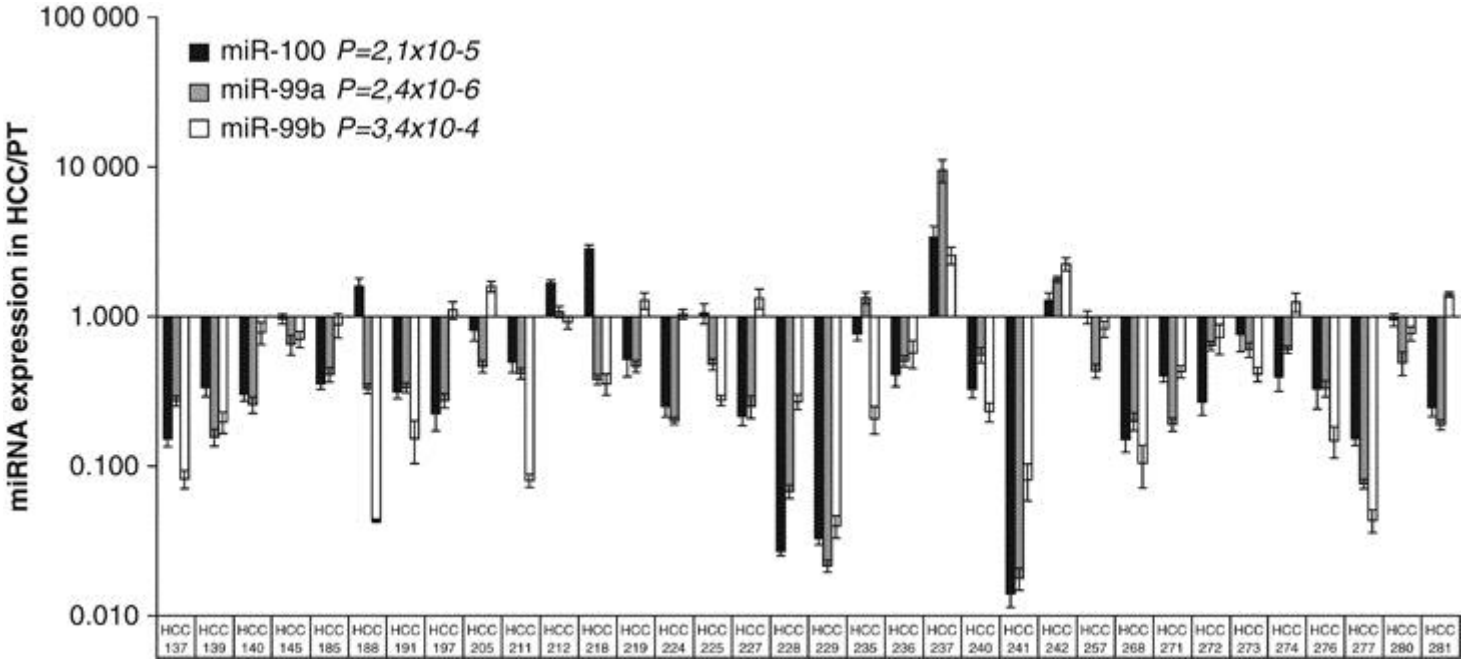

Figure 1.

The miR-100 family is downregulated in human HCCs. MiRNA expression was assessed by quantitative TaqMan RT–PCR in both the tumor (HCC) and peri-tumoral (PT) tissue for each patient. The levels were calculated as fold change difference between the expression in matched HCCs and PTs and reported in log scale. Values <1 represent decreased expressions in HCC compared with the corresponding PT. RNU48 was used as endogenous control. Error bars represent the standard deviation of technical triplicates. (*P* value=2.1 × 10^{-5} for miR-100; *P*=2.4 × 10^{-6} for miR-99a; and *P*=0.00034 for miR-99b).

