microRNA-141 is involved in a nasopharyngeal carcinoma-related genes network

Liming Zhang1, Tan Deng1, Xiaoy Li1, Huaying Liu, Houde Zhou, Jian Ma*, Minghua Wu, Ming Zhou, Shourong Shen1, Xiaoling Li, Zhaoxia Niu, Wenling Zhang, Lei Shi, Bo Xiang, Jianhong Lu, Li Wang, Dan Li, Hailin Tang and Guiyuan Li*

Cancer Research Institute, Central South University, Changsha, Hunan 410078, China and 1Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, China

1To whom correspondence should be addressed. Tel: +86 731 84805383; Fax: +86 731 84805466; Email: ligy@xysm.net
Correspondence may also be addressed to Jian Ma. Tel: +86 731 84805383; Fax: +86 731 84805466; Email: majian@mail.csu.edu.cn

microRNAs (miRNAs) are small non-coding RNAs and have been implicated in the pathology of various diseases, including cancer. Here we report that the miRNA profiles have been changed after knockdown of one of the most important oncogene c-MYC or re-expression of a candidate tumor suppressor gene SPLUNC1 in nasopharyngeal carcinoma (NPC) cells. Both c-MYC knockdown and SPLUNC1 re-expression can down-regulate microRNA-141 (miR-141), miR-141 is up-regulated in NPC specimens in comparison with normal nasopharyngeal epithelium. Inhibition of miR-141 could affect cell cycle, apoptosis, cell growth, migration and invasion in NPC cells. We found that BRD3, UBAP1 and PTEN are potential targets of miR-141, which had been confirmed following luciferase reporter assays and western blotting. BRD3 and UBAP1 are both involved in NPC carcinogenesis as confirmed through our previous studies and PTEN is a crucial tumor suppressor in many tumor types. BRD3 is involved in the regulation of the Rb/E2F pathway. Inhibition of miR-141 could affect some important molecules in the Rb/E2F, JNK2 and AKT pathways. It is well known that carcinogenesis of NPC is involved in the networks of genetic and epigenetic alteration events. We propose that miR-141- and tumor-related genes c-MYC, SPLUNC1, BRD3, UBAP1 and PTEN may constitute a gene-miRNA network to contribute to NPC development.

Introduction

It is well known that there will be more than one or even a network of genetic or epigenetic alteration events that could have happened in polygenetic inherited cancers such as nasopharyngeal carcinoma (NPC) (1). Searching for the susceptibility genes or oncogenes that are associated with NPC has attracted numerous research attentions. We have been working on identification of NPC-related genes through genome-wide scan, linkage analysis, expression profiling and position cloning (2–6). Based on this experience and many other literature evidence, we propose that NPC carcinogenesis is a stepwise, sequential process that involves multiple cancer-related genes.

By using suppression subtractive hybridization assay, we identified that the SPLUNC1 gene is a tissue-specific gene of nasopharyngeal epithelia and is down-regulated in NPC (5,7). SPLUNC1 protein is expressed in the serous glands and epithelium of the upper respiratory tract. It is an innate immunity-defensive secretory protein and can bind to bacterial lipopolysaccharide and inhibit Pseudomonas aeruginosa and Epstein-Barr virus, which is the most important environmental factor in NPC tumorigenesis (8).

BRD7 is a potential nuclear transcription factor and belongs to a bromodomain family, which includes BRD2 and BRD3 (9). BRD7 is absent or low expressed in NPC biopsies and over-expression of it can inhibit NPC cell growth and arrest cells in the G0-G1 phase (10). BRD2 and BRD3 could interact with BRD7 and have distinct roles in regulating cell apoptosis (11). Our previous study showed that c-MYC is a negative regulator of BRD7 (12). c-MYC is over-expressed in most human cancers and executes its multiple activities mostly through transcriptional regulation of its target genes (13). Several studies indicate that over-expression of c-MYC is a frequent genetic abnormality in NPC (14,15).

