MicroRNA-622 functions as a tumor suppressor by targeting K-Ras and enhancing the anticarcinogenic effect of resveratrol

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Aberrant expression of microRNA (miRNA) has been previously demonstrated to play an important role in a wide range of cancer types and further elucidation of its role in the mechanisms underlying tumorigenesis, anticarcinogenesis and potential chemotherapeutics is warranted. We chose the anti-benzo[a]pyrene-7,8-diol-9,10-epoxide-transformed human bronchial epithelial cell line 16HBE-T to study miRNAs involved in anticarcinogenesis. In resveratrol-treated cells, we found that miR-622 was upregulated, whereas it was downregulated in 16HBE-T cells, suggesting that miR-622 potentially acts as a tumor suppressor. Increasing the level of miR-622 by transient transfection-induced inhibition of proliferation and G0 arrest in 16HBE-T cells and the lung cancer cell line H460 as demonstrated by cell viability and cell cycle analysis. MiR-622 dramatically suppressed the ability of 16HBE-T cells to form colonies in vitro and to develop tumors in vivo. According to bioinformatics analysis, K-Ras messenger RNA was predicted as a putative miR-622 target; this was confirmed by western blot and luciferase reporter assays. Cell growth retardation was inhibited upon knockdown of K-Ras and an increase in the level of miR-622 in 16HBE-T cells. Furthermore, miR-622 inhibitor partially impaired the growth of 16HBE-T cells as demonstrated by luciferase reporter activity and K-Ras protein expression in 16HBE-T cells. In summary, miR-622 functions as a tumor suppressor by targeting K-Ras and impacting the anticancer effect of resveratrol. Therefore, miR-622 is potentially useful as a clinical therapy. MiR-622 impacts the K-Ras signal pathway and the potentially anticarcinogenic or chemotherapeutic properties warrant further investigation.

Introduction

Approximately 90% of cancer cases are due to environmental factors such as tobacco, diet and environmental pollutants (1). Benzo[a]pyrene is one of the most common carcinogenic polycyclic aromatic hydrocarbons, a very important class of environmental compounds. Benzo[a]pyrene is metabolized in cells to become anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (anti-BPDE), a molecule well known for its highly carcinogenic or tumorigenic properties. Accumulating evidence has demonstrated a link between benzo[a]pyrene exposure and the formation of lung cancer (2). The malignant transformation induced by anti-BPDE is related to the dysregulation of oncogenes and tumor suppressors. We recently demonstrated that anti-BPDE could transform normal human bronchial epithelial cell line 16HBE into malignant cells (called 16HBE-T) that exhibit the malignant properties of cancer (3,4). It has been previously reported that Ras (4), Tpx2 (5), miR-494 (6), miR-22 (7) and miR-106a (8) function as oncogenes in 16HBE transformation induced by anti-BPDE. Tumor suppressors, including tumor suppressor miRNAs, are known to act as important anticarcinogenic or chemopreventive agents inhibiting cancer development and progression.

MicroRNAs (miRNAs), ~22 nucleotide non-coding RNAs cleaved from long transcripts of primary miRNAs and precursor miRNAs, bind to complementary sequences in the 3' untranslated regions (3' UTRs) of their target messenger RNAs (miRNAs) and induce mRNA degradation or translational repression. According to current data, the human genome encodes ~1100 miRNAs (http://www.microrna.org/August 2010 release) that potentially target ~60% of mammalian genes (9,10) and are abundant in many human cell types (11). It has been previously reported that miRNAs regulate a variety of biological processes, including cellular differentiation (12), proliferation and death (13,14). Recent studies have been increasingly focused on the role of miRNAs as biomarkers and the potential for improved diagnosis, prevention, therapy and prognosis of a variety of cancers (15). Upregulated miRNAs have attracted a great deal of attention. The downregulated tumor suppressor miRNAs of the miR-34 family (14,16), the let-7 family (17–19), miR-101 (20,21) and miR-200b (22) are currently being explored as potential therapeutic agents.

The ability of natural agents, including resveratrol, to suppress carcinogenesis has attracted widespread attention in the field of cancer prevention and treatment. Numerous studies over the past decade have demonstrated the chemopreventive and chemotherapeutic potential of resveratrol (23,24). Resveratrol influences all three stages of carcinogenesis (initiation, promotion and progression) by modulating the signal transduction pathways that control cell growth, apoptosis, inflammation, angiogenesis and metastasis (25,26). It has been reported that resveratrol exhibits a protective effect against lung cancer via the transforming growth factor-β pathway (27). Recently, miRNAs have emerged as important gene regulators being critically involved in carcinogenesis and cancer chemoprevention (26,28). Recently, Tili et al. (29,30) reported that miR-663 is a tumor suppressor that impacts the anticancer activity of resveratrol. In a preliminary experiment, we found that miR-622 was highly expressed in both resveratrol-treated 16HBE-T cells and H460 lung cancer cells.