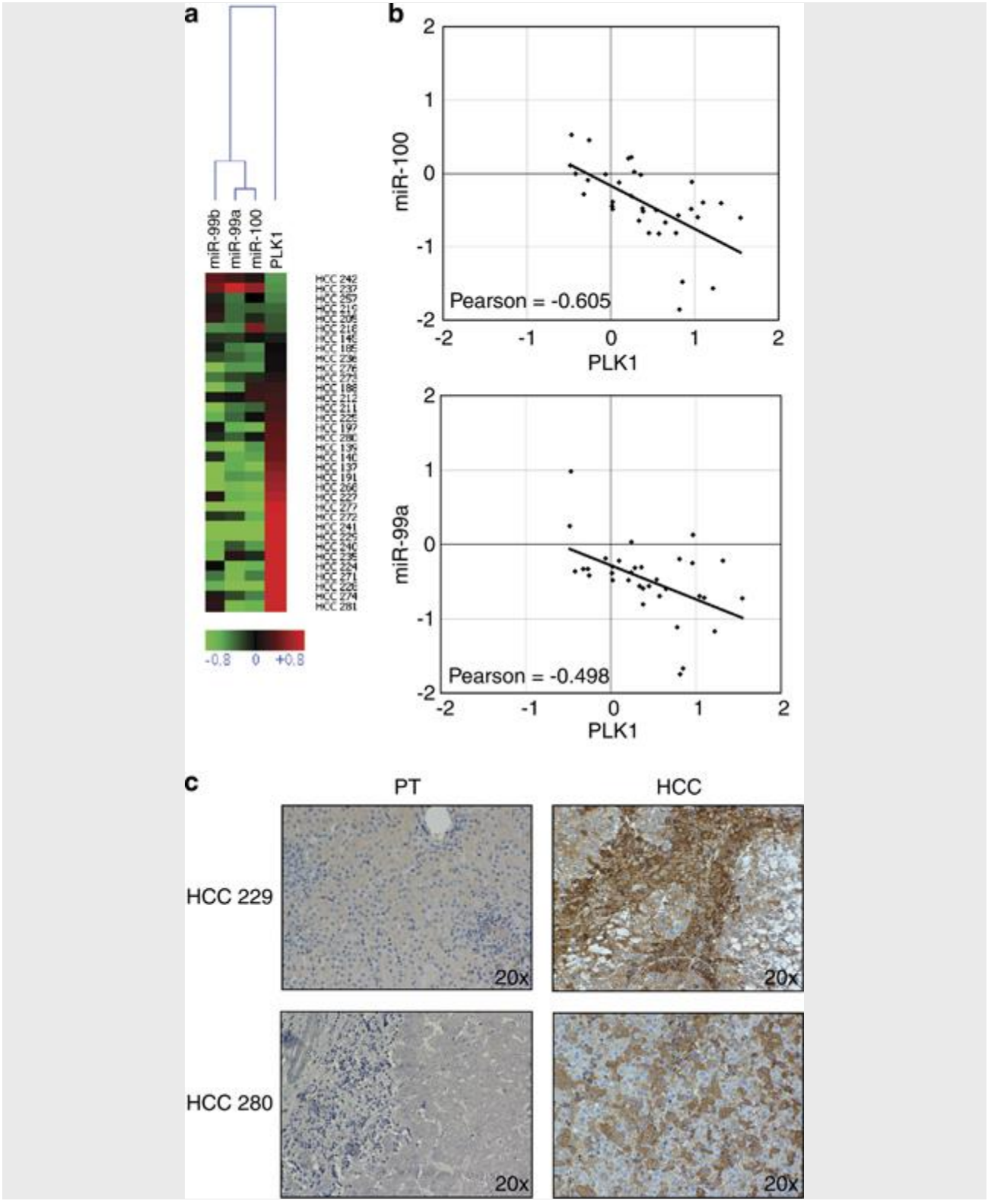

Figure 2.

MiR-100 family downregulation correlates with increased expression of PLK1. PLK1 expression was evaluated in HCC and PT for each patient using quantitative TaqMan RT–PCR and calculated as ratio between the expression in HCC and PT. Beta-actin was used as endogenous control. (**a**) The expression data of PLK1, miR-100, -99a and -99b were visualized exploiting the GEDAS software (Fu and Medico, 2007). As shown, low levels of miRNAs correlate with high levels of

PLK1. (**b**) Scatter plots representing the inverse correlation between miR-100 and PLK1 (upper panel, *P*=0.0001) or miR-99a and PLK1 (lower panel, *P*=0.0027) expression in HCCs. The values are reported as log fold change of HCC/PT. (**c**) IHC for PLK1 performed on two representative cases of human HCC samples (magnification 20 ×). In PT tissues PLK1 was barely detectable, whereas increased expression was observed in 27/28 tumors (see also Supplementary Figure 1).