UBAP1 gene is a putative NPC-related gene, which is located at chromosome 9p21-22, where loss of heterozygosity frequently occurs in NPC. UBAP1 is down-regulated in NPC specimens and contains two ubiquitin-associated domains (16–18).

MicroRNAs (miRNAs) are short (20–23 nucleotides), endogenous, single-stranded non-coding RNA molecules and fundamental regulatory elements of gene expression (19,20). miRNAs control gene expression by inhibiting protein translation or degrading cognate target miRNAs through binding to their 3′ untranslated regions (UTRs) with varying degrees of sequence complementarity. Global expression of miRNAs is seemingly deregulated in most cancer types, according to research by recent high-throughput miRNA microarray assays. The role of miRNAs in NPC carcinogenesis is poorly investigated. Sengupta et al. (21) reported that mir-29c is down-regulated in NPC and can up-regulate miRNAs encoding extracellular matrix proteins. Chen et al. (22) recently identified several classic tumor-related pathways that are targeted by dysregulated miRNAs in NPC. It becomes obvious that miRNAs, oncogenes and tumor suppressors all are members of the complex cancer pathway network.

In this study, we investigated the effect of c-MYC knockdown and SPLUNC1 re-expression in an NPC cell line on global miRNA expression profile and found some dramatic changes in miRNAs expression. One of them is microRNA-141 (miR-141), which is up-regulated in NPC. Through computational and luciferase reporter assays, we identified that BRD3, PTEN and UBAP1 are targets of miR-141. This finding suggested that miR-141 is involved in an NPC-related gene network.

Materials and Methods

Cell culture and stable transfection

5-8F cell lines (an NPC cell line with high tumorigenic and metastatic ability) were kindly provided by the Cancer Center of Sun Yet-Sen University (Guangzhou, China), 5-8F/Si-c-MYC cells, which knockdown c-MYC expression by stably expressed small interfering RNA (siRNA) targeting c-MYC, and its negative control (NC) cells (5-8F/Si-control), which is a negative mock siRNA vector, are introduced into 5-8F cells, as reported in previous literature (12). pcDNA3.1/SPLUNC1 expression vector and pcDNA3.1/control vector were stably expressed in 5-8F cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA)-mediated transfection according to the manufacturer’s protocol. G418 selection (80 μg/ml) began at 24 h post-transfection. Colonies were picked after 2 weeks and expanded into cell lines. We used SPLUNC1 antibody (23) to verify the positive clone. These two cell lines were named as 5-8F/SPLUNC1 and 5-8F/control. All of these cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. We also used the same technique to establish 5-8F cells, which can stably express BRD3.

Antibody and western blotting

Western blotting was carried out as described previously (24). Anti-β-ACTIN (A5441) was purchased from Sigma-Aldrich (St Louis, MO). Anti-CDK4

Abbreviations: 141I, microRNA 141 inhibitor; 141M, microRNA 141 mimic; INC, inhibitor negative control; miRNA, microRNA; miR-141, microRNA-141; MTT, 3(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NPC, nasopharyngeal carcinoma; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; RT, reverse transcription; siRNA, small interfering RNA; UTR, untranslated region.

1These authors contributed equally to this work.
miRNA microarray analysis
miRNA microarrays were obtained from CapitalBio Corporation (Beijing, China), corresponding to current release of Sanger miRNAs database (http://www.mirbase.org/; miBase Release 8.2). miRNAs were enriched from total RNA-extracted cells (5-8F/Li-c-MYC and 5-8F/Si-control; 5-8F/ SPLUNC1 and 5-8F/control) with mirVana miRNA Isolation Kit (Ambion, Foster City, CA) and labeled with mirVana Array Labeling Kit. Labeled miRNAs were used for hybridization on each miRNA microarray containing 509 probes in triplicate, corresponding to 434 human (containing 122 predicted miRNA sequences from a published reference), 196 rat and 261 mouse miRNAs, to determine differential expression between the cell lines. This procedure was repeated twice. The miRNA microarray from CapitalBio Corporation was a single-channel fluorescence chip; all oligonucleotide probes were labeled with Cy3 fluorescent dyes (green color). Fluorescence scanning was done using a double-channel laser scanner (LuxScan 10KA; CapitalBio). Then, the figure signals were transformed to digital signals using image analysis software (LuxScan3.0; CapitalBio). Raw data were normalized and analyzed using the Significance Analysis of Microarrays (SAM, version 2.1; Stanford University, CA) software.

