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A functional polymorphism in the *miR-146a* gene is associated with the risk for hepatocellular carcinoma

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A G > C polymorphism (rs2910164) is located in the stem region opposite to the mature miR-146a sequence, which results in a change from G:U pair to C:U mismatch in the stem structure of miR-146a precursor. Here, we elucidated the biological significance of this polymorphism, based on cancer association study and cell model system. The cancer association study included 479 hepatocellular carcinoma (HCC) and 504 control subjects. We found that the genotype distribution of this polymorphism in HCC cases was significantly different from that in control subjects ($P = 0.026$). The association between the genotype and the risk of HCC was further analyzed using multivariate unconditional logistic regression, with adjustment for sex, age and hepatitis B virus status. The results revealed that male individuals with GG genotype were 2-fold more susceptible to HCC (odds ratio = 2.016, 95% confidence interval = 1.056–3.848, $P = 0.034$) compared with those with CC genotype. We next examined the influence of this polymorphism on the production of mature miR-146a and found that G-allelic miR-146a precursor displayed increased production of mature miR-146a compared with C-allelic one. Further investigations disclosed that miR-146a could obviously promote cell proliferation and colony formation in NIH/3T3, an immortalized but non-transformed cell line. These data suggest that the G > C polymorphism in miR-146a precursor may result in important phenotypic traits that have biomedical implications. Our findings warrant further investigations on the relation between microRNA polymorphism and human diseases.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide (1). It has been shown that both genetic and environmental factors are involved in the etiology and prognosis of HCC (1,2).

MicroRNAs (miRNAs) are non-coding RNAs that inhibit the expression of protein-coding genes by either translational repression or messenger RNA degradation (3–5). Growing evidences have suggested that miRNAs regulate a wide range of biological processes, including development (6), cell differentiation (7), proliferation and apoptosis (8–10). It is well known that deregulations of these biological processes play critical roles during carcinogenesis (11,12).

Sequence variations have been found to affect the processing and/or target selection of human miRNAs. For example, Calin *et al.* (13) observed that a germ line mutation, located 7 bp downstream of the miR-16-1 precursor, resulted in dramatically reduced expression of mature miR-16-1. In another case, variation in the sequence of mature miR-376, caused by RNA editing, redirected miR-376 to silence a new set of target genes (14). Also, it was recently found that a single-nucleotide polymorphism (SNP) at the eighth nucleotide of the mature miR-125a sequence significantly blocked the production of mature miRNA (15). These findings indicate that sequence variations have important influence on the expression and function of miRNA.

SNP is the most common type of genetic variation in human genome. It has been well demonstrated that SNPs in protein-coding genes can affect the functions of proteins and in turn influence the individual susceptibility to cancers (16). In as much as the importance of miRNAs has only been recognized recently, little is known about the biological function of SNPs in the miRNA genes.

A G > C polymorphism (rs2910164) has been identified in the *miR-146a* gene. This polymorphism is located in the stem region opposite to the mature miR-146a sequence and results in a change from G:U pair to C:U mismatch in the stem structure of miR-146a precursor. In this study, we elucidated the biological function of this polymorphism, based on cancer association study and cell model system. We showed that male individuals with GG genotype were more susceptible to HCC compared with those with CC genotype. Furthermore, the G-allelic miR-146a precursor displayed increased production of mature miR-146a compared with the C-allelic one. Consistently, the miR-146a could obviously promote cell proliferation and colony formation. Our results indicate that SNPs in miRNA genes may result in important phenotypic traits that have biomedical implications.

Materials and methods

Study population

This case–control study included 479 HCC patients and 504 cancer-free controls. All studied subjects were unrelated ethnic Han Chinese who lived in Guangzhou or the surrounding regions, where HCC is prevalent and hepatitis B virus (HBV) infection has been demonstrated as main attributable risk factor (17,18). In our study cohort, HBV and hepatitis C virus (HCV) infections were identified in 88.9 and 1.9% of cases, respectively. HBV or HCV infection was diagnosed when HBV surface antigen or HCV antibody was detected by enzyme-linked immunosorbent assay in the serum isolated from peripheral blood. Patients with HCC were recruited from June 2000 to July 2005 at the Cancer Center of Sun Yat-Sen University in Guangzhou, People's Republic of China. All cases were diagnosed histopathologically. Control subjects were healthy checkup examinees without cancer history and were collected in the same period.

