MicroRNA-144 promotes cell proliferation, migration and invasion in nasopharyngeal carcinoma through repression of PTEN

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Abbreviations:
- EBV, Epstein–Barr virus
- FBS, fetal bovine serum
- miR-144, microRNA-144
- NPC, nasopharyngeal carcinoma
- PTEN, phosphatase and tensin homolog
- qPCR, quantitative PCR
- RIP, ribonucleoprotein immunoprecipitation
- RISC, RNA-induced silencing complex
- siRNA, small interfering RNA

Introduction

MicroRNAs (miRNAs) are a diverse class of 20–24-nucleotide non-coding RNAs. Initially discovered in 1993 (1), miRNAs have been shown to play important roles in multiple cellular processes as post-transcriptional regulators (2) and specifically have been shown to influence in cancer development and progression (3,4). Aberrant patterns of miRNA expression have been observed in multiple cancer types. Moreover, functional aberrant expression of miRNA can affect cancer cell proliferation (5), apoptosis (3), metastasis (6), chemosensitivity and radiation sensitivity (7) and could even define the cancer stem cell phenotype potentially (8).

Nasopharyngeal carcinoma (NPC) is a characteristic type of head and neck cancer, which is frequent in Southern China, with an incidence of 15–25 per 100 000 people (9). Genetic susceptibility, environmental factors and Epstein–Barr virus (EBV) latent infection, the three key etiological factors of NPC, were well established. However, the complicated molecular mechanism of NPC development and progression is not yet fully understood (10). The carcinogenesis of NPC is proposed to be a multistep process. One of the major events is inactivation of tumor suppressor genes (11). Different from other head and neck cancers, tumor suppressor genes mutation and deletion in NPC is uncommon. Conversely, downregulation of tumor suppressor genes expression by miRNA is increasingly recognized as an important mechanism of nasopharyngeal tumorigenesis (12–16).

In order to understand the function of miRNA in human NPC, we have conducted a miRNA profiling in previous study (17), wherein 50 miRNAs were found to be upregulated more than 2-fold in NPC compared with normal nasopharyngeal tissue. Notably, the human microRNA-144 (miR-144) was the most overexpressed miRNA among the 50 upregulated miRNAs. miR-144 has been originally identified as an erythroid-specific miRNA, which is required for subsequent survival and maturation of the erythroid lineage (18,19). In addition, it can increase anemia severity and decrease glutathione regeneration and antioxidant capacity by directly regulating a central regulator of cellular response to oxidative stress (20). In the field of tumor study, a comprehensive meta-analysis of miRNA expression microarray data sets revealed that miR-144 was downregulated in hepatocellular carcinoma, lung cancer and prostate cancer (21). However, another study showed that it can increase cell growth in Hela cells (22), indicating that miR-144 is probably a novel human cancer-related miRNA. To date, there is no evidence that miR-144 has a functional role in NPC tumorigenesis. In this study, we have characterized miR-144 in NPC and found upregulation of miR-144 promotes malignant progression of NPC cells by targeting crucial tumor suppressor gene phosphatase and tensin homolog (PTEN).

Materials and methods

Clinical specimens and NPC cell lines

Matched tumor specimens and non-tumor nasopharyngeal epithelial tissues from 16 confirmed NPC patients (age: 44–77 years; female: n = 4; male: n = 12) were obtained from Queen Mary Hospital in 2004 (Hong Kong). Tissue specimens were obtained by fiber optic nasopharyngoscopy directly on the tumor growth site and on the adjacent side with observed normal mucosal morphology. Informed consent was obtained from the patients involved, and ethical approval for this study was obtained from the hospital institutional review board at the University of Hong Kong. EBV-positive NPC cell line C666-1 was derived from NPC tissue harbored latent EBV infection (23,24), whereas EBV-negative NPC cell lines CNE2 and SUNE1 were derived from poorly differentiated NPC (25). NP460 is an immortalized nasopharyngeal epithelial cell line (26). CNE2, SUNE1 and C666-1 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). NP460 cells were cultured in defined keratinocyte serum-free medium. 293TN cells were cultured in Dulbecco’s modified Eagle’s medium.