Patient samples
For the miRNA expression study, we used 10 NPC tissues and 10 normal nasopharyngeal epithelium samples from biopsy-negative cases as control. Both samples were obtained from patients in the Second Xiangya Hospital and Hunan Province Tumor Hospital. All tissue samples were snap frozen in liquid nitrogen and stored at −80°C until laser-capture microdissection. We used LEICA CM 1900 for frozen section and used LEICA CTR 6500 to obtain the pure nasopharyngeal epithelial tissues. Written informed consent was obtained from all study participants. Collections and use of tissue samples were approved by the ethical review committees of the appropriate institutions.

Quantitative reverse transcription–polymerase chain reaction analysis
To confirm the miRNA microarray results, real-time reverse transcription (RT)–polymerase chain reaction (PCR) was used to detect cellular miRNAs, as described, but with minor modifications (25). The primers for RT–PCR to detect miRNAs were designed based on the miRNA sequences provided by the Sanger Center miRNA Registry and were synthesized and purified by the Shanghai Gene-Pharma Co. (Shanghai, China). The total RNAs were isolated from cells or tissues with TRIZOL reagent (Invitrogen). RT reactions were performed by means of the Script cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed on the BIO-RAD IQ™ Multicolor Real-Time PCR detection System (Bio-Rad). U6 RNA was used as an endogenous control for miRNA detection.

siRNAs transient transfection
siRNA duplex homologs in sequence with microRNA-141 mimics (141M) and microRNA-141 inhibitors (141I) were synthesized and purified by the Shanghai Gene-Pharma Co. (Shanghai, China). siRNA duplexes with non-specific sequences were used as NC as well as inhibitor negative control (INC). Hundred picomoles of different siRNAs (141M, 141I, NC and INC) were transfected separately into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Flow cytometry analysis
Cells were collected, washed twice with phosphate-buffered saline and fixed in 70% ethanol overnight. Cells were centrifuged at 1500 for 8 min, resuspended in 50 μg/ml propidium iodide (Sigma-Aldrich) in phosphate-buffered saline and immediately subjected to a FACStar flow cytometer (Beckton-Dickinson, Mountain View, CA). Appropriate settings of forward and side scatter gates were used to examine 10 000 cells per experiment. Data were analyzed with Modfit software (Verity Software House, Topsham, ME). Values were expressed as mean and error deviation of three independent experiments.

Wound-healing assay
Cells were plated and grown overnight to confluence in a six-well plate. Monolayers of cells were wounded by dragging a 10 μl pipette tip. Cells were washed to remove cellular debris and allowed to migrate for 24 h. Images were taken at 0 h and 24 h after wounding under the inverted microscope.

Transwell invasion assay
Cell invasion was measured by a Matrigel invasion chamber assay, which was performed using 6.5 mm and 8 μm pore size Transwell chambers (Corning, Corning, NY). 5-8F cells transfected with miRNA mimics or inhibitors were plated 12 h post-transfection in serum-free medium (1 × 10^5 cells per Transwell) and allowed to migrate toward a 10% fetal bovine serum gradient for 48 h. Cells that remained on the top of the filter were scrubbed off and cells that had migrated to the underside of the filter were fixed in methanol and stained with hematoxylin and eosin. Whole filters were manually counted under the inverted microscope.