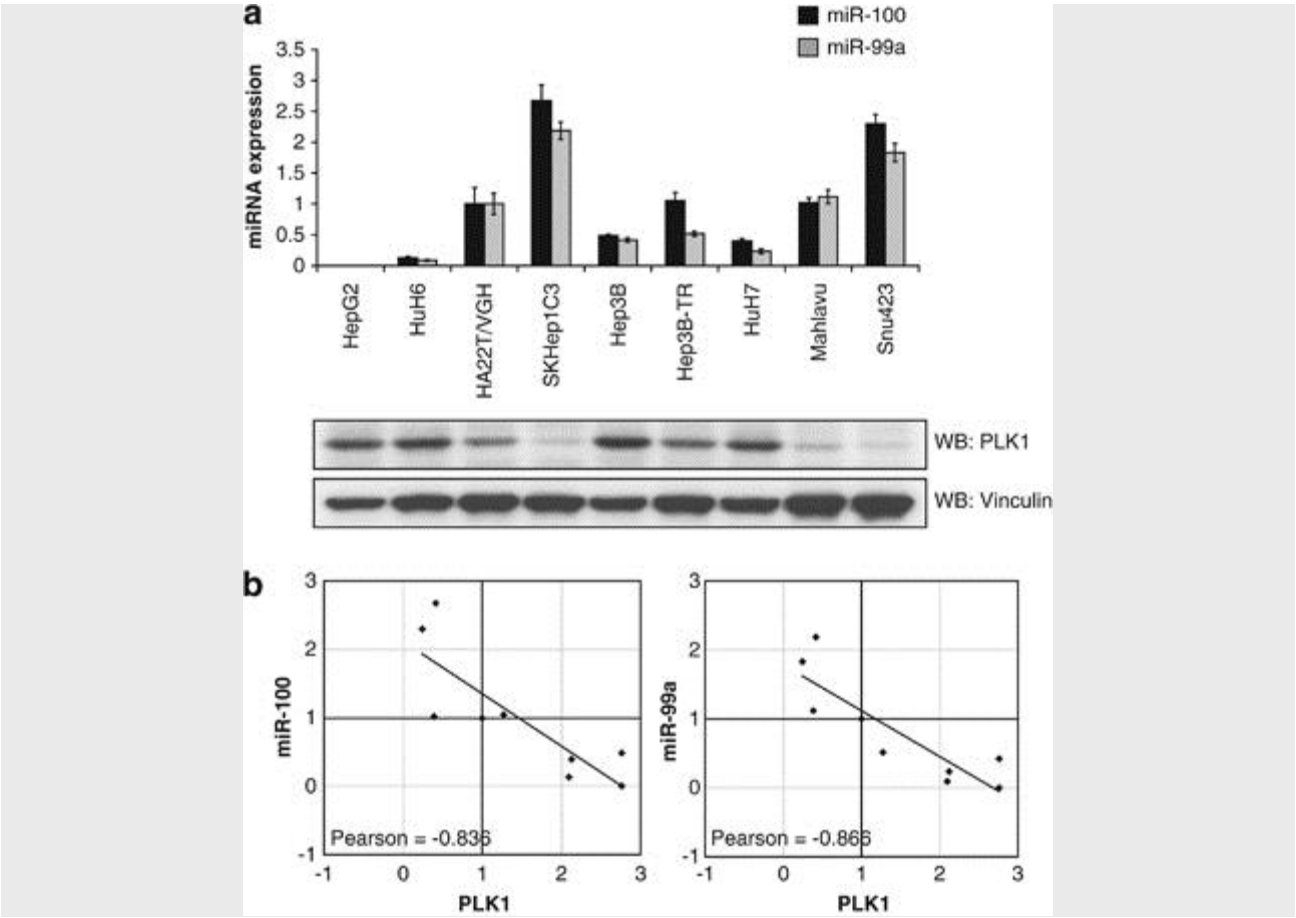

Figure 3.

MiR-100 and -99a anti-correlate with PLK1 expression in HCC cell lines. (**a**) miRNA expression was evaluated by quantitative TaqMan RT–PCR and reported as ratio between the levels of miRNA in each cell line and the level in HA22T/VGH cells that was set as 1. Error bars represent the standard deviation of TaqMan RT–PCR performed in technical triplicates. PLK1 expression was evaluated in the same cells by western blot. The bands of PLK1 were quantified using the ImageJ software and normalized to vinculin. MiRNA and PLK1 quantifications were reported in scatter plots (**b**) showing a statistically significant anti-correlation (*P*=0.0049 for miR-100 and *P*=0.0025 for -99a).

