miR-216b suppresses tumor growth and invasion by targeting KRAS in nasopharyngeal carcinoma

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Summary

MicroRNAs (miRNAs) are small noncoding RNAs that are involved in various diseases, including cancer. In the present study, we found that miR-216b was downregulated in nasopharyngeal carcinoma (NPC) cell lines and specimens. Decreased expression of miR-216b was directly related to advanced clinical stage and lymph node metastasis. miR-216b levels correlated inversely with levels of KRAS protein during nasopharyngeal tumorigenesis. Furthermore, we demonstrated that miR-216b can bind to the 3′ untranslated region (UTR) of *KRAS* and inhibit expression of the KRAS protein. Both in vitro and in vivo assays revealed that miR-216b attenuated NPC cell proliferation, invasion and tumor growth in nude mice. miR-216b exerts its tumor suppressor function through inhibition of the KRAS-related AKT and ERK pathways. Our findings provide, for the first time, significant clues regarding the role of miR-216b as a tumor suppressor by targeting KRAS in NPC.

Key words: microRNA-216b, KRAS, Nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy arising from the nasopharyngeal epithelium (NET) with an incidence in southern China of 20–30 cases per 100,000, a frequency 25-fold higher than that of other countries (Yu and Yuan, 2002). Many factors, including tumor suppressors and oncogenes, are involved in nasopharyngeal tumorigenesis (Deng et al., 1998; Lo et al., 1996; Qian et al., 2002; Sarac et al., 2001; Sheu et al., 1995; Xiang and Zhang, 2005; Xiong et al., 2004). Recently, the classical family of protein-encoding genes recognized as tumor suppressors and oncogenes has been expanded to include a type of non-protein-coding RNA molecule known as microRNA (miRNA) (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006). miRNAs are 19–24 nucleotides in length, and they regulate gene expression by imperfect base-pairing with complementary sequences located mainly, but not exclusively, in the 3′ untranslated regions (UTRs) of target mRNAs. Hence, miRNAs represent one of the major regulatory families of genes in eukaryotic cells, and they work by inducing translational repression and transcript degradation (Doench and Sharp, 2004; German et al., 2008; Pillai et al., 2007). miRNAs regulate genes involved in functions ranging from development, differentiation and proliferation to stress processes (German et al., 2008). Rapidly emerging evidence strongly suggests crucial roles of miRNAs in tumorigenesis (Slack and Weidhaas, 2008; Vasilatou et al., 2010). For example, miR-182 is upregulated in melanoma and promotes metastasis by repressing *FOXO3* and microphthalmia-associated transcription factor (MITF) (Segura et al., 2009). The let-7 family of miRNAs has been shown to regulate *KRAS* (Johnson et al., 2005), and let-7 levels have been correlated with prognosis in lung cancer (Takamizawa et al., 2004). miR-96 expression is decreased in pancreatic cancer, and it functions as a tumor suppressor by targeting *KRAS* (Yu et al., 2010).

Recently, Sengupta et al. reported that miR-216 was significantly downregulated (an 85% reduction) in NPC relative to NET when examined by a miRNA microarray analysis (Sengupta et al., 2008). Two members of the miR-216 family, miR-216a and miR-216b, are located at chromosome 2p16.1, a region frequently deleted in NPC (Shao et al., 2001). These findings suggest that deregulation of miR-216 is associated with nasopharyngeal carcinogenesis. However, to our knowledge, the role of miR-216 in tumorigenesis remains undefined.

Here, we determined that two members of miR-216 family (miR-216a and especially mir-216b) are downregulated in NPC, and we found an inverse correlation between the levels of miR-216b and KRAS protein. miR-216b directly targets the 3′-UTR of the *KRAS* transcript and elicits a specific and robust knockdown of KRAS protein; it also inhibits NPC cell proliferation, invasion and tumor growth in nude mice. miR-216b mediates its tumor suppressor function, at least in part, by suppressing pathways downstream of KRAS, such as the PI3K-AKT and MEK-ERK pathways. Our findings provide, for the first time, significant clues regarding the role of miR-216b as a tumor suppressor in NPC.

Results

miR-216b is downregulated in NPC cell lines and clinical specimens

Recently, Sengupta et al. identified eight miRNAs showing robust differential expression between 31 NPC and ten normal healthy NET samples by a miRNA microarray analysis. Among