Bioinformatics analysis
To find out the basic information about selected miRNAs, we chose miRBase (http://www.mirbase.org/search.shtml) for further research. To select possible targets to validate the significance of the detected miRNAs, we utilized both TargetScan 5.0 (http://www.targetscan.org/vert_70/) and PicTar (http://pictar.mdc-berlin.de/), which identifies binding sites targeted by single miRNA, as well as those that are co-regulated by several miRNAs in a coordinated manner. We used DAVID 2008 Functional Annotation Bioinformatics Microarray Analysis Tools (http://niaid.abc.ncifcrf.gov/) to classify the function of the target genes, which were predicted both from TargetScan 5.0 and PicTar. We used the Cancer Gene Census (http://www.sanger.ac.uk/genetics/CGP/Census/) and Tumor Suppressor Gene Database (http://www.cse.ufl.edu/~yy1/HTML-TSGDB/Homepage.html) to identify whether the target gene is an oncogene or a tumor suppressor gene.

3’ UTR luciferase reporter assays
The binding site of miRNAs and 3’ UTR of target genes were predicted from TargetScan 5.0 and PicTar. We synthesized two single strands of 3’ UTR of target genes that contained the binding site of miRNAs. Then two single strands of 3’ UTR of target genes that contained the binding site of miRNAs were synthesized as a mutant control. The oligonucleotides used in these studies were as follows:

3’ UTR of BRD3 (which contained the binding site of miR-141)
5’-CACTGTTGCAGGTCTGCTTCTTAAATTCAGTGTTATGATATCTTCACTGGTTTGCA-3’ and 5’-AGCTTGCAAAAAACTGGAAGATATCA-TAACACTGAAATGAGCAGACCTGCA-3’

3’ UTR of mutant BRD3 (which deleted five bases (AGTG) in the binding site of miR-141)
5’-CACTGTTGCAGGTCTGCTTCTTAAATTCAGTGTTATGATATCTTCAAACACTGGTTTGCA-3’ and 5’-AGCTTGCAAAAAACTGGAAGATATCA-TAACACTGAAATGAGGACAGACCTGCA-3’

3’ UTR of PTEN (which contained the binding site of miR-141)
5’-CACTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
5-8F cells were seeded in 24-well plates 24 h prior to transfection. The following day, 200 ng of reporter plasmid along with 10 pmol of miRNA mimic or mimic-NC were co-transfected into cells using Lipofectamine 2000. Luciferase activity was measured in cell lysates 24 h after transfection using a Luciferase Assay kit (Promega, Madison, WI). β-Galactosidase activity was measured in cell lysates by β-galactosidase Enzyme Assay System (Promega). Results are normalized against β-galactosidase activity.

Results

Changes of miRNA profile induced by c-MYC inhibition or SPLUNC1 re-expression in NPC cells

To investigate the role of c-MYC in NPC, we have established previously a stable NPC cell line 5-8F in which endogenous c-MYC was inhibited through siRNA technique (12). Figure 1a shows that endogenous c-MYC is significantly inhibited by siRNA-c-MYC stable transfection. c-MYC is a helix-loop-helix leucine zipper transcription factor that regulates an estimated 10–15% of genes in human genomes. Both c-MYC and miRNAs control the expression of globe genes. We thus sought to determine whether c-MYC is involved in NPC carcinogenesis through regulating miRNA expression in NPC cells.

An miRNA microarray capable of measuring expression of 434 human, 196 rat and 261 mouse miRNAs was used to analyze the miRNA profile changes between 5-8F/Si-control and 5-8F/Si-c-MYC cell lines (Figure 1b). Twelve human miRNAs were up-regulated and seven miRNAs were down-regulated (with fold change >3) in 5-8F/Si-c-MYC. Among those miRNAs, miR-200C and miR-141 were dramatically down-regulated with fold change >10 (supplementary Table 1 is available at Carcinogenesis Online). In order to exclude possibilities of false-positive results coming from miRNA microarray analysis, we performed quantitative RT–PCR analysis to verify the expression of miR-200C and miR-141. Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) results revealed that both miR-141 and miR-200c were down-regulated in...
the 5-8F/Si-c-MYC cells, which was in accordance with the results from miRNA microarray (Figure 1c and d).