Informed consent was obtained from each subject at recruitment. The study was approved by the Institute Research Ethics Committee at the Cancer Center of Sun Yat-Sen University.

Genotyping

Genomic DNA was extracted from peripheral blood samples. Genotypes were analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay. The primers, 5′-GGAGGGGTCTTTGCACCATC-3′ and 5′-TGCCTTCTGTCTCCAGTCTTCC-3′, were used to amplify a 210 bp fragment covering the G > C polymorphism site (rs2910164) in the miR-146a precursor. The PCR product was digested with restriction enzyme MamI (MBI Fermentas, Hanover, MD), and the resulting fragments were separated on a 15% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. The CC genotype produced four fragments (82, 51, 45 and 32 bp, respectively), whereas the GG homozygote and the GC heterozygote displayed three (82, 77 and 51 bp) and five bands (82, 77, 51, 45 and 32 bp), respectively. Three samples representing three distinct patterns of restriction fragment length polymorphism were applied to direct DNA sequencing to validate the genotype.

Statistical analysis

Pearson's chi-square test was used to evaluate the differences in the distribution of genotype between cases and controls. Association between the *miR-146a* polymorphism and the risk of HCC was analyzed by multivariate unconditional

Abbreviations: BrdU, bromodeoxyuridine; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miRNA, microRNA; OR, odds ratio; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

logistic regression, adjusted for age, sex and HBV status. Hardy–Weinberg equilibrium was assessed using goodness-of-fit chi-square test. A *P*-value <0.05 was considered statistically significant, and all statistical tests were two sided. All analyses were performed using SPSS software (version 13.0, SPSS, Chicago, IL).

Vector construction

All the plasmids, including pc3.0-miR-146G/C, pc3.0-miR-218-1 and pc3.0-HRas^{G12V}, were constructed based on the pcDNA3.0 (Invitrogen, Carlsbad, CA). The DNAs from normal blood donors with GG or CC genotype at the rs2910164 site were used as templates, and the PCR-amplified 386 bp DNA fragment encompassing the mature miR-146a sequence and its 5′ and 3′ flanking regions (179 and 185 bp, respectively) was cloned into the XhoI and XbaI sites of the pcDNA3.0. The yielded plasmid with GG genotype was designated pc3.0-miR-146G and the one with CC genotype named pc3.0-miR-146C. The pc3.0-miR-218-1, which contained the mature miR-218-1 sequence and its 5′ and 3′ flanking regions (202 and 157 bp, respectively), was used as a negative control in investigating the effect of G/C polymorphism on the maturation of miR-146a. The pc3.0-HRas^{G12V}, which comprised a HRas-coding sequence (ENSG00000174775) with an amino acid substitution at codon 12 (G to V), was used as a positive control in colony formation assays.

Northern blot analysis

To clarify the effect of G/C variance on the production of mature miR-146a, 293T cells were transfected with pc3.0-miR-218-1, pc3.0-miR-146G or pc3.0-miR-146C by calcium phosphate precipitation. The cells were harvested 24 h after transfection and then subjected to total RNA isolation using Trizol (Invitrogen). The small RNAs were enriched from the total RNA using PEG8000 as described previously (19). Ten microgram of small RNAs was separated on a 15% denaturing polyacrylamide gel, transferred to positively charged membrane (PerkinElmer, Boston, MA) using Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) and then immobilized by ultraviolet cross-linking and baking at 80°C under vacuum. The membranes were prehybridized and then hybridized at 37°C for 24 h to a γ-^{32}P-labeled DNA oligonucleotide that was complementary to the sequence of mature miR-146a (probe sequence: 5′-AACCCATGGAATTCAGTTCTCA-3′) or to *U6* small nuclear RNA (snRNA) (5′-AACGCTTCACGAATTTGCGT-3′). Thereafter, the membranes were washed and exposed to a Storage Phosphor Screen, which was subsequently read with Typhoon Phosphor Imager (Amersham Bioscience, Sunnyvale, CA).