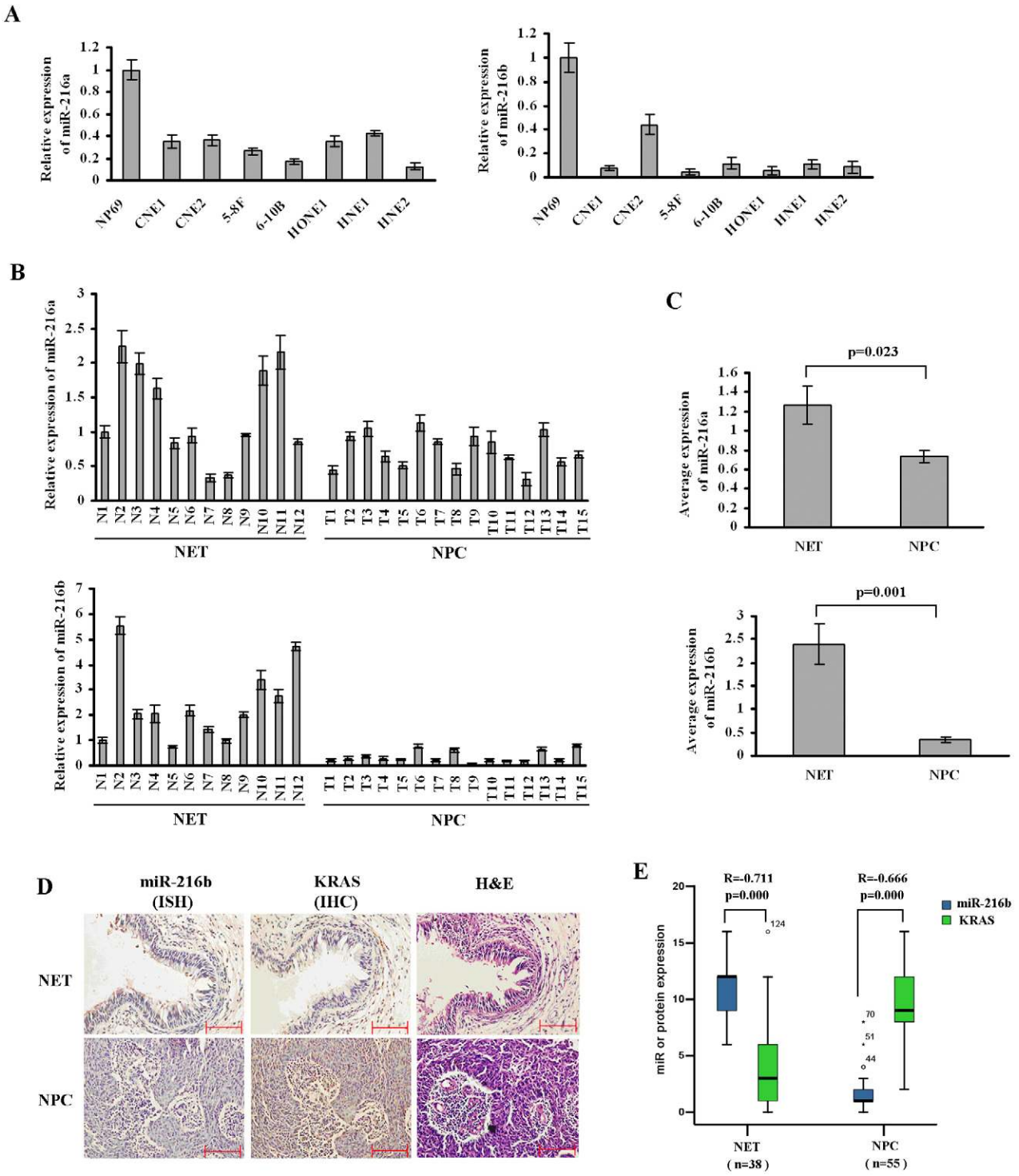

Fig. 1. miR-216b is downregulated and is inversely correlated with the levels of KRAS in NPC tissues and cell lines. (**A**) qRT-PCR analysis of the expression levels of miR-216 in multiple nasopharyngeal cell lines. The expression values of NP69 are set at 1 for both miR-216a and miR-216b. The relative expression levels of miR-216a and mir-216b were determined by using the $2^{-\Delta\Delta Ct}$ method. (**B**) qRT-PCR analysis of the expression levels of miR-216 in 15 NPC and (T) 12 non-matched normal NET (N) samples. The expression values of sample N1 are set at 1 for both miR-216a and miR-216b. The relative expression levels of miR-216a and mir-216b were determined by using the $2^{-\Delta\Delta Ct}$ method. (**C**) Average miR-216 expression levels in NPC and NET samples by qRT-PCR analysis. The levels are expressed as average ($2^{-\Delta\Delta Ct}$). (**D**) Representative expression levels of miR-216b (determined by in situ hybridization) and KRAS (determined by immunohistochemistry) in clinical specimens. Scale bars: 100 μm. (**E**) Pearson's correlation scatter plot of the levels of miR-216b (determined by in situ hybridization) and KRAS protein (determined by immunohistochemistry) during colorectal tumorigenesis. The number (n) of clinical specimens in given. All data are shown as means ± s.e.m.

those miRNAs, the level of miR-216 expression in cancer cells was reduced by 85% compared with the level of expression in normal epithelial cells. Here, we used quantitative real-time PCR (qRT-PCR) to measure mature miR-216a and -216b expression levels in eight NET cell lines, 15 NPC and 12 NET tissue samples. These two members of the miR-216 family showed reduced expression levels in all NPC cell lines with respect to benign immortalized NP69 NET cells (Fig. 1A). Similarly, both of them showed lower expression in NPC tumors relative to NET tissues (Fig. 1B). The overall average expression levels of miR-216a and -216b were lower in NPCs than in normal tissues (NET) (Fig. 1C). In particular, the difference in expression (between normal and tumor tissues) of miR-216b was several times higher than that of miR216a (miR-216a, fold difference=1.7, $P=0.023$; miR-216b, fold difference=6.7, $P=0.001$, Fig. 1C). For this reason, we focused on miR-216b in this study.

To determine how the downregulation of miR-216b might contribute to NPC phenotypes or clinical pathological features, we employed in situ hybridization to evaluate miR-216b levels in 38 NET and 55 NPC specimens. miR-216b expression levels were significantly decreased in NPC relative to normal tissues (Fig. 1D,E) and were inversely correlated with tumor metastasis and clinical stage ($P=0.003$ and 0.021, respectively; Table 1). These data suggest that downregulation of miR-216b contributes to NPC carcinogenesis.