Since the SPLUNC1 gene is a tissue-specific gene of nasopharyngeal epithelia and down-regulated in NPC, we sought to explore the downstream targets of the SPLUNC1 gene in NPC cells. We constructed an NPC cell line in which the SPLUNC1 gene was introduced into 5-8F cells through stable transfection. From 31 SPLUNC1-transfected clones and 11 mock transfection clones, we verified two positive clones S4 and S5 that expressed high levels of SPLUNC1 protein, whereas in mock transfection clones (P1-P4), endogenous SPLUNC1 was hardly to be detected (Figure 1e). We extracted total RNA from S4 and P1 and performed miRNA microarray analysis (Figure 1f). We found that 29 miRNAs were up-regulated and 25 miRNAs were down-regulated (with fold change >3) in Splunc1 re-expressed cells. Among those miRNAs, miR-202, miR-516-3P and miR-520b were greatly up-regulated, whereas miR-122a, miR-141 and miR-205 were dramatically down-regulated with fold change >10 (supplementary Table 2 is available at Carcinogenesis Online).

We also performed qRT–PCR to verify the expression of miR-141 and miR-205 and confirmed that both miR-141 and miR-205 were down-regulated in S4 cells and the differences were the same as the anticipated results from miRNA microarray (Figure 1g).

miR-141 is down-regulated through c-MYC inhibition or SPLUNC1 re-expression and up-regulated in NPC specimens

We were surprised that both c-MYC inhibition and SPLUNC1 re-expression can down-regulate miR-141 in 5-8F cells. Since c-MYC functions as an oncogene and SPLUNC1 is a candidate tumor suppressor gene in NPC, this suggests that miR-141 may be an important regulator in 5-8F cells through controlling its downstream targets. To explore the expression of miR-141 in the real world, 10 NPC specimens and 10 normal nasopharyngeal epithelium specimens were obtained to verify the miR-141 expression in these tissues by qRT–PCR. All tissue specimens were purified by laser-capture microdissection before the RNA extraction. Figure 2a showed that samples were microdissected by laser-capture microdissection system to remove the lymphocytes and interstitial cells. Figure 2b showed that the average expression levels of miR-141 are higher in 10 NPC specimens than in normal nasopharyngeal epithelium tissues ($P < 0.025$).

miR-141 plays a role as oncogene in NPC cell through affecting cell cycle, migration and invasion

To study the cellular function of miR-141 in NPC, we transfected 141M, 141I and siRNA duplexes with non-specific sequences as NC and INC into NPC cells separately. As expected, the levels of miR-141 were elevated by transfection of the mimics and decreased by transfection of the inhibitors (Figure 3a). We then performed an MTT assay to investigate whether miR-141 could affect the growth of 5-F cells. As shown in Figure 3b, at day 6, miR-141 inhibitor significantly decreased the viability of 5-8F cells, which suggested that the miR-141 level could regulate NPC cell viability. The reason 141M did not increase the viability of NPC cells is possibly due to there already being plenty of endogenous miR-141 in the 5-8F cells. We next assayed the effect of miR-141 on cell cycle regulation. Figure 3c shows that miR-141 inhibition resulted in a substantial increase in the G0–G1 phase (proportion percentage from 42% to 69%) and a reduction in the number of cells in the S phase (from 30% to 12%). Little effect was noticed for the G 2–M phase of the cell cycle. We also found that miR-141 inhibition slightly increased the apoptosis rate (Figure 3d).

In order to demonstrate the effect of miR-141 on the migration and mobility of 5-8F cells, an in vitro cell invasion assay was performed based on the principle of the Boyden chamber assay. Cells that had migrated through the Matrigel matrix were counted and the numbers are presented in Figure 3e. When treated with miR-141 inhibition, the number of 5-8F cells migrating through the Matrigel decreased significantly compared with the control group ($P < 0.05$). To provide further support on the effect of miR-141 on cell migration ability, the in vitro scratch wound-healing assay was performed. As shown in Figure 3f, 5-8F cells migrated significantly slower in miR-141

![Fig. 2.](https://academic.oup.com/carcin/article-abstract/31/4/559/2477087)
inhibition group ($P < 0.05$), whereas higher in 141M group ($P < 0.05$). Those results suggested that miR-141 is an important regulator of migration and invasion in 5-8F cells.