Bromodeoxyuridine incorporation assay

NIH/3T3 cells (1.2×10^4) were seeded in a 48-well plate and then transfected with 100 nM of Pre-miR-146a (Ambion, Austin, TX), si-miR-146a (GenePharma, Shanghai, China) or Pre-miR negative control (Pre-miR-NC, Ambion) using Lipofectamine RNAiMax (Invitrogen). The si-miR-146a was an RNA duplex (Figure 1) designed as described previously (20). The BrdU assay was performed at 36, 48 and 60 h after transfection, as reported previously (21). Briefly, transfected cells were incubated with 40 μM bromodeoxyuridine (BrdU) (Sigma, St Louis, MO) for 1 h at time points as indicated, washed with 1× phosphate-buffered saline, followed by fixation in 70% ethanol containing 20 mM glycine (pH 2.0) at room temperature for 45 min. Cells were then incubated sequentially with mouse monoclonal antibody against BrdU (BrdU-Ab-3, Neomarkers, Fremont, CA) and goat-anti-mouse Alexa-488-conjugated secondary antibody (Invitrogen, Eugene, OR), each for 1 h at 37°C. Three washes in 1× phosphate-buffered saline were performed after each incubation with antibody. BrdU incorporation was observed under a fluorescence microscope. At least 500 cells were counted for each sample.

Colony formation assay

NIH/3T3 cells (2.5×10^5) were seeded in six-well plate and then transfected with 2.5 μg of pcDNA3.0, pc3.0-HRas^{G12V}, pc3.0-miR-146G or pc3.0-miR-146C using Lipofectamine PLUS according to the manufacturer's protocol (Invitrogen). Twenty-four hours after transfection, aliquots of the transfected cells were placed in a 6 cm fresh dish and maintained in Dulbecco's modified Eagle's media containing 10% fetal bovine serum and 500 μg/ml G418 for 2–3 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 min.

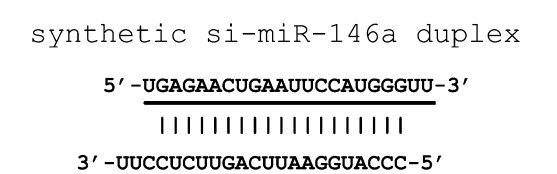

Fig. 1. The sequence of synthetic si-miR-146a duplex. The sequence for mature miR-146a is underlined.

Results

The miR-146a polymorphism is associated with the risk for HCC

In an attempt to explore the biomedical significance of the SNP in miRNA genes, we searched for SNPs that were located in the genomic regions comprising the stem–loop sequence of miRNA genes inferred from the miRBase (Release 8.2, http://microrna.sanger.ac.uk) (22) and an additional 10 bp each in the upstream and the downstream direction. Among the SNPs identified, data of allele frequency were available for 24 SNPs (supplementary Table I is available at *Carcinogenesis* Online, International HapMap Project Release 21, http://www.hapmap.org) (23). We were especially interested in those polymorphisms with high minor allele frequency in Chinese population, as we aimed to further conduct case–control study. The top three SNPs with high minor allele frequency (>40% in Chinese) were located in the genomic region of *miR-196a-2*, *miR-146a* and *miR-608*, respectively (supplementary Table I is available at *Carcinogenesis* Online).