Table 1. Analysis of the correlation between expression of miR-216b in primary NPC and its clinicopathological parameters

Variable	Number of cases	Median expression of miR-216b (±range)	*P*-value
Age (years)			
⩾60	23	1.59±0.18	0.479
<60	32	1.74±0.19	
Gender			
Male	30	1.70±0.09	0.823
Female	25	1.67±0.18	
Degree of differentiation			
Well and moderately differentiated	11	1.73±0.16	0.254
Poorly differentiated	44	1.57±0.11	
TNM stage			
Stage III or IV	15	2.03±0.12	0.021
Stage I or II	40	1.01±0.09	
Lymph node status			
Metastasis	39	0.98±0.05	0.003
No metastasis	16	3.34±0.21	

miR-216b directly targets and inhibits *KRAS*

To explore the function of miR-216b, three computational algorithms, TargetScan, PicTar and miRanda, were used to search for potential miR-216b target genes and a large number of different target genes were predicted. Among these candidate target genes, *KRAS*, which was predicted by all three algorithms, attracted our attention immediately. *KRAS* is a master regulator of cell growth, proliferation, differentiation and carcinogenesis. Previous studies have shown *KRAS* to be overexpressed in head and neck tumors including NPC (Field, 1992; Kiaris et al., 1995; Yarbrough et al., 1994). Moreover, a miR-216b-binding site was found in the 3′-UTR of *KRAS* mRNA. There was perfect base pairing between the seed sequence of mature miR-216b and the 3′-UTR of *KRAS* mRNA and these seed sequences were conserved across species (Fig. 2A). Indeed, there was an inverse correlation between the level of KRAS protein, indicated by immunohistochemistry staining, and the level of miR-216b expression assessed by in situ hybridization in the collection of human NET and NPC tissues during NPC tumorigenesis as used above (Fig. 1D,E; supplementary material Fig. S1). These data suggest that miR-216b could act as a tumor suppressor by targeting KRAS in NPC. Although levels of KRAS protein were consistently upregulated in NPC, levels of *KRAS* mRNA varied enormously. We did not find a correlation between miR-216b and *KRAS* mRNA levels (data not shown).

To verify whether *KRAS* is a direct target of miR-216b, we subcloned the full-length *KRAS* 3′-UTR into a luciferase reporter vector. Fig. 2B shows that addition of in-vitro-produced miR-216b, but not miR-216a, dramatically suppressed the luciferase activity of the KRAS 3′-UTR upon co-transfection of the luciferase vector (wild-type, mutant or negative control) with the in-vitro-produced microRNAs (miR-216a, miR-216b or scramble control) into NPC cells (Fig. 2B). We think this result is largely owing to differences in seed site sequence between miR-216a and miR-216b (see Fig. 2A). The profound inhibition was abolished when the seed sequences of the miR-216b target sequences were mutated in the Luc-mut vector (Fig. 2B). Moreover, inhibition of endogenous miR-216b by addition of anti-miR-216b (i.e. miR-216b inhibitors) in CNE2 cells, which have a high level of endogenous miR-216b (Fig. 1A), rescued luciferase expression (Fig. 2C).

To directly assess the effect of miR-216b on KRAS expression, we transfected miR-216b into 5-8F or HONE1 cells and found that overexpression of miR-216b reduced KRAS protein levels (Fig. 2D, left panel). Furthermore, anti-miR-216b transfection increased KRAS protein levels in CNE2 and NP69 cells (Fig. 2D, right panel). Much to our surprise, neither overexpression nor knockdown of miR-216b affected *KRAS* mRNA levels (Fig. 2E) although intracellular levels of miR-216b were altered significantly after treatment with in-vitro-produced miR-216b or anti-miR-216b (supplementary material Fig. S2). These results provide evidence that miR-216b directly recognizes the 3′-UTR of *KRAS* mRNA and inhibits *KRAS* translation.

miR-216b inhibits the AKT and ERKs pathways

KRAS activation can trigger several important signaling pathways, such as the PI3K–AKT and MEK–ERK pathways, most of which regulate cell proliferation, survival and invasion (Calvo et al., 2010; Campbell et al., 2007; Downward, 2003). Therefore, we investigated the possibility that miR-216b regulates those pathways by targeting KRAS. Upregulation of miR-216b, through transfection of miR-216b, in 5-8F and HONE1 cells decreased the phosphorylation levels of AKT and its downstream target GSK3β (Fig. 3A). Similarly, miR-216b suppressed the levels of phosphorylated ERK and its downstream effector c-Fos (Fig. 3B). We also observed that knockdown of miR-216b, through transfection of anti-miR-216b, in CNE2 cells increased the levels of phosphorylated AKT, GSK3β and ERK (Fig. 3C). These western blotting results demonstrated that miR-216b is negative regulator of AKT and ERK pathways.

Subsequently, rescue experiments were performed by overexpressing the KRAS vector (without an endogenous 3′-UTR) in miR-216b-treated cells: 5-8F cells were first transfected with miR-216b and then with *KRAS*-encoding vector 24 hours later. The miR-216b-induced downregulation of KRAS was rescued