Bioinformatics and experiments verification of putative miR-141 targets: Brd3, Ubap1 and Pten are among them

Since miR-141 plays a role as oncogene in NPC, we sought to search the possible targets of miR-141 by using Web-based bioinformatics software. The basic information of miR-141 is collected from miRBase: Sequences database, and the possible targets are predicted by the online software TargetScan and Pictar. TargetScan predicts that 429 genes are possible targets of miR-141, whereas Pictar predicts that 465 genes are targets of miR-141. Then, we used DAVID 2008 Functional Annotation Bioinformatics Microarray Analysis Tools to classify the function of the target genes, which are predicted from both TargetScan 4.2 and Pictar. The results revealed that most (>50%) target genes of miR-141 are involved in signaling pathways such as mitogen-activated protein kinase, Wnt, JAK-STAT, tight junction, cell cycle and adherens junction, which are important for tumorigenesis (Figure 4a). We were interested to know whether tumor
suppressor genes were potentially inhibited by miR-141. From the Tumor Suppressor Gene Database, we found that some miR-141-targeted genes, such as MAP2K4, MAP3K3 and DLC1, are considered as classical tumor suppressor genes. In the list of miR-141-targeted genes, three genes attracted our attention: PTEN, BRD3 and UBAP1. PTEN inhibits PI3K activity and then acts as a vital tumor suppressor gene involving multiple biological processes such as apoptosis, metabolism, cell proliferation and cell growth (26). BRD3 is a BRD7 (an NPC candidate tumor suppressor gene cloned in our laboratory) interacting protein and participates in the apoptosis process. UBAP1 was originally cloned in our laboratory and has been found to be down-regulated in NPC tissues. As a candidate tumor suppressor gene, the regulating mechanism of UBAP1 is largely unknown.

We cloned the 3'UTRs containing the miR-141-binding sites for the PTEN, BRD3 and UBAP1 genes into a vector downstream of a firefly luciferase gene. We named them as LR-blank, LR-PTEn and LR-PTEnm (mutant construct, predicted binding site of miR-141 was deleted); LR-BRD3 and LR-BRD3m and LR-UBAP1, LR-UBAP1m (Figure 4b). 5-8F cells were transfected with those constructs with 141M or NC. The 3'UTRs of PTEN, BRD3 and UBAP1 elicited significantly decreased luciferase activities (P < 0.05) in 141M transfected cells, whereas the mutant of PTEN, BRD3 and UBAP1 (which deleted the binding sites) diminishes the regulating function of miR-141. These results verified that the effect of miR-141 is due to direct interaction with the binding sites in 3'UTR of PTEN, BRD3 and UBAP1 (Figure 4c).

Since miRNAs down-regulate a specific target by affecting mRNA translation or mRNA stability, we want to detect whether transcription with 141I could affect the PTEN, BRD3 and UBAP1 protein levels. The result demonstrated that PTEN, BRD3 and UBAP1 were up-regulated upon miR-141 inhibition (Figure 4d). We also noticed that knockdown of c-MYC could up-regulate PTEN and BRD3, whereas re-expression of SPLUNC1 could up-regulate UBAP1 expression (Figure 4d). The above results confirmed that PTEN, BRD3 and UBAP1 are direct targets of miR-141.

Figure 4. Identification of targets of miR-141. (a) With the analysis of DAVID 2008 Functional Annotation Bioinformatics Microarray Analysis Tool, the pie chart showed the percentage of predicted target genes that may involve in different pathways. (b) Schematic representation of the seven reporter constructs that were named as LR-blank, LR-141/BRD3, LR-141/BRD3m, LR-141/PTEn, LR-141/PTEnm, LR-141/UBAP1 and LR-141/UBAP1m. 'm' indicates the mutant construct that predicted the binding site of miR-141 was deleted. (c) The '200 ng' indicated that firefly luciferase vector were cotransfected with indicated material (no treatment, 10 pmol NC, 10 pmol 141M) into 5-8F cells. Luciferase activities were measured after 48 h, and β-galactosidase is used to normalize for differences in transfection efficiency. Data are confirmed in duplicate experiments. The data are mean ± SEM of separate transfections (n = 6); *P < 0.05 compared with no treatment group. (d) BRD3, PTEN and UBAP1 protein expression levels were increased upon transfection with 141I compared with control group in 5-8F cells. Knockdown of c-MYC could up-regulate PTEN and BRD3 and re-expression of SPLUNC1 could up-regulate UBAP1 expression.