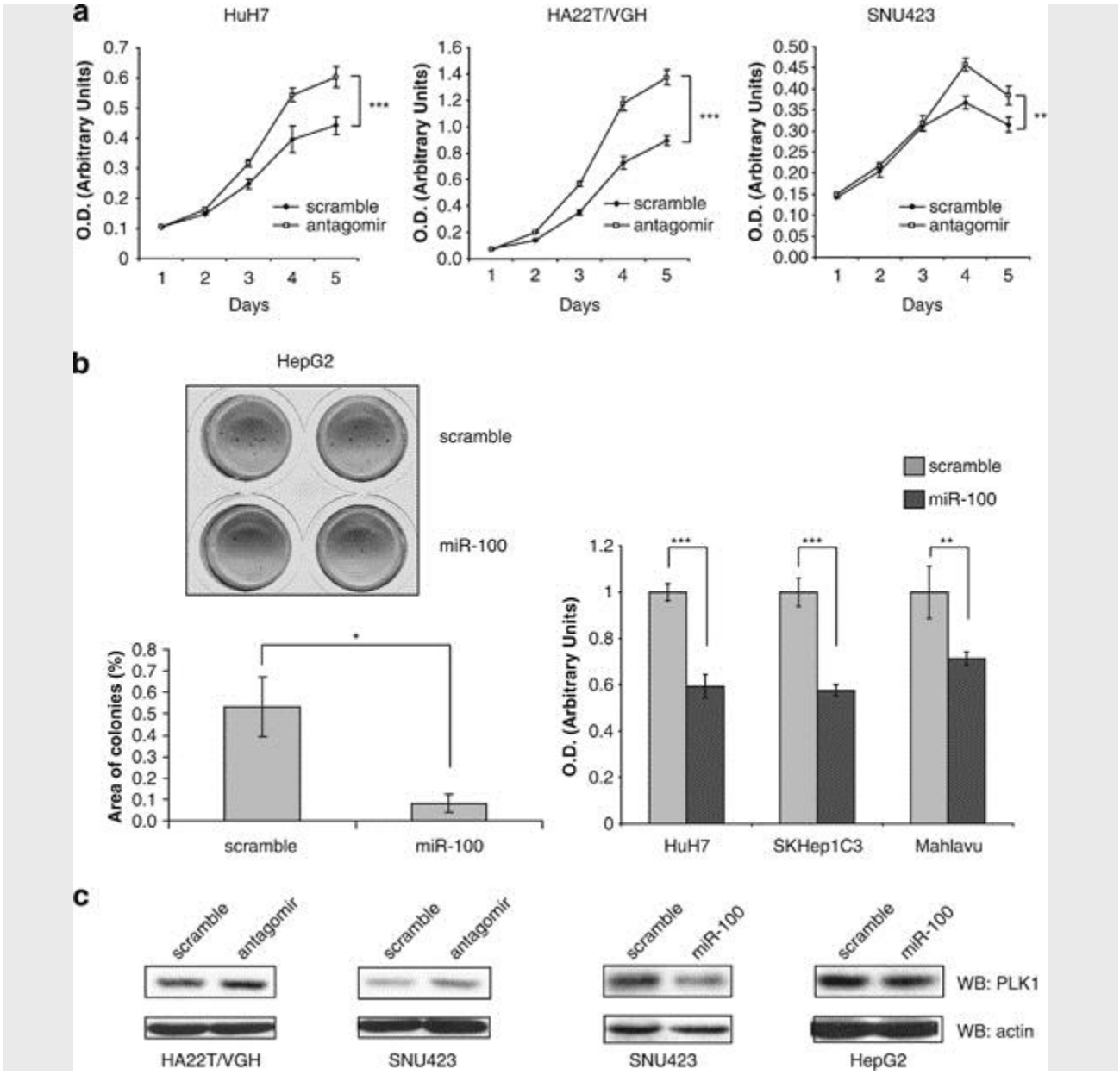

Figure 4.