We previously observed that the members of the Ras family (H-Ras, N-Ras and K-Ras) were activated in anti-BPDE-transformed cells (4). Accumulating evidence supports the importance of K-Ras in cellular transformation, tumorigenesis and maintenance of a malignant phenotype (31–33). K-Ras has become a focal point for potential cancer therapies and numerous strategies have targeted dysregulated K-Ras, including a number of clinical trials that have demonstrated encouraging antitumor activity. K-Ras is a key putative target for miR-622 in the present study.

In this study, we found that resveratrol upregulated miR-622 expression, reduced cell viability and dramatically suppressed the ability of 16HBE-T cells to form colonies in vitro and suppressed tumor development in nude mice. The ability of resveratrol to cause growth retardation is in part dependent on miR-622 expression. MiR-622 functions as a candidate tumor suppressor miRNA in 16HBE-T cells and is involved in the anticarcinogenesis or chemotherapeutic functions underlying anti-BPDE-induced transformation. Our results indicate that miR-622 targeting of the K-Ras signal pathway holds a great potential in cancer therapy.

Materials and methods

Cell culture

The anti-BPDE-transformed human bronchial epithelial cell line 16HBE-T was established in our previous study (3). Briefly, the human bronchial epithelial cell line 16HBE (kindly provided by Dr Jun Xu from the Guangzhou Institute for Chemical Carcinogenesis, State Key Laboratory of Respiratory Disease, Guangzhou Medical University, 195 Dongfengxi Road, Guangzhou 510182, People’s Republic of China and ¹Department of Physiology, Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA

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Abbreviations: anti-BPDE, anti-benzo[a]pyrene-7,8-diol-9,10-epoxide; cdk6, cyclin-dependent kinase 6; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MEM, minimum essential medium; miRNA, microRNA; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; 3' UTR, 3' untranslated region.
Institute of Respiratory Diseases) was treated with anti-BPDE (98.3% purity, NCI Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) at a concentration of 2.0 μM for 4 days followed by 30 passages in culture. As a control, the 16HBE cells were incubated with dimethyl sulfoxide (DMSO, Sigma–Aldrich, St Louis, MO) at a concentration equivalent to that used to create the 16HBE-T cells. The cells were expanded in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Fsiijing, Hangzhou, China) at 37°C in a humidified atmosphere of 5% CO₂. The human lung cancer cell line H460, which is sensitive to anticancer treatment, was chosen to be the cancer cell line H460, which is sensitive to anticancer treatment, was chosen to equivalent to that used to create the 16HBE-T cells. The cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO₂. Resveratrol (Sigma–Aldrich) was dissolved in DMSO and the final volume of DMSO did not exceed 0.1% of the total incubation volume. 16HBE-T and H460 cells were treated with 12.5, 25 and 50 μM resveratrol for up to 48 h and were then collected to investigate the anticarcinogenic activity of resveratrol. As controls, 16HBE-T and H460 cells were treated with the same concentration of DMSO used in the resveratrol treatment groups.

Transient transfection of miRNA oligonucleotides

16HBE-T and H460 cells were transiently transfected with 50 nmol of the miR-622 mimic or miR-622 inhibitor with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendation. The antisense oligonucleotides used in these studies were the miR-622 inhibitor: 5'-GCUCCCAACCUCCAGCAAGCUU-3' and the miRNA inhibitor-negative control (NC inhibitor): 5'-CCAGUUUUGUUGAUUAGCACA-3'. The miR-622 mimics used in these studies were the miR-622 mimic: 5'-ACAGUGUCUGAGGUAAGCAGG-3' and the NC mimic: 5'-UUCUUGAAAGCCAGUCUGU-3'. The antisense oligonucleotides were purchased from GenePharma (Shanghai, China).

RNA interference

Construction of the K-Ras-specific short hairpin RNA (shRNA) expression plasmid was conducted to knockdown the expression of K-Ras. The pGPU6/GFP/Neo siRNA plasmid vector that contains the neomycin resistance gene enabling G418 selection in mammalian cells was provided by GenePharma. Four DNA targets of K-Ras were designed and synthesized (Supplementary Table 2 is available at Carcinogenesis Online). The synthetic oligonucleotides were inserted between BbsI and BamHI sites, respectively, of the pGPU6/GFP/Neo vector. The recombinant plasmids were pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, and pGPU6-sh4 and pGPU6-NC. The empty vector control (pGPU6/GFP/Neo) was termed pGPU6. The constructed plasmids were confirmed by DNA sequencing. We used G418 to select cells that stably expressed the above-mentioned shRNAs. 16HBE-T cells were seeded (10 000 cells per well) in 12-well plates and cultured to 70% confluence prior to transfection. Next, the cells were transfected with pGPU6, pGPU6-NC, pGPU6-sh1, pGPU6-sh2, pGPU6-sh3 or pGPU6-sh4 using Lipofectamine 2000. The cells that stably expressed shRNAs were selected with medium containing 500 μg/ml G418 (Invitrogen) 48 h after transfection. The individual clones were trypsinized and maintained with 500 μg/ml G418 for two additional weeks. The stably transfected cells were termed Empty Vector, M-Sh-NC, M-K-Ras-sh1, M-K-Ras-sh2, M-K-Ras-sh3 and M-K-Ras-sh4, and transfection was verified by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and western blot analysis.