Effect of miR-141 on Rb/E2F and PTEN/AKT pathway is possibly through its targets

BRD3 is one of the BRD7 interacting proteins and BRD7 can regulate Rb/E2F pathway (11). In order to know whether BRD3 may also regulate Rb/E2F pathway, BRD3 recombinant vector was transfected into 5-8F cells. Over-expression of BRD3 in 5-8F cells down-regulated some critical molecules of Rb/E2F pathway including phos-Rb, cyclin D1 and E2F3 (Figure 5a). This result suggested that BRD3 negatively regulated the Rb/E2F pathway. We next explored the role of miR-141 in Rb/E2F pathway since inhibition of miR-141 can arrest NPC cells in the G0–G1 phase. Figure 5b showed that 141M up-regulated cyclin D1, phos-Rb, E2F3 and DP2 in 5-8F cells, which indicated that miR-141 is a promoter of cell cycle in 5-8F cells. We also found that inhibition of miR-141 can up-regulate the levels of JNK2 (Figure 5c). JNK2 activation is crucial for the induction of cancer cell apoptosis (27). Since PTEN is a target of miR-141, we then checked the phos-AKT level upon miR-141 inhibition. Figure 5d shows that miR-141 inhibition down-regulated phos-AKT expression. This result confirmed that miR-141 might regulate PTEN/AKT at least partly.

Discussion

NPC is a malignancy with high occurrence in Southern China and Southeast Asia. Previous studies indicate that multiple oncogenes, including c-MYC, and multiple tumor suppressor candidates, such as SPLUNC1, BRD7 and UBAP1, are abnormal in NPC. It is very possible that there is a complex NPC-related oncogenes–tumor suppressor genes network contributing to NPC tumorigenesis. But the mechanism of how those genes interact with each other to induce NPC development is far from being fully known.

The continued presence of c-MYC was required for cancer development, whereas inactivation of c-MYC resulted in sustained
regression of tumors (28, 29). Karlsson et al. (30) found that inactivation of c-MYC alone was sufficient to cause sustained tumor regression in c-Myc-induced hematopoietic tumors. Reducing c-MYC expression in MCF-7 breast cells by RNAi could significantly inhibit tumor growth both in vitro and in vivo (31). Several studies showed that c-Myc also can regulate some miRNAs (32–34), but the mechanism of how c-MYC interacts with miRNAs that cause the tumorigenesis of NPC is not known.

In this study, we assayed whether alteration of c-MYC and SPLUNC1 could affect the miRNA–gene network in NPC cells by employing miRNA microarray. A number of miRNAs were altered upon c-MYC knockdown or SPLUNC1 re-expression (fold change >3). Among the miRNAs with fold change >10, surprisingly, we found that miR-141 was down-regulated by both c-MYC knockdown and SPLUNC1 re-expression.

We then confirmed that miR-141 is expressed significantly higher in NPC tissues compared with normal nasopharyngeal epithelial tissues by qRT–PCR. We next found that inhibition of miR-141 significantly decreases the viability of 5-8F cells and cell cycle arrest in the G0–G1 phase and decreases cell migration and invasion ability. We also noted that miR-141 inhibition slightly increased apoptosis rate of 5-8F cells. All of these results imply that miR-141 might play an important role as oncogene in NPC tumorigenesis. The role of miR-141 in tumorigenesis is controversial: it functioned as tumor suppressor in renal cell carcinoma (35) and breast cancer cell lines (36), whereas in our research, it seemed to have the opposite effect on NPC cells. With the prediction of online tools such as Pictar and TargetScan, one miRNA was often found to target both oncogenes and tumor suppressor genes (37). As an example, miR-175 and miR-20a can behave as an oncogene (38) or a tumor suppressor (33) in different cell types. A recent study showed that miR-141, which belongs to the miR-200 family, was found to be up-regulated in ovarian carcinoma (39), prostate cancer (40), adrenocorticotrophic hormone pituitary tumor (41) and cholangiocarcinoma (42) and down-regulated in renal cell carcinoma (35) and breast cancer (36). Since miR-141 was either up-regulated or down-regulated in different cancers, we could draw a conclusion that miR-141 may play different roles as an oncogene or a tumor suppressor gene in different cancer types.