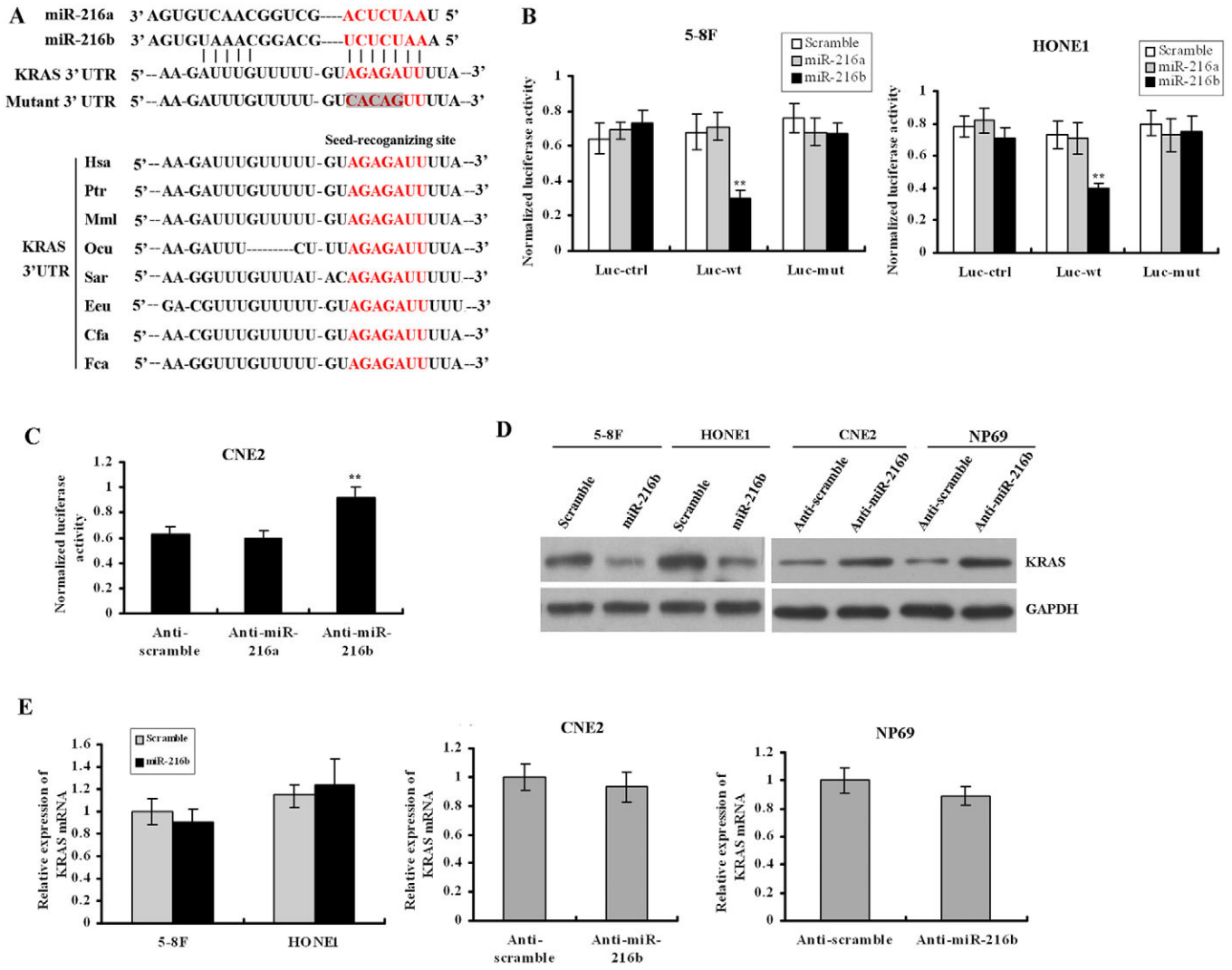

Fig. 2. *KRAS* is a direct target of miR-216b. (**A**) Sequence alignment of microRNAs of the miR-216 family and the *KRAS* 3′-UTR. There are differences in seed site sequence between miR-216a and miR-216b. The *KRAS* 3′-UTR contains one predicted miR-216b-binding site. The seed regions of miR-216 and the seed-recognizing sites in the *KRAS* 3′-UTR are indicated in red, and all nucleotides in seed-recognizing sites are completely conserved in several species. Hsa, *Homo sapiens*; Ptr, *Pan troglodytes*; Mml, *Mus musculus*; Ocu, *Oryctolagus cuniculus*; Sar, *Sorex araneus*; Eeu, *Erinaceus europaeus*; Cfa, *Canis lupus familiaris*; Fea, *Felis catus*. (**B**) Luciferase assay on 5-8F cells (left-hand side) and HONE1 cells (right-hand side), which were co-transfected with miR-216 and a luciferase reporter containing the full length of KRAS 3′-UTR (Luc-wt) or a mutant (Luc-mut) in which the first five nucleotides of the miR-216b-binding site were mutated. An empty luciferase reporter construct was used as a negative control (Luc-ctrl). Luciferase activities were measured 48 hours post transfection. miR-216b markedly suppressed luciferase activity in Luc-wt reporter constructs. The data are means ± s.e.m. for separate transfections (n=4). (**C**) Luciferase assay on CNE2 cells co-transfected with a luciferase reporter (Luc-wt) and either anti-miR-216a or anti-miR-216b. Inhibition of endogenous miR-216b rescued the luciferase expression. The data are means ± s.e.m. for separate transfections (n=4). (**D**) miR-216b or anti-miR-216b transfection affects KRAS protein levels. 5-8F and HONE1 cells were transfected with miR-216b or scramble control, and CNE2 and NP69 cells were transfected with anti-miR-216b or anti-scramble. (**E**) *KRAS* mRNA levels were analyzed upon miR-216b or anti-miR-216b transfection by qRT–PCR. All data are shown as means ± s.e.m. **P<0.01.

upon the introduction of KRAS (Fig. 3D), and the phosphorylation levels of AKT and ERK were altered in a similar manner. The downregulation of phosphorylated AKT and phosphorylated ERK by miR-216b could be rescued by re-expression of KRAS (Fig. 3D). These observations suggest that miR-216b inhibits the AKT and ERK pathways by targeting KRAS.

miR-216b suppresses NPC cell proliferation and invasion by targeting KRAS

In light of the above findings, we decided to explore the biological significance of miR-216b in NPC tumorigenesis. We found that overexpression of miR-216b in CNE2 and 5-8F cells markedly attenuated cell proliferation and that re-expression of KRAS (without an endogenous 3′-UTR) rescued this inhibition, suggesting that miR-216b targets KRAS specifically (Fig. 4A). Overexpression of miR-216b inhibited the in vitro invasive potentials of 5-8F and HONE1 cells, whereas re-expression of KRAS rescued this inhibition (Fig. 4C). In contrast with in-vitro-produced miR-216, anti-miR-216b induced cell growth and invasive phenotypes in CNE2 cells (Fig. 4B,D).