It has been reported that miR-146a and miR-146b, which display high similarity in their sequences, are overexpressed in papillary thyroid carcinoma (24) and HCC (25). Moreover, Cheng *et al.* (26) have found that inhibition of miR-146a in Hela and A549 cell lines by antisense RNA causes a reduction in cell number. These data indicate the potential involvement of miR-146a in tumorigenesis. Therefore, the SNP in *miR-146a* was selected for detailed study. It is a G > C polymorphism (rs2910164) lying in the stem region opposite to the mature miR-146a sequence, which results in a change from G:U pair to C:U mismatch in the stem structure of miR-146a precursor (Figure 2). The biological function of this polymorphism was evaluated based on cancer association study and cell model system.

First, a case–control study including 479 case patients and 504 control subjects was conducted. The baseline characteristics of the studied objects are shown in Table I. The distributions of age and sex did not differ significantly between the patients and the controls, suggesting adequate matching on the basis of these two variables ($P = 0.958$ and 0.245, respectively). The incidence of HBV infection

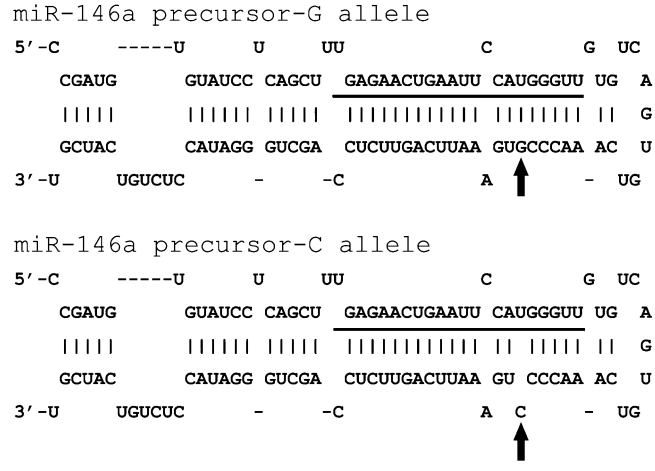

Fig. 2. Schematic diagram of the hairpin loop structure of the G- and C-allelic miR-146a precursor. The sequence for mature miR-146a is underlined. The polymorphism site is indicated by arrow. The G > C polymorphism located in the stem region opposite to the mature miR-146a sequence and results in a change from G:U pair to C:U mismatch in the stem structure of precursor.

in the HCC group (88.9%) was much higher than that in the control subjects (16.1%), implying HBV infection as a main risk factor for HCC development in our study population.

The observed genotype distribution of the *miR-146a* polymorphism in both case and control groups conformed to Hardy–Weinberg equilibrium ($P = 0.543$ and $P = 0.119$, respectively), providing no evidence of population stratification within the dataset. We found that the genotype distribution of this polymorphism in HCC cases was statistically significantly different from that in control subjects ($\chi^2 = 7.291$ and $P = 0.026$). Furthermore, the GG genotype in the patients was more frequent than that in the controls (16.7 versus 11.5%, Table II). A similar trend was also observed in the frequency of GC genotype (50.3 versus 48.4%). The association between the genotype and the risk of HCC was further analyzed using multivariate unconditional logistic regression, with adjustment for sex, age and HBV status. We observed a borderline statistically significant association of the GG phenotype with the increased risk for HCC [odds ratio (OR) = 1.724, 95% confidence interval (CI) = 0.960–3.096, $P = 0.069$ and Table II). When further stratification was performed, the increased risk associated with the GG genotypes became more evident in male individuals (OR = 2.016, 95% CI = 1.056–3.848, $P = 0.034$ and Table II). Moreover, the ORs for HCC risk in males were higher in the recessive model (GG versus GC + CC, OR = 1.691 and 95% CI = 0.911–3.141) than that in the dominant model (GG + GC versus CC, OR = 1.192 and 95% CI = 0.768–1.851), indicating a recessive role of the G allele in the prediction for HCC risk. Interestingly, no significant association between the SNP and the risk for HCC was observed in the female cohort (data not shown).