miR-141 inhibition can regulate the Rb/E2F pathway, which is critical for normal cell cycle progression from G1 into S phase (Figure 5b). In the presence of extracellular growth-stimulatory signals, cyclin D and its kinase partner CDK4 form activated complex and phosphorylate RB protein or RB family members, thus inactivating RB and allowing E2F/DP transcription factor to exert their transactivation activity. This leads to transcription of a number of genes essential for DNA replication and entry into S phase (43).

Bioinformatics and experimental results confirmed that BRD3, PTEN and UBAP1 act as direct targets of miR-141. Our previous study indicated that BRD7 inhibited G1–S progression through Rb/E2F and RAS/MEK/ERK pathways (10). Here, we also showed that BRD3 also negatively regulated Rb/E2F pathway (Figure 5a).

Phosphorylation of AKT plays an important role to result in the activation of cascade of different protein targets involved in cell growth, proliferation and invasion, and promote tumorigenesis. PTEN is the most important negative regulator of AKT signaling pathway (44–47). MiR-141 might increase the AKT phosphorylation levels through the inhibition of PTEN expression (Figure 5d).

Some bioinformatics tools employed in the PROSITE database identified two tandem UBA domains within UBAP1 protein (18). UBA domain is involved in conferring target specificity to multiple enzymes of the ubiquitination system. The ubiquitin-dependent pathway has a decisive role in understanding pathological states including abnormal cellular proliferation and tumor growth.

Here we draw a hypothesis schema of the relationship between c-MYC, SPLUNC1, BRD3, UBAP1, PTEN and miR-141 as well as the possible regulatory mechanism of NPC tumor inhibition when knockdown of c-MYC or re-expression of SPLUNC1 occurs (Figure 6). miR-141 played a role as an oncogene to affect NPC cell cycle, migration and invasion by positive regulation of Rb/E2F and AKT pathway. Inhibition of miR-141 also affected the JNK2 pathway as well. BRD3, UBAP1 and PTEN are direct targets of miR-141, which are involved in tumorigenesis. The possible relationship between miR-141- and tumor-related genes c-MYC, SPLUNC1, BRD3, UBAP1 and PTEN may constitute a gene–miRNA network to contribute to NPC development. Our finding might shed light on a question: how did miRNA and genes work together in tumorigenesis? The research of miRNA may be a good method to solve the puzzle. However, to completely answer the puzzle, it is necessary to verify other

**Fig. 5.** (a) BRD3 recombinant vector was constructed and transfected into 5-8F cells. Over-expression of BRD3 in 5-8F cells down-regulated some critical molecules of Rb/E2F pathway including cyclin D1, phos-RB and E2F3. But there are no changes of DP2, CDK2 and CDK4 observed by western blotting. (b) 141M can up-regulate the protein expression levels of critical molecules of Rb/E2F pathway including cyclin D1, phos-RB, E2F3 and DP2. (c) miR-141 inhibition increased JNK2 protein expression. (d) miR-141 inhibition down-regulated phos-AKT expression in 5-8F cells.

**Fig. 6.** Hypothetical model of miR-141-related genes network in NPC carcinogenesis.
target genes and detect the expression of miRNAs in great amount of tumor tissues in the future study.

Supplementary material

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding

China National Key Scientific Technology Programs (2006CB910502); 111 Project (111-2-12); National 863 High Technology Program (2007AA02Z170); National Nature Scientific Foundation (30772481).

Conflict of Interest Statement: None declared.

References