Cell viability assay

16HBE-T and H460 cells were seeded (3000 cells per well) in 96-well plates and transfected with the miR-622 mimic or the miR-622 inhibitor for 48 h. Cell counting and colony assays (Dojindo, Japan) were used to measure cell viability according to the manufacturer’s instructions. Each cell type was analyzed in triplicate. To determine whether K-Ras is integral to miR-622 and cell growth, we used the cell line M-K-Ras-sh4 in which K-Ras is knocked down. M-K-Ras-sh4 cells were transfected with the miR-622 mimic or NC mimic for 48 h, then collected and examined for cell proliferation using the cell counting kit-8 assay.

Cell cycle analysis

16HBE-T and H460 cells were seeded (10 000 cells per well) in six-well plates and transfected with miR-622 or miR-622 inhibitor for 48 h. Cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4), trypsinized and collected, fixed in 75% cold ethanol, incubated with propidium iodide (Sigma–Aldrich), counted and analyzed using a FACS Calibur (Becton Dickinson, Mountain View, CA) instrument. All experiments were repeated three times.

RNA extraction and qRT-PCR

Total RNA was extracted from the cultured cells using TRIzol(r) reagent and miRNA expression was analyzed using the TaqMan(r) MicroRNA Assay (Ambion, Austin, TX) to detect mature miRNAs. Experiments were performed using an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA) with the SDS analysis software package (version 2.0.1). Applied Biosystems and the comparative method (ΔCt) were used to calculate ΔΔCt. A two-step SYBR(r) Green II qRT-PCR was used to expression level of K-Ras mRNA. Production of complementary DNA with oligo(T) primers was performed according to the protocol supplied with the PrimerScript(tm) RT Kit (TaKaRa, Tokyo, Japan). SYBR(r) Green II qRT-PCR was performed using a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) and Rotor-Gene 6000 software. A new analytical software (AAGC) of 7; Corbett Technologies. A two-step SYBR(r) Green II qRT-PCR was used to expression level of K-Ras mRNA. Production of complementary DNA with oligo(T) primers was performed according to the protocol supplied with the PrimerScript(tm) RT Kit (TaKaRa, Tokyo, Japan). SYBR(r) Green II qRT-PCR was performed using a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) and Rotor-Gene 6000 software. A new analytical software (AAGC) was used for normalization. The oligonucleotides used as PCR primers were synthesized from Invitrogen K-Ras as follows: forward, 5'-GGACTGGG-GAGGGCTTTCT-3' and reverse, 5'-GCCGTTTTGTGCTACTGTCTTCT-3' and 18S rRNA: forward, 5'-GTACCCGTTGAACCCATT-3' and reverse, 5'-CCATCCACTCGGTAGTACGG-3'. Each experiment was performed three times in triplicate.

Soft-agar colony formation assay

16HBE-T cells transfected with miR-622 mimic and NC mimic were expanded. A 1.5 ml base layer of agar (0.6% in MEM with 10% FBS; Difco, Detroit, MI) was allowed to solidify in a six-well flat-bottomed plate prior to the addition of 2 ml of cell suspension containing 1000 cells in 0.3% agar in MEM with 10% FBS. Colonies were allowed to grow for 28 days at 37°C in a humidified atmosphere of 5% CO₂ before imaging and counting with an inverted microscope (Olympus IX71, Olympus, Japan). Each experiment was performed in triplicate. To observe the effects of resveratrol on the colony formation of 16HBE-T cells after down-regulating the miR-622 levels. 16HBE-T cells transfected with miR-622 inhibitor, NC inhibitor, or cells not transfected were propagated. A 1.5 ml base layer of agar (0.6% in MEM with 10% FBS) was allowed to solidify in a six-well flat-bottomed plate prior to the addition of 2 ml of cell suspension containing 1000 cells in 0.3% agar or without 50 μM resveratrol in MEM with 10% FBS. For the 50 μM resveratrol treatment condition, 500 μl MEM with 10% FBS and 50 μM resveratrol was added to each well after the top layer of agar solidified and the medium was changed every 3 days. To determine whether the effect of miR-622 on cell colony formation occurs via K-Ras, we used the K-Ras knock-down cell line M-K-Ras-sh4. 16HBE-T cells, M-NC-sh cells and M-K-Ras-sh4 cells underwent transfection by miR-622 mimic or NC mimic, or no transfection, for 24 h and were then propagated in preparation for the soft-agar colony formation assay.