Transfections of NPC cell lines

Lentiviral constructs expressing anti-miR-144 (miRZip™ lentiviral-based microRNA inhibition; System Biosciences) or pre-miR-144 (Lenti-miRTM microRNA precursor clone collection; System Biosciences) were packaged using the pPACKH1 lentivector packaging kit (System Biosciences) in 293TN cells. Anti-miR-144-expressing lentivirus was used to transfect NPC cells CNE2 and C666-1 to establish miR-144-repressed cells [CNE2/miR-144(−)], whereas pre-miR-144-expressing lentivirus was used to transfect miR-144-repressed NPC cells to establish miR-144-restored cells [CNE2/miR-144(+)]. The NPC cells transfected with empty vector [CNE2/C666-Vec or CNE2/C666(−)/−Vec) were used as control. Otherwise, we transiently transfected constructs expressing pre-miR-144 or empty vector into nasopharyngeal epithelial cells NP460 [NP460/144(+)/− or NP460-Vec] and small interfering RNA (siRNA)-PTEN or scrambled siRNA (siNC) into CNE2-144(−) cells [144(−)/siP(1)/siP(2) or 144(22)-siNC]. Lipofectamine 2000 (Invitrogen) was used for all the transient transfection experiments according to the manufacturer’s instructions.
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RNA isolation, reverse transcription and quantitative real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and reversely transcribed using miRCURY LNA Universal microRNA PCR cDNA Synthesis Kit (System Biosciences). To quantitate miR-144 expression, quantitative PCR (qPCR) was performed using miRCURY LNA Universal RT microRNA PCR SYBR Green master mix (System Biosciences). Moreover, in order to measure the miRNA levels of PTEN, total RNA was reversely transcribed using Transcript High Fidelity cDNA Synthesis Kit (Roche) and qPCR was performed using SYBR Green PCR master mix (Applied Biosystems) on an ABI 7900HT System. The primers were listed in Supplementary Table S1, available at Carcinogenesis Online. SNORD48 or β-actin was used as an endogenous control. All samples were normalized to endogenous controls and fold changes were calculated by relative quantification (27).

Tumorigenic function of miR-144

For cell proliferation assay, 1×10^3 cells were plated in 96-well plates and the cell growth rate was detected using XTT cell proliferation kit II (Roche) according to the manufacturer’s instruction. Triplicate independent experiments were done. For foci formation assay, 800 cells were seeded onto 6-well plates. After 10 days culture, surviving colonies (>50 cells/colony) were counted with 1% crystal violet staining. Colony formation in soft agar was carried out by growing 5×10^4 cells in 0.4% bactoagar on a bottom layer of solidified 0.6% bactoagar in 6-well plates. After 3 weeks, colonies consisted of >50 cells were counted and expressed as the means ± SD of triplicate within the same experiment.

Tumor formation in nude mice

In vivo tumorigenic ability of miR-144 was investigated by tumor xenograft experiment. Female BALB/c nude mice aged 4–5 weeks were purchased from The Laboratory Animal Unit of the University of Hong Kong. Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of the University of Hong Kong. Briefly, empty vector or anti-miR-144-transfected CNE2 or C666-1 cells were injected subcutaneously into the dorsal flank of nude mice. Each group contained five mice. Tumor size was measured every 3 days. After 4 weeks, mice were killed and tumors were dissected. Tumor volumes were calculated as follows: volume = (D × d^2)/2, where D is the longest diameter and d is the shortest diameter.

Cell-cycle analysis

To analyze cell-cycle distribution, cells were cultured in medium containing 10% FBS in 6-well plates. Serum was withdrawn when cells were 70% confluent. After 72h, 10% FBS was added in the medium for an additional 12h. Cells were fixed in 70% ethanol, stained with propidium iodide, and DNA content was analyzed by Cytomics FC (Beckman Coulter).