As shown above, the association between the GG genotype and the risk for HCC in males is statistically significant even after adjustment with HBV status. To further confirm that the observed association was an independent marker for the risk of HCC but not a predisposition to HBV infection, the genotype distributions were compared between the individuals with and without HBV infection within the control or the case group. The results revealed no obvious differences in genotype distributions between HBV-infected and non-infected subjects ($P = 0.565$ within cases and $P = 0.765$ within controls). When further stratification on HBV status was performed, we still observed the similar trend of association between GG genotype and the risk for HCC in males (OR = 2.153, 95% CI = 0.893–5.193 and $P = 0.088$ in HBV-infected cohort and OR = 1.866, 95% CI = 0.720–4.840 and $P = 0.199$ in HBV non-infected cohort), although the results did not reach the statistically significant level owing to the small sample size after stratification. Taken together, these findings suggest *miR-146a* polymorphism as an independent marker of the risk for HCC.

Table I. Baseline characteristics of the studied cases and controls.

	Cases ($n = 479$)	Controls ($n = 504$)	P^a
	No. (%)	No. (%)	
Age (year)			
Mean ± SD	45.2 ± 12.1	44.6 ± 14.4	0.471
≤40	173 (36.1)	181 (31.4)	0.958
41–50	158 (33.0)	162 (25.7)	
51–60	77 (16.1)	80 (23.3)	
>60	71 (14.8)	81 (19.6)	
Gender			
Male	433 (90.4)	444 (88.1)	0.245
Female	46 (9.6)	60 (11.9)	
HBV			
Presence	426 (88.9)	81 (16.1)	<0.001
Absence	53 (11.1)	426 (83.9)	

[a]*P*-values were calculated by student's *t*-test for comparison of mean age and by χ^2 test for comparison of age and gender distributions.

The polymorphism affects the maturation of miR-146a

To gain insight into the mechanism underlying the observed association, we next investigated the influence of this polymorphism on the production of mature miRNA. Expression vector containing the miR-146a precursor with either GG or CC genotype was transiently transfected into 293T cells, which showed low endogenous level of miR-146a. The miR-218-1 expression vector based on the same backbone was used as a negative control. The expression of neomycin, which was derived from the vector backbone, was detected by reverese transcription–PCR and served as a control to correct the differences in cell transfection and harvesting efficiencies. Interestingly, although the expression levels of C- and G-allelic miR-146a precursors were very similar, the level of mature miR-146a produced from the G-allelic precursor was much higher than that from the C-allelic one (Figure 3, lane 2 and 3). These data suggest that this G > C polymorphism affects the production of mature miR-146a.

miR-146a promotes cell proliferation and colony formation

Based on our above findings that individuals with GG genotype were more susceptible to HCC development and the precursor with GG genotype produced more mature miR-146a, we hypothesized that miR-146a may promote cell proliferation. To test this hypothesis, we employed NIH/3T3 cell line, which is immortalized but non-transformed, to investigate the role of miR-146a on cell proliferation and colony formation.

Small interfering RNA (siRNA)-like miRNA duplex was used for miRNA overexpression. Principally, the miR-146a duplex contained a RNA strand with sequence identical to the mature miR-146a and an artificial strand that was partially complementary to the mature miR-146a sequence (Figure 1). Therefore, transfection of miR-146a duplex allowed us only to investigate the function of the *miR-146a* gene, but not to distinguish differential effects of the G- and C-allelic miR-146a precursors.

The effect of miR-146a on cell proliferation was analyzed using BrdU incorporation assay. NIH/3T3 cells were transiently transfected with miRNA duplex (Pre-miR-146a, si-miR-146a or Pre-miR-NC). Pre-miR-NC, used as a negative control, was a miRNA duplex with scrambled sequence that was non-homologous to any human genome sequence. The rate of cell proliferation was represented by the percentage of BrdU-positive cells. In comparison with the Pre-miR-NC transfectants, cells transfected with miR-146a displayed increased cell proliferation, seen as ~100% increase in the rate of BrdU-positive cells at 60 h after transfection (Figure 4A). Furthermore, similar