Tumor xenograft model study

The five-week-old BALB/c nude mice used to examine tumorigenesis were purchased from Medical Animal Experimental Center of Guangdong Province. All animal protocols were approved by the Animal Care and Use Committee. 16HBE-T cells transfected with the miR-622 mimic or the NC mimic were propagated and 5 × 10⁶ cells were injected subcutaneously into the bilateral inguino-abdominal flanks of seven nude mice; four mice were injected with the NC mimic on the right-hand side and the miR-622 mimic on the left-hand side and three mice were injected with the miR-622 mimic on the right-hand side and the NC mimic on the left-hand side. Tumor size was measured, and tumor volumes were estimated as:

\[ V = \frac{a \times b^2}{2}, \]

where \( V \) represents the volume of the tumor (in cubic centimeters), \( a \) represents the long diameter of the tumor (in centimeters), and \( b \) represents the short diameter of the tumor (in centimeters). For end-point experiments, tumors were removed, weighed and photographed 5 weeks after the cells were injected.

miRNA target prediction

Targetscan 5.1 (http://www.targetscan.org/), computer-based RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/mahybrid/submission.html) and RNA22 miRNA (http://chces.watson.ibm.com/mi22_targets.html) target detection programs were used to predict the miRNA targets on putative miRNAs. The DNA sequence of the 3’ UTR of the putative target miRNA was obtained from Genbank of the National Center for Biotechnology Information webpage (http://www.ncbi.nlm.nih.gov/).

Luciferase assays

For luciferase analysis, the first 24 000 bases of K-Ras 3’ UTR that contained all putative miR-622 recognition sequences were subcloned downstream of the Renilla luciferase gene in psiCHECK-2 Vector (Promega, Madison, WI). generating a psiCHECK-2-K-Ras-3’ UTR vector (termed wt-K-Ras). We also
constructed a mutant psiCHECK-2-K-Ras-3 UTR vector (termed mt-K-Ras) in which all the seed sequences were completely mutated (details in Supplementary Figure 1, available at Carcinogenesis Online). The 16HBE-T cells (2.5 × 10^5) were seeded in each well of a 48-well plate 1 day before transfection. Cells were transfected with psiCHECK-2, wt-K-Ras vector, mt-K-Ras vector together with either 50 nmol of the miR-622 mimic, miR-622 inhibitor, NC mimic or NC inhibitor using Lipofectamine 2000. After 48 h, cells were lysed and luciferase activity was measured according to the manufacturer’s instructions. All experiments were performed in triplicate and the results were normalized to the activity of the Renilla luciferase gene that is contained within the psiCHECK-2 vector as an internal control.

To observe the effects of resveratrol on luciferase activity, the 16HBE-T cells (2.5 × 10^5) were seeded in each well of a 48-well plate 1 day before transfection. Cells were transfected with psiCHECK-2, wt-K-Ras vector, mt-K-Ras vector together with either 50 nmol of the miR-622 inhibitor, NC inhibitor or blank control using Lipofectamine 2000. The untransfected cells and transfected cells were treated with 50 μM resveratrol for another 48 h and lysed to determine luciferase activity.

Western blot analysis

Cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA). Lysates were sonicated and centrifuged (12 000 g for 10 min at 4°C). Proteinaceous components of the whole cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (40 μg/lane) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). For immune detection, membranes were incubated with the specific antibodies K-Ras (Abcam, Cambridge, UK), cyclin-dependent kinase 6 (cdk6) (Santa Cruz Biotechnology, Santa Cruz, CA), c-myc (Invitrogen), GAPDH (Guangzhou Jetway Biotech, Guangzhou, China), E2F1 (Biorworld Technology, Minneapolis, MN) and β-actin (Cell Signaling Technology). The membranes were incubated with secondary antibodies Alexa Fluor® 680 goat anti-mouse IgG (Invitrogen) and IRDye® 800CW conjugated anti-rabbit IgG (Li-Cor, Lincoln, NE) diluted in 5% fat-free dried milk in PBS-T buffer (PBS containing 0.1% Tween 20). Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor).

Statistics

Values were expressed as mean ± SD from three separate experiments, unless indicated otherwise, and analyzed with SPSS software (version 17.0 for Mac OS X; SPSS, Chicago, IL). Comparisons of tumor volume and tumor weight indicated otherwise, and analyzed with SPSS software (version 17.0 for Mac OS X; SPSS, Chicago, IL). The results were analyzed using Student’s t-test (two-tailed). Unless otherwise indicated, multiple group comparisons were assessed using one-way analysis of variance. Differences were considered statistically significant at P < 0.05.