MiR-100 overexpression impairs HCC cell growth. (**a**) HuH7, HA22T/VGH and SNU423 cells were transiently transfected with a specific miR-100 inhibitor (antagomir) or a scrambled siRNA (scramble) as a control and then seeded in 96-well plates to perform a growth curve assay (see Materials and methods for details). MiRNA inhibition significantly enhanced cell proliferation (O.D.=Optical Density at 590 nm; ***P<0.0005;**P<0.001). (**b**) HepG2, HuH7, SKHep1C3 and Mahlavu cells, constitutively expressing miR-100, were seeded in soft agar to evaluate anchorage-independent growth; at the end of the assay, either colony area (left panel) or the ability to metabolize Alamar blue (right panel) was evaluated (see Materials and methods for details). MiRNA overexpression significantly impaired HCC cells ability to grow in anchorage-independent conditions (***P<0.0005; **P<0.005; *P<0.05). (**c**) HCC cells were transiently transfected with either a specific miR-100 inhibitor (antagomir) or pre-miR-100 or a scrambled siRNA, as a control, and then processed for western blot analysis. In the presence of the miRNA, PLK1 was downregulated, whereas an increase was detected upon miR-100 inhibition.

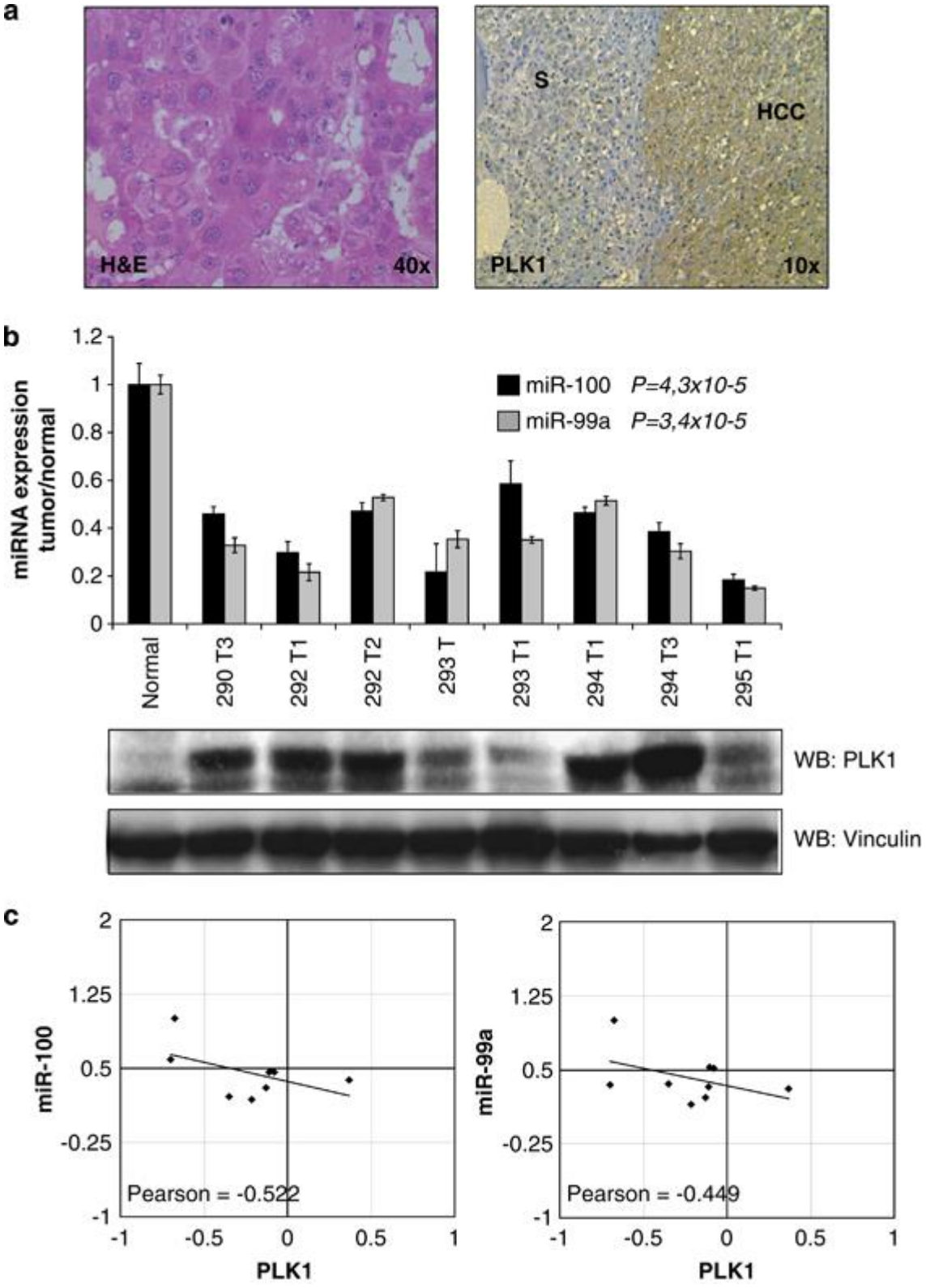

Figure 5.

MiR-100 and -99a downregulation correlates with increased expression of PLK1 in a rat model of liver tumorigenesis. (**a**) Representative microphotography illustrating a HCC developed in rats exposed to the R-H model. Left panel: H&E (original magnification 40 ×); right panel: IHC staining for PLK1 (10 ×). HCC: tumoral tissue; S: surrounding tissue. (**b**) Expression levels of miR-100 and -99a were evaluated by TaqMan RT–PCR in rat liver carcinoma samples (290T3 to 295T1) and

compared with normal rat liver (Normal). The 4.5S RNA was used as endogenous control. The two miRNAs were significantly downregulated in rat liver tumors (upper panel). Western blot analysis (lower panels) showed an increased expression of PLK1 in the same tumors, compared with normal tissue. (**c**) PLK1 bands were quantified using the ImageJ software and normalized to vinculin. The values obtained were compared with miRNA expression, as shown in the scatter plots.