Next, we tested whether miR-216b could play a role in tumorigenesis by using nude mice xenograft models. Nude mice

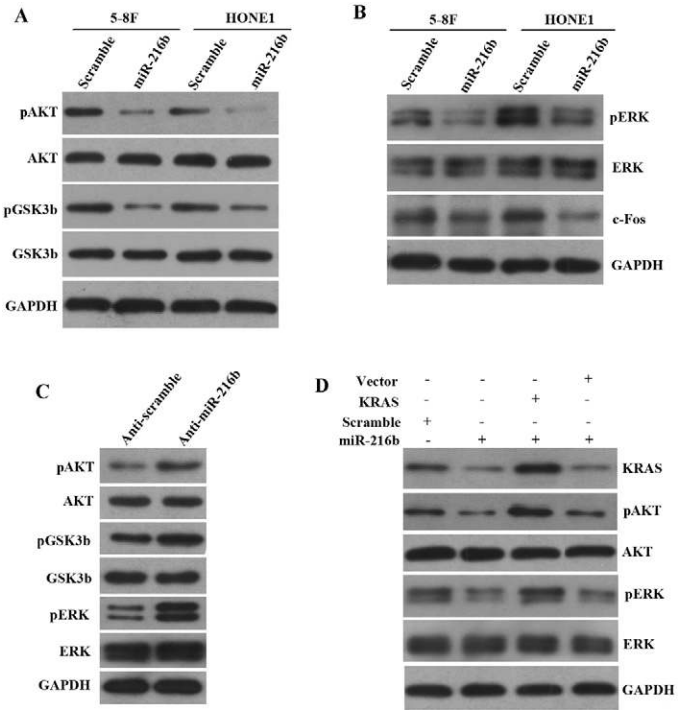

Fig. 3. miR-216b inhibits AKT and ERK pathways by targeting KRAS. (**A**,**B**) miR216b overexpression reduced the activity of AKT (A) and ERK pathways (B) in 5-8F and HONE1 cells. (**C**) Knockdown of miR-216b by anti-miR-216b increased the activity of AKT and ERK signaling in CNE2 cells. (**D**) miR-216b (or scramble control) transfection followed by KRAS (or mock vector) transfection 24 hours later in 5-8F cells affects AKT and ERK signaling. AKT pathway activity was measured by examining expression of phosphorylated AKT (pAKT) and phosphorylated GSK3β (pGSK3b), whereas EKR pathway activity was measured by examining expression of phosphorylated ERK (pERK) and c-Fos.

transplanted with 5-8F cells transfected either with scramble or miR-216b developed solid tumors in 25 days. However, tumor growth (including tumor volume and weight) was significantly reduced when the miR-216b was stably expressed in 5-8F cells ($P<0.05$, Fig. 4E, left-hand panels). CNE2 cells infected with the miR-216b-knockdown lentivirus or the control lentivirus were also transplanted into nude mice. After 25 days, tumor growth (including tumor volume and weight) was significantly increased in the miR-216b-knockdown CNE2 cells ($P<0.05$, Fig. 4E, right-hand panels). In addition, we confirmed that changes in miR-216b expression altered KRAS protein expression levels in lysates from mouse tumors (Fig. 4F; supplementary material Fig. S3).

Our findings demonstrate that miR-216b has properties consistent with tumor suppressor function. The ability to modulate KRAS protein levels might explain, at least in part, why miR-216b can inhibit cell proliferation and invasion in NPC.

Discussion

Although the first microRNAs were discovered in *C. elegans* several years ago [lin-4 in 1993 (Lee et al., 1993) and let-7 in 2000 (Reinhart et al., 2000)], it is only more recently that the study of this class of small regulatory RNAs in humans has become widespread (Ruvkun et al., 2004). Until now, only a handful of studies have identified specific miRNAs involved in human tumorigenesis and metastasis. Therefore, we believe more effort should be made, not only towards the identification of relevant miRNAs but also into the specific mechanisms by which they accomplish their specific functions, particularly with regard to the oncogenesis of different types of tumors.

A recent report has shed light on which miRNAs are involved in NPC tumorigenesis. Sengupta et al. reported that miR-216 expression levels were significantly downregulated (an 85% reduction) in NPC relative to NET, although little is known about the function and mechanism of miR-216 in tumorigenesis (Sengupta et al., 2008). Here, we used qRT-PCR and in situ hybridization to assay expression of mir-216 family of miRNAs in NPC cell lines and clinical specimens and found that these miRNAs were indeed downregulated in NPC tumor tissues. Importantly, the decrease in levels of miR-216b was several times greater than that of the levels of miR-216a in NPC samples as opposed to in normal tissues (Fig. 1C), and the levels of miR-216b were found to be inversely correlated to tumor metastasis and clinical stage (Table 1). In the same samples, the copy numbers of miR-216 and miR-216b showed a positive correlation, even although they were not exactly equal, implying that their mechanisms of regulation are somehow different (data not shown).