Results

Expression of miR-622 is upregulated in resveratrol treated 16HBE-T cells

The effects of different concentrations of resveratrol (12.5–50 μM) on cell viability were examined in 16HBE-T, H460 and 16HBE cells (Figure 1A). In concordance with a previous study (34), resveratrol exhibited no measurable effects on cell viability and proliferation of human normal 16HBE cells. Resveratrol induced growth inhibition of 16HBE-T and H460 cells in a dose-dependent manner and exhibited the greatest inhibition at a concentration of 50 μM over a treatment period of 48 h. We compared the miRNA expression profiles of 16HBE-T and H460 cells with and without 50 μM resveratrol over a treatment period of 48 h using The Illumina MicroRNA Profiling Assay. Among the 735 human miRNAs analyzed, 105 exhibited a significantly different expression level between resveratrol-treated and -untreated 16HBE-T cells (Supplementary Table 1a is available at Carcinogenesis Online). We selected the four most highly upregulated miRNAs for further study: mir-150, miR-622, miR-302d and miR-151. MiR-150, miR-302d and miR-151 displayed a lesser effect on proliferation of 16HBE-T cells compared with miR-622 (data not shown). Moreover, miR-622 was among the nine most highly upregulated miRNAs in resveratrol-treated H460 cells (Supplementary Table 1b is available at Carcinogenesis Online). We verified the expression levels of miR-622 using a PCR Taqman MicroRNA assay and we demonstrated that miR-622 is upregulated by 3.54 ± 0.40-fold and 2.08 ± 0.16-fold upon treatment with 50 μM resveratrol for 48 h in 16HBE-T and H460 cells, respectively (Figure 1B). Subsequently, we assessed the expression level of miR-622 using the PCR Taqman MicroRNA assay in 16HBE-T and H460 cells compared with control 16HBE cells. The expression level of miR-622 in 16HBE-T and H460 cells was ~3- and 16-fold less, respectively, compared with the control 16HBE cells (Figure 1C). These data suggest that miR-622 is upregulated by the chemopreventive agent resveratrol. Therefore, miR-622 was chosen for further study.

miR-622 inhibits proliferation of 16HBE-T cells

Although the low expression level of miR-622 in 16HBE-T and H460 cells was clearly upregulated by resveratrol, the mechanisms underlying the function of miR-622 concerning the proliferation inhibitory effect of resveratrol remained unclear. Prior to studying the effects of miR-622, we used the miR-622 mimic and miR-622 inhibitor to elevate and depress the miR-622 expression levels. The levels of miR-622 in 16HBE-T and H460 cells increased or decreased significantly following transfection with the miR-622 mimic or the miR-622 inhibitor (Supplementary Figure 2A and B is available at Carcinogenesis Online), suggesting that the oligonucleotides were introduced efficiently into 16HBE-T and H460 cells and thus enhanced or repressed the expression of miR-622.
Initially, we investigated the effects of miR-622 on the viability of 16HBE-T cells. Upregulation of the expression level of miR-622 in 16HBE-T cells induced inhibition of cell growth, which in turn reduced the average cell viability to 72.18 ± 2.01% (Figure 2A). The effect of increased miR-622 was also observed in H460 cells, in which the average cell viability was reduced to 77.13 ± 6.00% (Figure 2B). The cell cycle distribution was analyzed by flow cytometry to determine the cell cycle phase at which miR-622 exerts its growth inhibitory effect. We found that miR-622 induced cell cycle arrest at the G0 phase; 68.96 ± 1.75% of 16HBE-T cells transfected with the miR-622 mimic were in the G0 phase after 48 h transfection compared with 56.86 ± 3.24% of untransfected control cells, whereas the proportion of cells in the S phase decreased from 28.76 ± 1.15% to 18.05 ± 1.22% upon transfection with the miR-622 mimic (Figure 2C); additionally, miR-622 induced cell cycle arrest in the G0 phase in H460 cells (Figure 2D). The G0 phase cell cycle arrest induced by exogenous expression of miR-622 in 16HBE-T cells can be seen in Figure 2E.

Because the original level of miR-622 in 16HBE-T and H460 cells was very low, use of miR-622 inhibitor did not affect cell proliferation or cell cycle progression (data not shown). MiR-622 exhibited no noticeable effect on growth of normal human bronchial epithelial 16HBE cells as demonstrated by the cell viability assay and cell cycle analysis (data not shown); this is potentially because the original level of miR-622 in 16HBE cells was higher, causing a saturation of targets in 16HBE cells.

MiR-622 suppresses colony formation in vitro and suppresses tumorigenicity in vivo

Because resveratrol induces miR-622 expression, and exogenous expression of miR-622 inhibits proliferation of 16HBE-T cells, we decided to explore the potential effect of miR-622 on tumorigenesis. Using miR-622 mimic, we assessed the colony formation ability of 16HBE-T cells. Cells transfected with the miR-622 mimic exhibited fewer and smaller colonies compared with the NC mimic or untransfected groups (Figure 3A). We further examined the effect of miR-622 on tumorigenicity in vivo. 16HBE-T cells transfected with the NC mimic or the miR-622 mimic were injected subcutaneously into the bilateral inguino-abdominal flanks of nude mice. Among the seven inoculated mice, the average gross tumor weight was 0.18 ± 0.03 g (Figure 3B) and average tumor volume was 0.58 ± 0.14 cm³ (Figure 3C); animals transfected with the miR-622 mimic exhibited lighter and smaller tumors, respectively, compared with the NC mimic control group (0.35 ± 0.10 g and 1.09 ± 0.24 cm³, respectively). The tumors grown in these seven mice are illustrated in Figure 3D, where it is evident that tumors of the miR-622 mimic-transfected group are smaller than those in the NC mimic-transfected group.