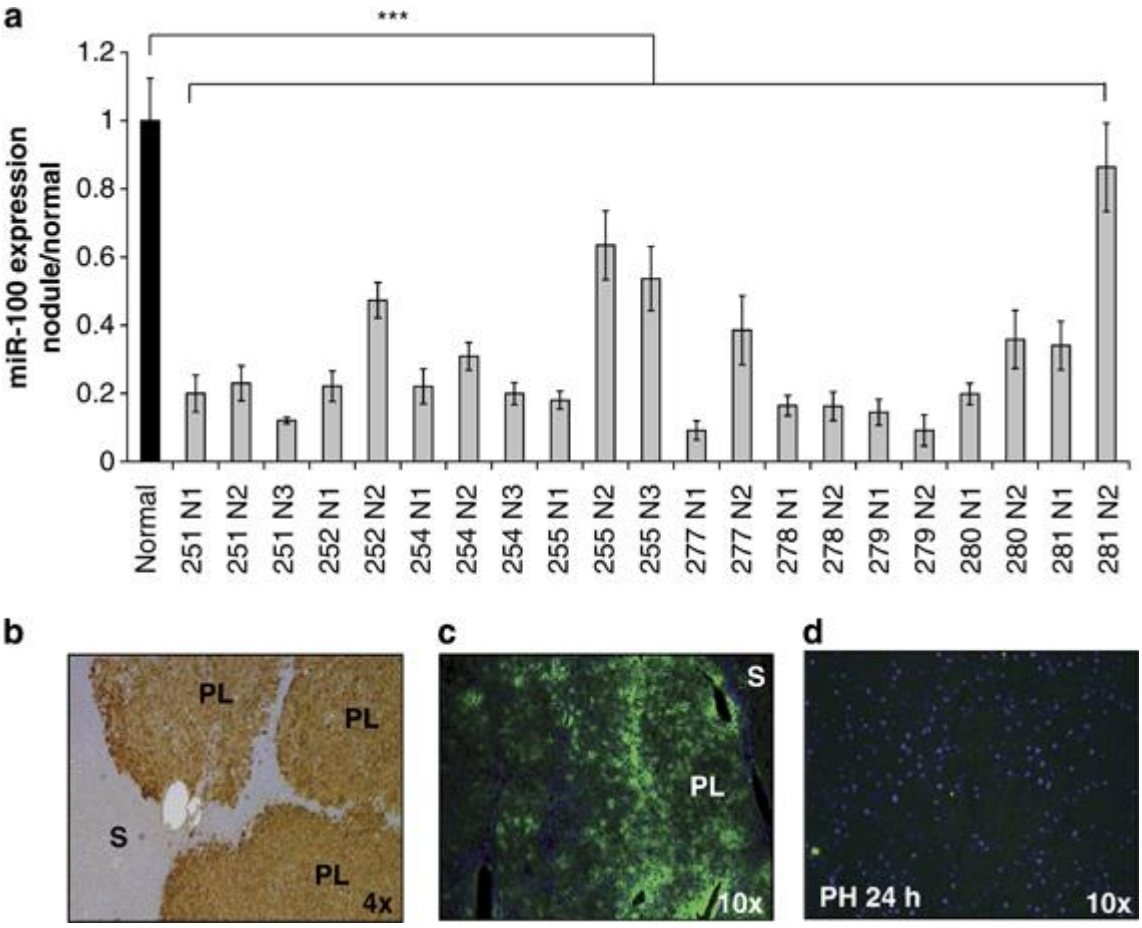

Figure 6.

MiR-100 downregulation correlates with increased expression of PLK1 in rat preneoplastic lesions. (**a**) MiR-100 expression was evaluated in 21 laser microdissected rat nodules obtained from different animals (251N1 to 281N2) and compared with normal liver. A striking downregulation of the miRNA was detected in the preneoplastic lesions (***P<0.0001). (**b**) GSTP staining of preneoplastic lesions. PL: preneoplastic lesions; S: surrounding tissue (4 ×). (**c**) Dual-color immunofluorescence staining with DAPI (blue) and PLK1 (green) in preneoplastic