We next explored the possible targets of miR-216b in NPC cells through different computational algorithms. Silicon analysis revealed KRAS as a candidate target of miR-216b, which attracted our attention immediately. Like other members of the RAS family, KRAS is a GTPase and an early player in many signal transduction pathways. Active GTP-bound KRAS associates with a wide variety of effectors, including Raf, PI3K, Ral-GDS, Rho GTPases and other molecules, to transmit downstream signals controlling distinct cellular events, including cell proliferation, survival, differentiation and invasion (Campbell et al., 2007; Downward, 2003). Active point mutations in the *KRAS* gene have been found at high rates in a wide range of human tumors (Almoguera et al., 1988; Anderson et al., 1992; Burmer and Loeb, 1989). For example, ~90% of pancreatic cancers contain a *KRAS* point mutation, as do ~50% of colon cancers and thyroid cancers (Bos, 1989). Interestingly, *KRAS* gene mutations are rare in breast cancer (Slamon et al., 1984), but its upregulation has been found in 60% of the tumors analyzed (Thor et al., 1986). The same situation has been observed in head and neck tumors, including NPC. Hence, *KRAS* mutations do not appear to play a major role in these types of tumors, but several studies have revealed that KRAS

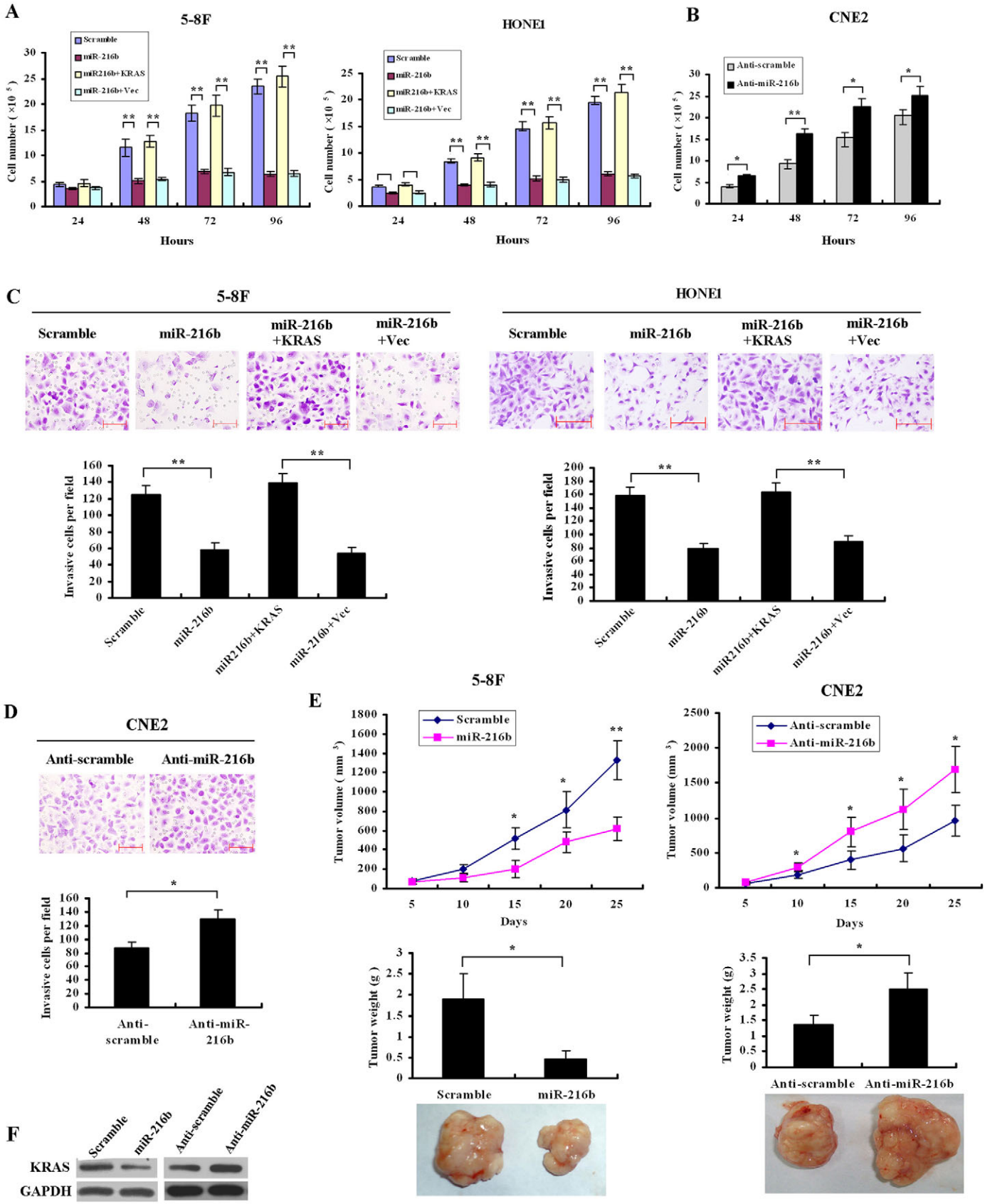

Fig. 4. See next page for legend.

overexpression is a frequent event in these tumors (Field, 1992; Kiaris et al., 1995; Yarbrough et al., 1994). We observed substantial upregulation of KRAS protein in NPC tissues (Fig. 1D,E), and KRAS protein levels were inversely correlated with miR-216b levels (Fig. 1E). Several miRNA target prediction programs suggested *KRAS* as a putative target of miR-216b, which was further confirmed in luciferase activity assays and miR-216b-mediated KRAS expression analysis. The luciferase activity assays demonstrated that miR-216b could bind to the 3′-UTR of *KRAS* mRNA (Fig. 2B,C). Moreover, when we transfected miR-216b or inhibiting oligonucleotides into NPC cells, an inverse expression pattern was observed between miR-216b and KRAS protein. That is, when miR-216b expression was high, KRAS expression was low and vice versa (Fig. 2D). All of these data suggest that miR-216b directly targets KRAS expression in NPC cells. miRNAs regulate gene expression through suppressing translation or inducing degradation of the target mRNAs. In current study, we also investigated the effect of miR-216b on *KRAS* mRNA levels and found that miR-216b does not modulate *KRAS* mRNA levels (Fig. 2E), which implies that miR-216b targets KRAS through inhibiting translation, not by degrading mRNA.