K-Ras is a direct target of miR-622

We investigated the ability of miR-622 to inhibit the growth of 16HBE-T cells in vitro and in vivo; additionally, we explored the target genes that miR-622 regulates. As described above, miR-622 caused cell cycle arrest at the G0 phase, which prompted us to investigate possible target proteins involved in the G0–S checkpoint transition. We studied the proteins: K-Ras, E2f1, cyclin E1, cyclin E2, cyclin D2, Bcl2, Cdc25a, c-myc and Cdk6, which all play a critical role during this transition. We examined the predicted targets of miR-622 using the Targetscan, RNA22 and RNAhybrid algorithms. The analysis predicted that there are complementary sites that recognize the seed region of miR-622 on the K-Ras, E2f1, c-myc and Cdk6 mRNA 3' UTR regions. We performed western blot analysis to determine whether miR-622 represses any of these putative targets in

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**Fig. 2.** Upregulation of miR-622 expression inhibited cell proliferation. (A) 16HBE-T and (B) H460 cells were seeded and transfected, and cell growth was monitored with the cell counting kit-8 assay at 48 h after transfection. Each experiment was performed in triplicate. († P < 0.05, #P < 0.01 all versus NC mimic). (C) 16HBE-T and (D) H460 cells were transfected, harvested at 48 h and stained with propidium iodide for cell cycle analysis. Exogenous expression of miR-622 induced cell cycle arrest in the G0 phase († P < 0.05, #P < 0.01 all versus NC mimic). (A–D) Mean ± SD, n = 3. (E) A representative histogram demonstrating exogenous expression of miR-622 inducing G0 phase cell cycle arrest in 16HBE-T cells; the percentage of cells in G0 and S phases is indicated.
miR-622 functions as a tumor suppressor

16HBE-T cells transfected with the miR-622 mimic. As shown in Figure 4A and B, the miR-622 mimic did not affect the levels of E2f1, c-myc or Cdk6 proteins but exhibited a significant 0.35 ± 0.08-fold reduction in the level of K-Ras protein in 16HBE-T cells transfected with the miR-622 mimic, indicating that miR-622 potentially regulates K-Ras. However, the miR-622 mimic did not affect the K-Ras mRNA expression levels, suggesting that miR-622 regulates K-Ras mRNA translation without affecting accumulation levels (Figure 4C). These results demonstrated that K-Ras is a potential target of miR-622; we verified this with the luciferase reporter assay. K-Ras has three binding sites in the 3′ UTR (Figure 4D). Sites 1 and 2 are conserved in mammals and the Site 3 is not. We constructed the psiCHECK-2-wild-K-Ras-3′ UTR reporter plasmid (wt-K-Ras) or psiCHECK-2-mutant-K-Ras-3′ UTR reporter plasmid (mt-K-Ras), in which portions of the 3′ UTR encompassed all of the predicted and mutant binding sites, in order to verify whether K-Ras is directly regulated by miR-622 (Figure 4E and Supplementary Figure 1 is available at Carcinogenesis online). The reporter plasmids were cotransfected with miR-622 mimic or miR-622 inhibitor. The wild-type reporter wt-K-Ras plasmids cotransfected with miR-622 mimic exhibited inhibited luciferase activity compared with the vector control group or the NC mimic group, whereas plasmids cotransfected with miR-622 inhibitor exhibited increased luciferase activity compared with the vector control group or NC inhibitor group. In contrast, the luciferase activity was not affected by mutant type mt-K-Ras plasmid cotransfection with miR-622 mimic and miR-622 inhibitor, potentially because miR-622 was unable to recognize the complete mutant binding sites of K-Ras mRNA, thereby disrupting the interaction between miR-622 and K-Ras (Figure 4F). These data indicate that K-Ras is targeted directly by miR-622.

Knockdown of K-Ras affects the growth retardation caused by miR-622
Our results indicated that K-Ras is a favorable target for miR-622. However, the role of K-Ras concerning the growth retardation caused by miR-622 remains unclear. Because K-Ras is a key oncogene that controls the growth of cancer, we speculated that K-Ras is a functional target for miR-622. To test this hypothesis, we used RNA interference techniques to knock down endogenous K-Ras expression in 16HBE-T cells. We constructed vector control cells and M-Sh-NC, M-K-Ras-sh1, M-K-Ras-sh2, M-K-Ras-sh3 and M-K-Ras-sh4 cells, which stably expressed NC shRNA and four shRNAs targets for K-Ras mRNA for a minimum of 2 months. As shown in Figure 5A, the level of K-Ras mRNA and protein in M-K-Ras-sh4 cells was reduced by over 80% compared with NC shRNA cells. The suppression of K-Ras expression was not observed in the vector control and negative control. M-K-Ras-sh4 cells were chosen for subsequent experiments.

We determined the cell viability of vector control cells, M-sh-NC cells and M-K-Ras-sh4 cells and found that the cell viability of M-K-Ras-sh4 cells was reduced to 53.33 ± 5.56% compared with NC shRNA cells after 48 h (Figure 5B). The K-Ras knockdown of M-K-Ras-sh4 cells transfected with miR-622 mimic did not decrease the cell viability compared with cells transfected with NC mimic (Figure 5C). Additionally, M-K-Ras-sh4 cells developed fewer colonies in soft agar compared with M-sh-NC cells (Figure 5D), whereas colony formation in M-K-Ras-sh4 cells transfected with miR-622 mimic was not affected compared with colony formation in cells transfected with NC mimic (Figure 5E). Moreover, The Ras inhibitor FTS prevented proliferation inhibition and cell cycle arrest at the G0 phase in 16HBE-T cells exhibiting exogenous expression of miR-622 (Supplementary Figure 3A–E is available at Carcinogenesis online). Taken together, these results indicated that K-Ras is a functional target in the growth retardation induced by miR-622.