Cell migration and invasion assays

For wound-healing migration assay, cells were cultured in 24-well plates until confluence. The culture medium was replaced by serum-free medium 24h before wound creation. A yellow pipette tip was used to create wounds at intervals of approximately 1.5 mm. After wounding, the medium was changed to fresh serum-free medium to remove cellular debris. Serial photographs were obtained at 0, 24 and 72h. Invasion of cells was assessed using the Matrigel Invasion Chamber (BD Biosciences). Cells (1×10^5) were seeded on trans-well chambers with Matrigel in medium without FBS. Medium containing 10% FBS in the lower chamber served as the chemoattractant. The invasive cells attached to the lower surface of the membrane insert were fixed and stained, then counted under a microscope.

Luciferase assay

A 246-bp fragment of PTEN 3'UTR containing the binding site of miR-144 was amplified by PCR (Supplementary Table S1, available at Carcinogenesis Online) and cloned into pGL3 Basic vector (Promega). Reverse sense of the fragments was also cloned by reversing the sequences of the 246-bp fragment. pGL3-control vector (Promega) was used as a positive control. CNE2/C666-Vac and CNE2/C666-144(−) were transfected with the reporter constructs containing either the target binding site of PTEN or reversed sequence of PTEN (antisense). Luciferase activity was measured after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Ribonucleoprotein immunoprecipitation assay

Ribonucleoprotein immunoprecipitation (RIP) was performed using the RiboCluster Profiler RIP-Assay Kit (MBL) following the manufacturer’s protocol. Briefly, miR-144-repressed cells [CNE2/144(−)] and control cells (CNE2-Vac) were lysed in complete RIP lysis buffer and precleared by protein G agarose beads, after which 500 μl of precleared cell lysate was incubated with RIP buffer containing agrose beads coated with human anti-Agornate 2 (Ago2) antibody (MBL, Code No. RN003M) or isotype control normal mouse IgG (MBL, Code No. M076-3) and rotated for 3 h at 4°C. Subsequently, the immune complexes were washed three times with samples (10 μl) were analyzed by western blotting to check immunoprecipitation efficiency. The remaining samples were used for RNA isolation. cDNA was synthesized using Transcript High Fidelity cDNA Synthesis Kit (Roche) and quantitative real-time PCR was performed to determine PTEN enrichment in the RNA-induced silencing complex (RISC); the average value of RISC-associated GAPDH, B2M and GUSB was used for normalization (Supplementary Table S1, available at Carcinogenesis Online), as described (28).

RNA interference

siRNA (20 μM) against TAT (Ambion) was transfected into cells in 6-well plates using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. At 48h after transfection, the effects of gene silencing were measured via RT–PCR and western blot analysis.

Western blot analysis

Western blot analysis was performed with the standard method with antibodies to rabbit-anti-PTEN, pAkt (Ser473), total Akt and cyclin D1 (Cell Signaling Technology), as well as mouse anti-E-cadherin (Cell Signaling Technology) and β-actin (Abcam). β-Actin was used as a protein-loading control.

Statistical analysis

SPSS 13.0 software was used for statistical analysis. Data were presented as means ± SD of at least 3 independent experiments. Two-tailed Student’s t test was used for comparisons of two independent groups. The relationship between PTEN and miR-144 expression was explored by Spearman’s correlation. P values of <0.05 were considered statistically significant.

Results

miR-144 was upregulated in NPC clinical specimens and human NPC cell lines

The expression level of miR-144 was initially evaluated in 16 pairs of NPC specimens and their matched non-tumor nasopharyngeal epithelial tissues by qPCR. The results showed that the average expression level of miR-144 was significantly higher in NPC specimens than in their normal counterparts (Figure 1A; P < 0.001). Next, we examined the expression level of miR-144 in three human NPC cell lines: C666-1 (EBV positive), CNE2 (EBV negative) and SUNE1 (EBV negative). Consistent with the data obtained from NPC clinical specimens, miR-144 was upregulated in all three NPC cell lines compared with the immortalized nasopharyngeal epithelium cell line NP460 (Figure 1B). Taken together, the initial findings indicate that upregulation of miR-144 may play an important role in NPC tumorigenesis.