lesions or (**d**) in regenerating liver (PH) (10 ×). As shown, PLK1 is highly expressed in PL but not in the non-tumorigenic liver and in proliferating hepatocytes.

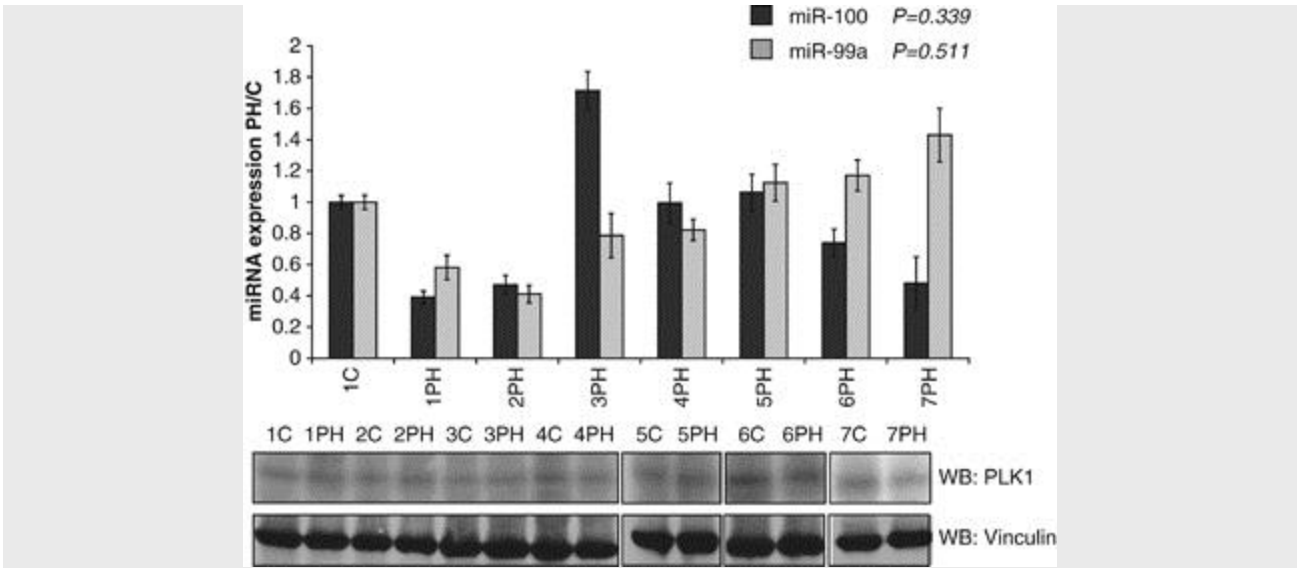

Figure 7.

Expression of miR-100, -99a and PLK1 is not significantly modified upon PH. MiR-100 and -99a expression was assessed 24 h upon PH in regenerating livers (PH samples) from different animals and compared with the same liver before surgery (C samples). No significant modification of the miRNAs was observed in regenerating livers. PLK1 expression was not significantly modified (lower panels).