Downregulation of miR-622 affects the growth inhibition enhanced by resveratrol
Our findings indicated that resveratrol caused cell proliferation inhibition and upregulation of miR-622 that in turn enhanced growth inhibition by the functional target K-Ras; however, the mechanisms...
underlying the growth inhibition effects induced by miR-622 and resveratrol warranted further elucidation. We investigated the effects of resveratrol on 16HBE-T cells following a decrease in miR-622 levels upon transfection with miR-622 inhibitor. The cell viability of the miR-622 inhibitor group increased to 1.53 ± 0.12 (A450/A650) compared with the NC inhibitor group (1.03 ± 0.03 A450/A650) (Figure 6A), suggesting that decreased miR-622 levels potentially impair the proliferation inhibition caused by resveratrol. The 16HBE-T cells of the miR-622 inhibitor group developed a greater number of colonies (46.33 ± 2.89 per well) compared with the cells of the NC inhibitor group (31.33 ± 3.11 per well) (Figure 6B). After downregulation of miR-622 levels with the miR-622 inhibitor, the cell viability and colony formation levels in resveratrol-treated cells did not reach the levels in untreated control groups (Figure 6A and B). This finding suggests that the growth inhibition of 16HBE-T cells enhanced by resveratrol was in part dependent upon miR-622. Resveratrol treatment decreased the wt-K-Ras 3’ UTR luciferase activity in 16HBE-T cells (Figure 6C). Transfection with the miR-622 inhibitor significantly increased the wt-K-Ras 3’ UTR luciferase activity in resveratrol-treated 16HBE-T cells (Figure 6C). However, transfection with the miR-622 inhibitor showed no effect on the mt-K-Ras 3’ UTR luciferase activity in resveratrol-treated 16HBE-T cells (Figure 6C). This indicates that resveratrol is able to affect the binding between miR-622 and the K-Ras mRNA 3’ UTR. Moreover, the K-Ras protein levels were decreased in the resveratrol group compared with the control group, and the protein levels were upregulated in the resveratrol + miR-622 inhibitor group (Figure 6D). However, K-Ras mRNA was not affected by resveratrol treatment (Supplementary Figure 4 is available at Carcinogenesis Online), indicating that resveratrol exerts its effects primarily at the translation level. These results suggest that miR-622 mimic and resveratrol both affect K-Ras mRNA translation without influencing K-Ras mRNA expression levels. In addition, miR-622 is modulated by resveratrol. Taken together, our findings indicate that resveratrol, potentially through the miR-622-K-Ras pathway in part, inhibits 16HBE-T cell proliferation and growth.

Discussion

We conducted in vitro studies on the anti-BPDE-transformed human bronchial epithelial cell line 16HBE-T to elucidate the molecular mechanisms of anti-BPDE carcinogenesis (3,4,6–8). We demonstrated that N-Ras (4), miR-494 (6), miR-22 (7) and miR-106a (8) are carcinogenic factors in the 16HBE transformation induced by anti-BPDE. Previous studies have demonstrated that miRNA exhibits the potential for preventive and therapeutic activity against cancer; thus, in this study, we investigated miR-622 expression levels and found upregulated expression in resveratrol-treated 16HBE-T and H460 cells, whereas the expression of this miRNA was lower in 16HBE-T cells and H460 lung cancer cells compared with control 16HBE cells. Using bioinformatic analysis, we found that MIR622, the miR-622 coding gene, is mapped to chromosome 13q31.3. The genomic locus of this chromosomal region exhibits a high frequency
of allelic loss or deletion and has been reported in cancers of the colon (35), ovary (36) and head and neck (37). Allelic loss or deletion could be the potential cause for the change of miRNA levels. Some predicted targets for miR-622, including E2f1, Pdgfb, Map4k4, Cdk6, K-Ras, cyclin D2 and c-myc, are oncogenes. This suggests that miR-622 functions as a tumor suppressor and is potentially involved in tumorigenesis. However, the precise biological function of miR-622 and its targets remains elusive.

Our findings indicated that miR-622 exhibits a growth inhibitory effect on malignant transformed 16HBE-T cells via targeting of the oncogene K-Ras, which suggests that miR-622 itself plays a role as a tumor suppressor in malignant transformation or lung cancer. In a recently reported study of 57 paired gastric neoplastic tissues, the downregulated expression level of miR-622 correlated with the matched adjacent non-neoplastic tissue regulation of cellular invasion and tumor metastasis through the targeting of oncogene ING1 (38); this finding is in concordance with our study, which suggests that miR-622 acts as a tumor suppressor.