miR-144 promotes NPC cell growth in vitro and in vivo

To assess the biological function of miR-144, CNE2 and C666-1 cells were stably transfected with anti-miR-144 [CNE2/C666-144(−)] by lentiviral infection. Empty vector transfected cells (CNE2/C666-Vac) were used as controls (Figure 2A). Furthermore, miR-144 expression in stable anti-miR-144-expressing transfecteds was restored by transfection of miR-144 mimic [CNE2/C666-144(+)/144(+) (Figure 2A). Also, NP460 without endogenous miR-144 expression were transiently transfected with pre-miR-144 and empty vector to generate the NP460-144(+) and NP460-Vec cells, respectively (Figure 2B).

To explore the effect of miR-144 on cell growth, we performed a series of biological experiments in vitro, including XTT, foci formation and soft agar assay. In Figure 3A, XTT results showed that anti-miR-144 significantly inhibited cell growth rates in CNE2-144(−) and C666-144(−) cells, whereas pre-miR-144 significantly promoted cell growth rates in NP460-144(+) cells. In addition, after restoring miR-144 in anti-miR-144-infected NPC cells [CNE2/C666-144(−)/144(+)], the cell growth rates were nearly restored. In contrast, the CNE2/C666-Vac or CNE2/C666-144(−)/Vec control cells showed no effect on cell growth, indicating that the role of miR-144 in cell growth promotion was highly specific. Foci formation and soft agar assays also showed that anti-miR-144-infected cells [CNE2/C666-144(−)] displayed much fewer and smaller colonies compared with antimiumpyt vector infected cells (CNE2/C666-Vac). However, when
we restored the miR-144 expression in miR-144 knockdown cells [CNE2/C666-144(−)/144(+)], the pre-miR-144-restored cells showed increased clonogenicity compared with pre-empty vector treated miR-144 knockdown cells [CNE2/C666-144(−)/Vec] (Figure 3B and C).

In vivo tumor formation assay was performed by subcutaneous injection of CNE2/C666-144(−) cells into nude mice, whereas CNE2/C666-Vec cells were used as controls. Within 4 weeks, solid tumors were readily visible in left hind legs of all 10 mice (injected with CNE2/C666-Vec cells), but only observed in 4/5 mice injected with CNE2-144(−) or C666-144(−) cells, respectively. As compared with controls, the average tumor volume of the anti-miR-144-treated group was markedly reduced by more than 75% (Figure 3D, P < 0.01).

miR-144 enhances NPC cell migration and invasion

Given the functional annotation term enrichments of miR-144-repressed targets having functional attribution to migration, we next assessed the potential role of miR-144 in NPC cell migration by wound-healing assay. The results showed that miR-144 depletion was able to significantly inhibit cell motility in NPC cells (Figure 4A). To quantify the effect observed in the wound-healing assay, we further measured cell migration as well as invasion using trans-well assays in miR-144-depleted and miR-144-restored cells. Statistically significant decreases were observed in the CNE2/C666-144(−) cells compared with their control counterparts. In contrast, restoring miR-144 expression significantly increased cell migration, confirming the migration and invasion enhancer function of miR-144 (Figure 4B).

miR-144 directly targets PTEN in NPC cells

The above phenotypic data thus far indicate that miR-144 has a strong tumorigenic ability for NPC. To identify the potential mRNA targets of miR-144, which could contribute to its tumorigenic functions, we used online bioinformatics algorithms (miRbase, Pictar and Targetscan) to predict the mRNA targets of miR-144. A total of 430 targets were predicted by any two of the three bioinformatics tools.
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PTEN was identified as one of the overlapped candidate effectors of miR-144 based on the putative target sequence on 2906–2925 bp of PTEN 3′-UTR.