Table II. Association between the *miR-146a* polymorphism and the risk for HCC

Genotype	All HCCs ($n = 479$) versus all controls ($n = 504$)				Male HCCs ($n = 433$) versus male controls ($n = 444$)			
	Cases, no. (%)	Controls, no. (%)	OR[a] (95% CI)	P^a	Cases, no. (%)	Controls, no. (%)	OR[a] (95% CI)	P^a
CC	158 (33.0)	197 (39.1)	1 (reference)		139 (32.1)	174 (39.2)	1 (reference)	
GC	241 (50.3)	249 (48.4)	1.172 (0.784–1.751)	0.439	219 (50.6)	221 (49.8)	1.192 (0.768–1.851)	0.434
GG	80 (16.7)	58 (11.5)	1.724 (0.960–3.096)	0.069	75 (17.3)	49 (11.0)	2.016 (1.056–3.848)	0.034

[a]OR and *P*-values were calculated by multivariate unconditional logistic regression, adjusted for age, sex and HBV status.

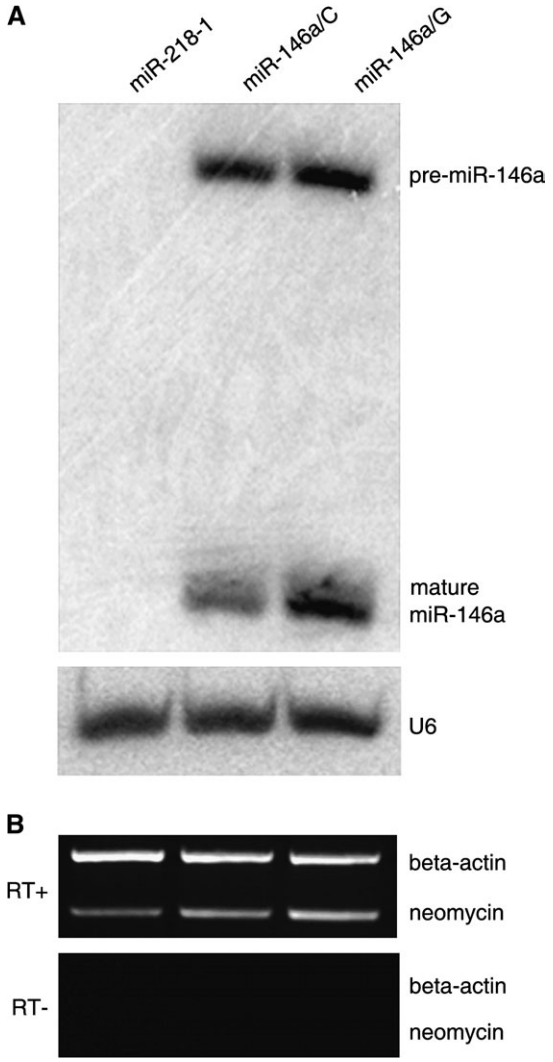

Fig. 3. Influence of *miR-146a* polymorphism on the production of mature miRNA. (**A**) Expression level of the miR-146a. Small RNAs isolated from 293T cells transfected with pc3.0-miR-218-1 (lane 1, negative control), pc3.0-miR-146C (lane 2) or pc3.0-miR-146G (lane 3) were analyzed by northern blot. The same membrane was sequentially hybridized with probes for miR-146a (upper panel) and U6 (lower panel). The level of cell endogenous U6 was used as loading control. The fragments for miR-146a precursor and mature miR-146a were indicated on the right. (**B**) Expression level of the neomycin. RNAs extracted from the same sample as Figure 3A were analyzed. Neomycin derived from the plasmid backbone was amplified by reverese transcription–PCR and used as control for transfection and harvesting efficiencies. Beta-actin was amplified in the same reaction as neomycin and served as an internal control for reverese transcription–PCR. RT+ and RT− indicate amplification with (upper panel) and without (lower panel) reverse transcriptase, respectively. The fragments representing beta-actin and neomycin were indicated on the right. The results revealed that the G-allelic miR-146a precursor expressed a higher level of mature miR-146a compared with the C-allelic one, and this result was reproducible in three independent experiments.