Our study indicated that the miR-622-K-Ras axis of regulation plays an important role in the preventive and therapeutic activity of resveratrol. The properties of resveratrol as a natural anticarcinogenic agent against various human cancers have been widely investigated (23–26). Although the molecular mechanisms of the preventive effects of resveratrol have been extensively investigated, very little information is currently available regarding miRNAs concerning the potential for a preventive and therapeutic role in lung cancer. A recent report showed that miRNAs were differentially expressed in A549 lung cancer cells following treatment with resveratrol (39).

**Fig. 5.** Knock-down of K-Ras affects the proliferation inhibition caused by miR-622. 16HBE-T cells stably expressing pGPU6-sh1, pGPU6-sh2, pGPU6-sh3, pGPU6-sh4, and pGPU6-NC were selected with G418 and termed M-K-Ras-sh1, M-K-Ras-sh2, M-K-Ras-sh3, M-K-Ras-sh4 and M-Sh-NC. (A) The relative level of K-Ras mRNA was measured by qRT-PCR and calculated relative to the vector control group \((P < 0.05, #P < 0.01\) all versus M-Sh-NC group). The K-Ras protein level in 16HBE-T cells after transfection was measured by western blot with ß-actin used as a loading control. A representative result from three independent experiments is shown. The intensity for each band was densitometrically quantified. The value above each lane indicates the fold change in relative K-Ras expression level in M-K-Ras-sh1, M-K-Ras-sh2, M-K-Ras-sh3, M-K-Ras-sh4 and M-Sh-NC relative to the vector control group. (B) Cell growth of the vector control cells, M-Sh-NC cells and M-K-Ras-sh4 cells was examined using the cell counting kit-8 assay at 48 h \((P < 0.05\) versus the vector control or M-Sh-NC group). (C) The M-K-Ras-sh4 cells were transfected with NC mimic and miR-622 mimic. Cell growth was monitored 48 h posttransfection using the cell counting kit-8 assay. (D) Colony formation of the vector control cells, M-Sh-NC cells and M-K-Ras-sh4 cells was inspected in soft agar. The number of colonies \((\geq 50\) cells per colony) in each dish of triplicate wells was counted \((P < 0.05\) versus vector control or M-sh-NC group). (E) M-K-Ras-sh4 cells transfected with miR-622 mimic and NC mimic were examined in soft agar. The number of colonies \((\geq 50\) cells per colony) in each dish of triplicate wells was counted. (A–E) Mean ± SD, \(n = 3\).
Unfortunately, no direct evidence has demonstrated that miRNAs are regulated in a specific manner and play a role in the effects of chemopreventive agents such as resveratrol. In this study, we demonstrated that miR-622 and its target K-Ras do indeed play a role in the chemopreventive effects of resveratrol.

It is well established that K-Ras, like other Ras genes, encodes a family of small guanosine triphosphate-binding proteins that control signaling pathways, including the mitogen-activated protein kinase, signal transducer and activator of transcription and phosphoinositide 3-kinase signaling cascades, which are key regulators of several aspects of cell growth, differentiation, apoptosis and malignant transformation (40,41). K-Ras expression is aberrant in most human tumors due to activating mutations in the RAS genes or to alterations in upstream or downstream signaling components. Previous studies have demonstrated that human lung cancer specimens frequently harbor activating K-Ras mutations not found in corresponding normal tissues, and these findings have been confirmed by observations in human cell lines (41,42). A K-Ras mutation is rare in a non-smoker; it is reasonable to assume that carcinogens in tobacco smoke directly cause the mutation and increased levels (43,44), which promotes lung neoplastic initiation, progression and lung tumorigenesis. In our previous study, K-Ras mRNA and protein levels are high in 16HBE-T cells (4). These results indicate that K-Ras potentially plays an important role in anti-BPDE-induced malignant transformation, which further strengthens the potential role of K-Ras in chemically induced carcinogenesis and suggests the suitability of its application in chemoprevention and chemotherapy against lung cancer. It was recently reported that K-Ras is inversely regulated by let-7a (17), mir-18a (45) and miR-96 (46), and interestingly, the oncogenic miR-21 was found to regulate K-Ras-dependent lung tumorigenesis (47). Our study demonstrated that miR-622 inhibits the growth of 16HBE-T cells by targeting K-Ras and enhancing the anticarcinogenic effect of resveratrol. Rational therapies that target the Ras pathways could potentially inhibit tumor growth, survival and spread. Although previous K-Ras targeted therapies have shown promise in the clinical setting, the therapeutic effect remains unsatisfactory (27,48). Thus, our study demonstrates potential new aspects regarding K-Ras targeted therapy in lung cancer.

In summary, we demonstrated that miR-622 is upregulated in 16HBE-T cells treated with resveratrol and induced cell growth retardation via targeting K-Ras. Therefore, miR-622 targeting of the K-Ras signal pathway is a potentially promising new direction in the development of therapeutic treatments for lung cancer.

**Supplementary material**

Supplementary Tables 1 and 2 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/
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Conflict of Interest Statement: None declared.

References