Activation of the KRAS signal has been well documented in various tumor types (Anderson et al., 1992; Jancik et al., 2010). It is well known that active RAS transduces signals from cell surface receptors into the cytoplasm through specific effector pathways and regulates diverse cellular processes. The MEK–ERK and the PI3K–AKT are the best characterized pathways downstream of KRAS (Calvo et al., 2010; Campbell et al., 2007; Downward, 2003). The ability to target KRAS transcripts signifies that miR-216b might be a potential regulator of PI3K–AKT and MEK–ERK pathways in NPC cells. Our results showed that miR-21b can inhibit the constitutive activity of AKT and ERK pathways through targeting KRAS, whereas expression of exogenous KRAS (without an endogenous 3′-UTR) can rescue this inhibition (Fig. 3).

Given that KRAS plays a significant role in regulation of cell proliferation, survival and invasion (Anderson et al., 1992), we investigated the effect of miR-216b on these phenotypes of NPC cells. miR-216b overexpression in CNE2 and 5-8F cells markedly attenuated the ability of the cells to proliferate (Fig. 4A,C), whereas overexpression of KRAS (without an endogenous 3′-UTR) rescued the inhibition of cell growth and invasion caused by miR-216b, suggesting target specificity (Fig. 4A,C). Meanwhile, inhibition of miR-216b by anti-miR-216b induced cell growth and promoted invasive phenotypes (Fig. 4B,D). We also used nude mouse xenograft models to confirm that miR-216b could suppress NPC cells tumorigenicity in vivo (Fig. 4E).

KRAS is an upstream modulator of many pathways, including AKT and ERK, through which it controls cell proliferation, invasion and tumorigenesis. Our findings suggest that miR-216b functions as a tumor suppressor, affecting NPC cell proliferation and invasion by targeting KRAS and subsequently suppressing downstream AKT and ERK signaling pathways. However, to completely clarify the function of miR-216b in tumorigenesis, future studies will have to detect more candidate targets over more types of cancer.

Fig. 4. miR-216b inhibits cell proliferation, invasion and tumor growth. (**A**) Overexpression of miR-216b arrested cell proliferation, but this was rescued upon coexpression of exogenous KRAS in 5-8F and HONE1 cells. Cells were plated in six-well plates at the desired cell concentrations after transfection and were counted at the indicated time points in triplicate. (**B**) Knockdown of miR-216b accelerated cell proliferation in CNE2 cells. (**C**) miR-216b overexpression decreased cell invasion in 5-8F and HONE1 cells. Cells were transfected with miR-216b (or scramble) followed by KRAS (or mock) transfection. All cells were subjected to a Matrigel invasion assay. (**D**) Anti-miR-216b increased the invasiveness of CNE2 cells. (**E**) miR-216b attenuated nasopharyngeal tumor growth in mouse xenograft models. The left-hand panels show tumor formation upon subcutaneous injection of 5-8F cells that were stably transfected with the miR-216b-expressing or control vector into nude mice. The right-hand panels show tumor formation upon transplantation of CNE2 cells infected with the lentivirus expressing antisense miR-216b or control sequence into nude mice. Tumor size was measured every 5 days. After 25 days, the mice were killed, necropsies were performed and tumors were weighed. (**F**) KRAS expression levels were measured by western blotting in tumor tissues extracted from nude mice 25 days after cancer cell injection. All data are shown as means ± s.e.m. *$P<0.05$; **$P<0.01$.

Materials and Methods

Cell lines

This study employed human nasopharyngeal epithelial cell line NP69 and NPC cell lines, including 5-8F, 6-10B, CNE1, CNE2, HNE1 and HNE2. CNE1 and CNE2 cells were obtained from the Chinese Academy Medical Science (Beijing). 5-8F and 6-10B cells were obtained from Sun Yat-Sen University (China). HNE1 and HNE2 cells were established in our laboratory. All NPC cell lines were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. NP69 cells were cultured in keratinocyte-SFM medium (Invitrogen) supplemented with burine pituitary extract and rEGF (recombinant EGF). All cells were maintained at 37℃ under an atmosphere of 5% CO_2 and 95% air.

Patient samples

NPC specimens and non-tumor nasopharyngeal epithelial tissues were collected from Xiang-Ya Hospital (Hunan, China). Written informed consent was obtained from all study participants. Collections and using of tissue samples were approved by the ethical review committees of the appropriate institutions.

Quantitative RT-PCR analysis (qRT-PCR)

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen). For the detection of *KRAS* mRNA, cDNA was synthesized from 1 μg of total RNA by means of the reverse reaction kit according to the manufacturer's instructions (Promega). Human *GAPDH* was amplified in parallel as an internal control. For miR-216, reverse transcription and qRT-PCR reactions were performed by means of a qSYBR-green-containing PCR kit (GenePharma, Shanghai, China), and U6 snRNA was used as an endogenous control for miRNA detection. Expression of each gene was quantified by measuring cycle threshold (Ct) values and normalized using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) relative to U6 snRNA or *GAPDH*.