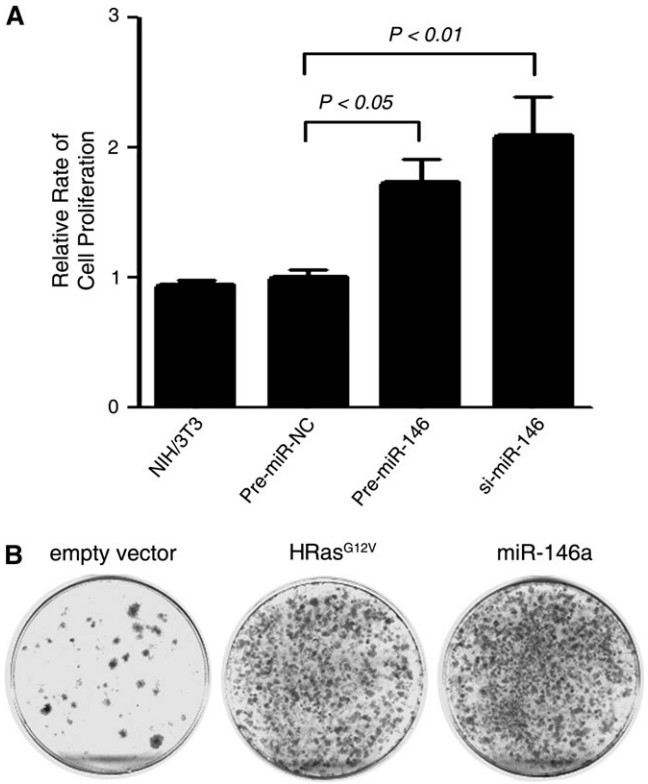

Fig. 4. Promotion of cell proliferation and colony formation by miR-146a. (**A**) Analysis of cell proliferation. Cell proliferation was evaluated by BrdU incorporation assay, which was performed 60 h after transfection with Pre-miR-NC, Pre-miR-146a or si-miR-146a, as indicated. NIH/3T3 indicates non-transfected cell. Values represent the mean ± SD of at least three independent experiments. Relative rate of cell proliferation: the mean percentage of BrdU-positive cells among the Pre-miR-NC-transfected NIH/3T3 was set as relative rate of 1. *P*-values were calculated by one-way analysis of variance. (**B**) Analysis of colony formation. NIH/3T3 cells were transfected with pcDNA3.0 (indicated as empty vector, used as negative control), pc3.0-HRas^{G12V} (indicated as HRas^{G12V}, used as positive control) and pc3.0-miR-146G (indicated as miR-146a). The results revealed that miR-146a promoted colony formation in a similar extent as oncogenic HRas^{G12V}, and this result was reproducible in three independent experiments.

proliferation rates were observed in the cells transfected with miR-146a duplex from either Ambion (Pre-miR-146a) or GenePharma (si-miR-146a, Figure 4A). These results suggest that miR-146a may possess the capability of promoting cell proliferation.

To confirm the above finding, we further examine whether miR-146a can promote colony formation of NIH/3T3 cells. Cells were transiently transfected with an expression vector containing a 386 bp DNA fragment encompassing the mature miR-146a sequence and its 5′ and 3′ flanking regions (179 and 185 bp, respectively). The pc3.0-HRas^{G12V}, which expressed an oncogenic HRas protein (27), was employed as a positive control, whereas the pcDNA3.0 empty vector was used as a negative control. We found that overexpression of miR-146a substantially promoted colony formation in NIH/3T3 cells. Furthermore, the size and number of colonies derived from miR-146a-transfected cells were similar to those of HRas^{G12V}-transfected ones (Figure 4B). Interestingly, the cells overexpressing G-allelic-miR-146a precursor did not display obvious difference from those transfected with C-allelic precursor in their ability of forming colonies (data not shown).