To verify the in silico prediction, we first evaluated the expression level of PTEN and miR-144 in 13 pairs of clinical NPC specimens by qPCR. The result revealed that the expression of miR-144 was upregulated and mRNA expression of PTEN was downregulated in 10 NPC specimens. In contrast, two NPC tissues with low expression of miR-144 observed high level of PTEN. Only one case showed both overexpression of PTEN and miR-144 (Figure 5A). Furthermore,
Spearman’s correlation analysis demonstrated that miR-144 was significantly inversely correlated with PTEN in NPC specimens (Figure 5B, \( r = -0.5029; P < 0.01 \)). A similar inverted correlation was also observed in all three NPC cell lines (Figure 5C), indicating mRNA expression of PTEN could be repressed by miR-144 in NPC. Next, we evaluated whether miR-144 could negatively regulate PTEN expression in miR-144 knockdown [CNE2/C666-144(−)] and knock-in [CNE2/C666-144(−)/144(+) or NP460-144(+)] cells. As shown in Figure 5D, PTEN mRNA expression was increased in miR-144 knockdown NPC cells but decreased to control level when restoring miR-144 in miR-144 knockdown cells. Western blotting further confirmed the changes of PTEN protein expression in these cells (Figure 5E). Moreover, a luciferase reporter assay was performed to determine whether miR-144 had an effect on the 3′-UTR of PTEN in NPC cells. The target sequence of PTEN 3′-UTR (sense) or the reverse sequence (antisense) was cloned into a luciferase reporter vector (Figure 6A). miR-144 knockdown cells CNE2-144(−) and their control counterpart CNE2-Vec were then transfected with sense or antisense 3′-UTR vector. The results showed that sense 3′-UTR vector in CNE2-144(−) led to a 1.8-fold increase of luciferase activity when compared with control cells. However, the activity of antisense 3′-UTR vector was unaffected. Similar results were observed in the C666-144(−) cells (Figure 6B).

Next, we demonstrated the direct binding of miR-144 to endogenous
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PTEN by immunoprecipitating Ago2, a core component of RISC. Western blotting of immunoprecipitated samples showed that Ago2 was specifically isolated with the anti-Ago2 antibody, but not with mouse IgG, in both miR-144 knockdown cells CNE2-144(−) and control cells CNE2-Vec (Figure 6C). In addition, PTEN transcripts were detected by real-time PCR using specific primers. Although the level of Ago2 proteins immunoprecipitated with anti-Ago2 antibody was nearly the same from CNE2-144(−) and CNE2-Vec lysates, the enrichment of PTEN transcripts significantly decreased in miR-144-attenuated cells (Figure 6D), strongly suggesting that PTEN was indeed a bona fide target of miR-144 in NPC cells.

PTEN was involved in miR-144-induced proliferation, migration and invasion in NPC cells

To test whether downregulation of PTEN might function in miR-144-induced NPC cell proliferation, migration and invasion, we inhibited PTEN expression with siRNA in miR-144 depletion cells (Supplementary Figure S1A and B, available at Carcinogenesis Online). The XTT and foci formation results revealed that both PTEN and miR-144-depleted cells showed increased proliferation compared with those cells with only depleted miR-144 and phenocopied the proliferation promoting effect of miR-144 (Supplementary Figure S1C and D, available at Carcinogenesis Online). Moreover, both wound-healing and trans-well assays showed that PTEN silencing could significantly enhance migration and invasion in miR-144-depleted cells (Supplementary Figure S1E and F, available at Carcinogenesis Online). Collectively, these results proved that miR-144 promotes tumorigenicity of NPC cells via directly targeting PTEN.

miR-144 promotes NPC cell proliferation, migration and invasion mainly via the PI3K/Akt signaling pathway

Following observation of miR-144-mediated growth promotion, we detected cell-cycle distribution in miR-144 knockdown and knock-in cells. Compared with CNE2-Vec control cells, CNE2-144(−) and/or CNE2-144(−)/Vec cells displayed an increased percentage of cells in G1 phase and reduced percentage of cells in S phase, whereas miR-144-restored cells [CNE2-144(−)/144(+)] showed no difference in cell-cycle distribution. These results suggested that the growth-suppressive effect of anti-miR-144 was partly due to a G1-phase arrest (Figure 7A).