In situ hybridization and immunohistochemistry

Tissue slides included 38 normal nasopharyngeal epithelial tissues and 55 primary NPC samples. In situ hybridizations were performed overnight at 52℃ after the addition of 50 nM DIG-labeled locked nucleic acid (LNA)-based probe specific for mir-216b (Exiqon, Vedbaek, Denmark). Next, washing and scanning were carried out according to the manufacturer's protocols. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue using anti-KRAS antibody (Sigma-Aldrich) and the standard streptavidin–peroxidase staining method described previously (Fan et al., 2006). In situ hybridization and immunohistochemistry results were scored by intensity (0–4) and percentage of positive cells score 0: no staining; 1: <10% positive cells; 2: 11–50% positive cells; 3: 51–75% positive cells; 4: >75% positive cells. Relative expression was obtained by multiplying intensity by percentage.

Constructs

luc-UTR vectors

The full-length KRAS 3′-UTR was cloned into the *Sac*I and *Mlu*I sites of the pMIR-REPORT luciferase vector (Ambion, Austin, TX, USA) using the PCR-generated fragment. The luc-mut vector, in which the first five nucleotides complementary to the miR-216b seed-region were mutated by site-directed mutagenesis (Stratagene), was constructed as a mutant control.

Constructs for miR-216 overexpression and knockdown

Synthesized RNA duplexes of scramble miRNA, miR-216a and miR-216b, and their inhibitors anti-scramble and anti-miR-216b were obtained from Ambion. To construct a vector expressing miR-216b, the precursor sequence of miR-216b (MI0005569) was synthesized, annealed and then inserted into the *Bam*HI–*Hin*dIII fragment of the pGCsi/U6 vector (GeneChem, Shanghai, China). A construct including the nonspecific miRNA was used as a negative control. miR-216b-knockdown lentiviruses were purchased from SunBio (Shanghai, China).

KRAS-expressing vector

Full-length *KRAS* cDNA entirely lacking the 3′-UTR was purchased from GeneCopeia (Rockville, MD, USA) and subcloned into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). The empty pcDNA3.1(+) vector was used as a negative control.

Cell transfection and infection

5-8F cells were transfected with the miR-216b-expressing vector or the control vector expressing a scrambled miRNA, using Lipofectamine 2000 (Invitrogen). All cells were selected with 400 mg/l G418 to generate two stable monoclonal cell lines (a stable cell line expressing miR-216b, 5-8F-miR-216b, and a control stable cell line, 5-8F-miR-scramble). In-vitro-produced miR-216a and miR-216b and the negative scramble control were transfected into 5-8F and HONE1 cells. The inhibitors anti-miR-216b and anti-scramble were also transfected into CNE2 and NP69 cells.

For miRNA and pcDNA3.1KRAS combination experiments, 5-8F and HONE1 cells were transfected with pcDNA3.1-KRAS and empty vector 24 hours after transfection of the in-vitro-produced miR-216b. To establish stable miR-216b-knockdown cell lines, CNE2 cells were transduced with the miR-216b knockdown lentivirus or the control lentivirus and selected with 5 mg/l puromycin.

Luciferase assay

Luc-wt, Luc-mut and Luc-ctrl were co-transfected with in-vitro-produced miR-216a or miR-216b into 5-8F and HONE1 cells. In addition, Luc-wt was co-transfected with miR-216b inhibitor into CNE2 cells. The pMIR-REPORT β-galactosidase control vector was transfected as a control. Luciferase activity was measured in cell lysates 48 hours after transfection using the Dual-Light luminescent reporter gene assay kit (Applied Biosystems). Results were normalized against β-galactosidase activity.

Western blotting

Western blotting was carried out as described previously (Zhou et al., 2008). Anti-KRAS antibody was obtained from Sigma-Aldrich. Antibodies against ERK, phosphorylated ERK, AKT, phosphorylated AKT, GSK3β, phosphorylated GSK3β and c-Fos antibodies were obtained from Cell Signaling Technology. Anti-GAPDH antibody was from Santa Cruz Biotechnology.

Cell proliferation assay

After transfection, cells were plated into six-well plates at the desired cell concentrations. Cell counts were estimated by trypsinizing the cells and performing analysis in triplicate with a Coulter counter (Beckman Coulter, Fullerton, CA) at the indicated time points.

Cell invasion assay

At 48 hours after transfection, cells were seeded onto the basement membrane matrix (EC matrix, Chemicon, Temecula, CA) present in the insert of a 24-well culture plate. Fetal bovine serum was added to the lower chamber as a chemoattractant. After a further 48 hours, the non-invading cells and EC matrix were gently removed with a cotton swab. Invasive cells located on the lower side of the chamber were stained with Crystal Violet, air-dried and photographed.

In vivo tumorigenesis

Five-week-old male nude athymic BALB/c nu/nu mice were used for examining tumorigenicity. To evaluate the role of miR-216b in tumor formation, 5-8F cells stably overexpressing miR-216 or scramble control were propagated and inoculated subcutaneously into the dorsal flanks of nude mice (2×10^6 cells in 0.2 ml volume). CNE2 cells infected with the miR-216b-knockdown lentivirus or the control lentivirus were also transplanted into nude mice (1.5×10^6 cells in 0.2 ml volume). Tumor size was measured every 5 days. After 25 days, the mice were killed, necropsies were performed and the tumors were weighed. Tumor volumes were determined according to the following formula: $A\times B^2/2$, where A is the largest diameter and B is the diameter perpendicular to A. The experiments were performed using five or six mice per group, and all animal procedures were performed in accordance with institutional guidelines.

Statistical analysis

Student's unpaired *t*-tests were used to evaluate statistical significance. Spearman's correlation tests were used to evaluate the pair-wise expression correlation between miR-216b and KRAS. Data are expressed as means ± s.e.m. $P<0.05$ was considered statistically significant.

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