Taken together, our results suggest that miR-146a can promote cell proliferation as well as colony formation.

Discussion

Although the association between the SNPs in protein-coding genes and the risk of cancer has been investigated extensively, very few cancer association studies concerning the SNPs in miRNA genes have been reported. Here, we analyzed the influence of *miR-146a* polymorphism on individual susceptibility to HCC and its underlying mechanisms and found that male individuals with GG genotype of *miR-146a* gene were in increased risk for HCC compared with those carrying CC genotype. Furthermore, we showed that GG genotype

conferred a higher expression level of mature miR-146a by *in vitro* cell model. In combination with our finding that miR-146a can promote cell proliferation, we speculate that individuals with GG genotype of *miR-146a* gene may have increased level of mature miR-146a and are therefore more susceptible to carcinogens that promote cell proliferation. This contention is supported by the report that cells with GG genotype display higher level of endogenous miR-146a (3.9-fold), compared with those harboring CC genotype (28). Meanwhile, the functions of miR-146a target genes also support our hypothesis. Two potential targets of miR-146a, including tumor necrosis factor receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1 (29), are key adapter molecules downstream of the Toll-like and cytokine receptors in the signaling pathways that play crucial roles in cell growth and immune recognition.

While this manuscript was in preparation, Jazdzewski *et al.* (28) reported that the GC genotype of *miR-146a* was associated with an increased risk for papillary thyroid carcinoma when compared with both homozygous genotypes, which differs from our finding that individuals with GG genotype predisposed to HCC. This discrepancy may reflect the difference of the etiological factors for papillary thyroid carcinoma and HCC.

Our results showed that GG genotype of *miR-146a* gene was associated with the risk for HCC in male individuals. However, the genotype distributions did not display significant difference between cases and controls in the female cohort ($P = 0.818$). Furthermore, analysis using multivariate unconditional logistic regression revealed that neither GG nor GC genotype conferred increased risk for HCC in females, compared with CC genotype ($P = 0.586$ and 0.951, respectively). The observed gender difference may result from the interaction between the polymorphism and the sexual hormones during carcinogenesis, which has been exemplified by the case of *MDM2* SNP309 (30). On the other hand, the number of female HCC cases in our study cohort is too few (only 46 cases) to get solid conclusion. Therefore, the correlation between the *miR-146a* polymorphism and the risk for HCC in females requires further investigation in a larger cohort in the future.

Our finding that G to C variation in the miR-146a precursor results in reduced production of mature miRNA is consistent with the report by Jazdzewski *et al.* (28). The effect of this polymorphism on the expression of mature miR-146a may be stemmed from the change of G:U pair to C:U mismatch in its stem region of the precursor. This hypothesis is supported by the evidences from other studies (15,31). For example, the expression of mature miR-125a is decreased when a G:C match is replaced by a U:C mismatch in the stem region (15). Furthermore, introduction of artificial mutations to the stems of miR-30 and miR-21 precursors revealed that large bulges in the stem region were detrimental to the production of miRNA (31). These results suggest that sequence variations disturbing the secondary structure of miRNA precursor may affect the maturation process of miRNA.

Interestingly, we found that the G- and C-allelic-miR-146a precursor did not display obvious difference in their ability to promote colony formation in non-transformed cell line. This observation is understandable because the amount of mature miR-146a produced from overexpression of the C-allelic-miR-146a precursors may be high enough for promoting colony formation, even though the C-allelic-miR-146a precursors produced less mature miR-146a compared with the G-allelic one. On the other word, the overexpression strategy used in this assay may not allow closely control of the mature miR-146a level that is required for detecting the different capacity of G- and C-allelic-transfectants in colony formation.

Our findings underscore the significance of natural genetic variations in miRNA genes and warrant further investigations on the association of miRNA polymorphisms with human diseases.

Supplementary material

Supplementary Table I can be found at http://carcin.oxfordjournals.org/

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