To further explore the mechanism underlying the observed cellular phenotypic changes, pAkt, a centrally important downstream effector of PTEN was evaluated by western blotting (29). The results showed that suppression of miR-144 reduced pAkt expression, whereas restoring miR-144 rescued the expression of pAkt, suggesting that miR-144 could regulate pAkt via PTEN in NPC cells. Moreover, we examined two well-known downstream effectors of PTEN-Akt, cyclin D1 and E-cadherin. We found that cyclin D1 was profoundly decreased in miR-144 suppressing cells, but increased in miR-144 restoring cells. In contrast, E-cadherin was significantly increased in miR-144 suppressing cells, but decreased in miR-144 restoring cells (Figure 7B). Taken together, our findings strongly suggest that miR-144 promote proliferation, migration and invasion via the PTEN/PI3K/Akt/cyclin D1 or E-cadherin signaling pathway (Figure 7C).

Discussion

Knowledge about the precise molecular mechanisms underlying NPC tumorigenesis is crucial in the development of better therapeutic strategy for NPC patients. In the past several years, studies about molecular mechanisms of NPC tumorigenesis were mainly focused on the oncogenes or tumor suppressor genes. With the development of functional genomics, gene’s expression regulation was gradually extended from single gene linear regulation to multiple genes, gene clusters, even the whole genomic regulation networks. Among the various regulation and control factors of mRNA translation, miRNA was increasingly
taken into account. It has been put forth that oncomirs as the new direction in cancer studies. Our preliminary study of NPC miRNA expression profiles has shown that miR-144 was the most upregulated miRNA in NPC tissues when compared with their non-tumor counterparts (17). Moreover, miR-144 is located at chromosome 17q11.2, which is a frequently amplified region in NPC (30). Previous studies have reported that miR-144 was associated with human cancers. As early as 2005, a study showed that inhibition of miR-144 caused a decrease in cell growth of HeLa cells (22). Two years later, another study reported that introduction of miR-144 affected the activation of caspase cascade in human breast cancer cells (31). Recently, a comprehensive meta-analysis of miRNA expression microarray data sets revealed that miR-144 was downregulated in hepatocellular carcinoma, lung cancer and prostate cancer (21). In addition, miR-144 was perceived to inhibit colorectal cancer tumor growth through a Notch-1-dependent mechanism (32). These controversial results suggested that the role of miR-144 was possibly tumor specific and highly dependent on its targets in different cancer cells. However, no information about the function or molecular mechanism of miR-144 in NPC has been reported.

In this study, we found that miR-144 is frequently upregulated in NPC tissues and all three NPC cell lines (Figure 1). We then selected two undifferentiated cell lines C666-1 (EBV positive) and CNE2 (EBV negative), which exhibited similar clinical phenotypes of NPC, as the most suitable model for further characterization of miR-144 (Figure 2). The tumorigenic function of miR-144 demonstrated by both in vitro and in vivo assays further supports its role as an oncomir in the development and progression of NPC. The results showed that knockdown of miR-144 could inhibit cell proliferation, decrease foci formation, suppress cell mobility and invasion and inhibit tumorigenesis in nude mice. However, after restoring miR-144 in NPC cells, all the tumor suppressive functions exhibited by anti-miR-144 could be nearly rescued (Figures 3 and 4).

To better understand the underlying mechanisms of miR-144-induced NPC cell growth, migration and invasion, we identified PTEN as a potential target gene of miR-144 using bioinformatics method. PTEN is a phosphoinositide phosphatase, which is originally identified as a multifunctional tumor suppressor frequently loss in various human cancers (33–36). It functions as a negative regulator of the PI3K/Akt pathway via dephosphorylation of PI(3,4,5)P3, ultimately participating in regulation of the cell cycle, proliferation, apoptosis, cell adhesion, migration, invasion and metastasis during cancer progression (29). It has been reported that PTEN was downregulated in NPC patients by immunohistochemistry assay, making it partially responsible for the upregulation of the PI3K/Akt pathway in NPC (37,38). Interestingly, accumulating evidence revealed that downregulated PTEN, rather than PTEN mutations or deletions, represents a critical factor in the development and progression of NPC (39). Downregulation of PTEN could be caused by epigenetic alterations to the PTEN genome (i.e. promoter hypermethylation). However, PTEN hypermethylation has been demonstrated in laryngeal and thyroid cancer but not in NPC (40,41).

PTEN has also been shown to be a direct target of miR-26a in high-grade glioma (42), miR-22 and the miR-106b~25 cluster in prostate tumorigenesis (43), miR-155 in hepatic carcinogenesis (44), miR-214 in ovarian cancer and miR-21 in multiple cancers (45–47). These studies revealed that multiple miRNAs probably contribute to the loss of PTEN expression in specific types of cancers; however, whether miR-144 can directly target PTEN in NPC is still unknown. Here, our findings demonstrated that miR-144 was significantly inversely correlated
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MicroRNA-144 promotes NPC with PTEN mRNA and protein level in clinical NPC specimens as well as in NPC cell lines (Figure 5). We subsequently confirmed that PTEN was a direct target of miR-144 in NPC cells by luciferase assay and RIP assay (Figure 6). In addition, we found that knockdown of PTEN in NPC cells with miR-144 repression could promote cell growth, migration and invasion, which was similar to the functional phenotypes induced by miR-144 restoration (Supplementary Figure S1, available at Carcinogenesis Online). These results are in line with a study showing that miR-144 targeted PTEN in mouse breast carcinoma cell line 4T1 (48). Downregulation of PTEN has been reported to be able to activate the PI3K/Akt pathway in NPC (49). With the development of new technologies in miRNA studies, more novel miRNAs targeting the PI3K/Akt signaling pathway have been disclosed, contributing to the new knowledge about the fundamental mechanisms regulating this pathway. Our present study indeed showed that miR-144 exerts a drastic tumorigenic effect on NPC cells.

Fig. 7. miR-144 promotes NPC cell proliferation, migration and invasion mainly via activating the PI3K/Akt signaling pathway. (A) Representative charts for cell-cycle distribution in anti-miR-144-repressed [144(−)] and pre-miR-144-restored [144(−)/144(+)] CNE2 cells. CNE2-Vec or CNE2-144(−)/Vec cells were used as control. (B) Western blotting for p-Akt (Ser473), total Akt, cyclin D1, E-cadherin and loading control β-actin in CNE2 [CNE2-Vec, CNE2-144(−), CNE2-144(−)/Vec and CNE2-144(−)/144(+)], NP460 [NP460-Vec and NP460-144(+)] and CNE2-144(−) [CNE2-144(−)/siNC, CNE2-144(−)/siP1 and CNE2-144(−)/siP2] cells. (C) Schematic diagram of activation of PTEN-dependent PI3K/Akt signaling pathway by miR-144 in NPC. miR-144 suppressed PTEN expression to promote NPC cell proliferation by increasing p-Akt and cyclin D1, whereas to promote NPC cell migration and invasion by decreasing E-cadherin.
by directly targeting PTEN. Once inhibited, PTEN is not able to block the PI3K/Akt pathway, resulting in upregulated pAkt (Ser473) and cyclin D1, and downregulated E-cadherin in NPC cells (Figure 7). It is noteworthy that PTEN and E-cadherin can affect each others’ stability; repress E-cadherin could conversely reduce the expression of PTEN (50).

In summary, we have identified a critical oncomir, miR-144, in NPC, which was frequently overexpressed in NPC tissues and cell lines. The miR-144 played an important role in the malignant progression of NPC cells by directly targeting PTEN and positively regulating the PI3K/Akt pathway. Although miRNA-based therapeutics is still in their infancy, our findings are encouraging and miR-144 may be used as a potential therapeutic target for the treatment of patients with NPC.

Supplementary material
Supplementary Table S1 and Figure S1 can be found at http://carcin.oxfordjournals.org/

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References

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