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High *miR-196a* levels promote the oncogenic phenotype of colorectal cancer cells

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Abstract

AIM: To analyze the relevance of the microRNA *miR-196a* for colorectal oncogenesis.

METHODS: The impact of *miR-196a* on the restriction targets *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* was analyzed by reverse transcription polymerase chain reaction (RT-PCR) after transient transfection of SW480 cancer cells. The *miR-196a* transcription profile in colorectal cancer samples, mucosa samples and diverse cancer cell lines was quantified by RT-PCR. Transiently *miR-196a*-transfected colorectal cancer cells were used for diverse functional assays *in vitro* and for a xenograft lung metastasis model *in vivo*.

RESULTS: *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* were restricted by *miR-196a* in a dose-dependent and gene-specific manner. High levels of *miR-196a* activated the AKT signaling pathway as indicated by increased phosphorylation of AKT. In addition, high levels of *miR-196a* promoted cancer cell detachment, migration, invasion and chemosensitivity towards platin derivatives but did not impact on proliferation or apoptosis. Furthermore, *miR-196a* increased the development of lung metastases in mice after tail vein injection.

CONCLUSION: *miR-196a* exerts a pro-oncogenic influence in colorectal cancer.

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Key words: Micro-RNA; Cancer; Colorectal; *miR-196a*; Migration; Homeobox

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INTRODUCTION

Survival in colorectal cancer (CRC), one of the three most prevalent malignancies in western countries, is delineated by local recurrence, lymphatic and distant dissemination[1-3]. Molecular determinants occurring during the adenoma-carcinoma sequence of sporadic CRC include mutations in certain tumor-suppressor genes (*APC*, *DCC*, *Smad-2*, *Smad-4*, *p53*) and oncogenes (*K-ras*) that have been summarized by Fearon and Vogelstein[4-6]. However, as only 8% of CRCs harbor concomitant mutations of *APC*, *K-ras* and *p53*, it seems very likely that additional pathogenic alterations are instrumental in promoting progression and metastasis of colorectal cancer[7].

A recently discovered class of non-protein-coding small RNAs, microRNAs (miRNAs), extend our understanding of oncogenesis. miRNAs are endogenous small RNA molecules of 20-25 nucleotides length, regulating gene expression by inhibiting transcription,

inducing direct cleavage of the targeted mRNAs or blocking translation through their complementarity *versus* targeted mRNAs at 3' untranslated regions[8-13].

More than 50% of all known miRNA genes are located in cancer-associated regions or in fragile sites of the genome, indicating that miRNAs might play an important role in oncogenesis[14]. Supporting evidence is the close location of miRNAs, as *miR-196a*, in homeobox (*Hox*) gene clusters[14]. Hox proteins are major transcription factors that play a crucial role during embryogenesis, organogenesis and oncogenesis[15].

While some miRNAs can function as oncogenes, others act as tumor suppressors. Specific miRNAs, such as *let-7*, are under-expressed in cancer and function as tumor suppressors by regulating oncogenes in normal tissue. New evidence indicates that down-regulation of *let-7* transcription is a relevant step during oncogenesis which is significantly associated with shortened postoperative survival in lung cancer[16-18]. *Let-7* negatively regulates the expression of oncogenes *Ras* and *Myc* by targeting their mRNAs for translational repression in diverse malignancies[19].

In contrast, over-expressed miRNAs, such as *miR-17-92*, function as oncogenes promoting cancer development through inhibition of tumor suppressor genes. The expression of miRNA *miR-17-92* is significantly increased in small-cell lung cancer[20]. Interestingly, the known targets of *miR-17-92* include the two well-known tumor suppressor genes, *PTEN* and *RB2*[21].

The miRNA *miR-196a*, encoded at three locations in the mammalian Hox clusters A, B, and C, depicts evolutionarily conserved complementarity to mRNA of *HoxB8*, *HoxC8*, and *HoxD8*[22]. Interestingly, *miR-196a*-directed cleavage of *HoxB8* was detected in mouse embryos, and additional *in vivo* experiments revealed a down-regulation of *HoxB8*, *HoxC8*, *HoxD8* and *HoxA7* in mammalian cells. These results indicate a miRNA-mediated regulation of *Hox* gene expression during vertebrate embryogenesis[22].

Matching these observations, Hornstein and colleagues describe that *miR-196a* acts upstream of *HoxB8* and *sonic hedgehog* (*Shh*) *in vivo* during limb development[23]. Analyzing the miRNA expression pattern in pancreatic adenocarcinoma by large-scale miRNA chip analyses, Croce and colleagues found that 75% of tumors expressed *miR-196a* at a high level, predicting poor patient survival and linking *miR-196a* to human oncogenesis (14.3 mo *vs* 26.5 mo)[24].

As we had previously investigated the relevance of *Hox* genes for gastrointestinal cancer progression and observed a tumor-suppressive function of high *HoxC8* expression levels, we hypothesized that *miR-196a* might exert a pro-oncogenic influence in human cancer cells.

MATERIALS AND METHODS

Cell culture and human tissue

The human colorectal cancer cell lines SW480, SW620 and HT29 and the human gastric cancer cell line Snu16 were cultured in RPMI-1640 (Invitrogen, Germany) supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin (Cambrex, Germany) and 1 mmol/L L-glutamine (Invitrogen, Germany).

Colorectal cancer and mucosal tissue has been collected from the resectate of seven patients undergoing elective surgery for colorectal cancer after obtaining patients' written informed consent and approval by the local ethics committee.

miRNA isolation and quantitative reverse transcription polymerase chain reaction (RT-PCR)

miRNA isolation was performed from four cancer cell lines, and from seven colorectal cancer and matching mucosal samples using the MirVana miRNA Isolation Kit according to the manufacturer's recommendations (Ambion, Austin, USA). HSA-*miR-196a* and *U6* primer sets were commercially acquired and applied for quantitative RT-PCR using the MirVana QRT-PCR miRNA Detection Kit with Super *Taq* Polymerase (Ambion). For amplification, an Applied Biosystems 7900 HT Fast Realtime PCR System (Applied Biosystems, Foster City, USA) was used.

miR-196a transfection

3×10^5 SW480 colon cancer cells were plated in a six-well plate and cultured as described before. SW480 cells were used, as they had the lowest *miR-196a* transcription levels (see below). *miR-196a* was commercially synthesized (MWG Biotech, Germany) and applied at different concentrations (0, 20, 40, 80, 160 and 240 nmol/L). Transfection was performed with Lipofectamine siRNAmax (Invitrogen, Carlsbad, CA, USA) according to the recommendations of the manufacturer. Cells were harvested 24-48 h after transfection and either applied in the functional assays, in a xenograft bioassay or collected for RNA/protein extraction, respectively.

Proliferation assays

6×10^3 transiently transfected SW480 cells (mock or 160 nmol/L *miR-196a*) were plated in 96-well plates and cultured as described above. The start of analyses was 24 h after transient transfection. The number of cells per well was determined daily by absorbance (MTT). Absorbance was quantified with an ELISA reader. Each condition was performed in quadruplicate.

Adhesion assay

For adhesion assays, SW480 cells were used. Transient transfection (mock or 160 nmol/L *miR-196a*) was performed 48 h prior to assay start. Ninety-six-well plates had been prepared with laminin (10 μg/mL, 30 min, room temperature, Sigma, Germany), fibronectin (10 μg/mL, 30 min, room temperature, Sigma) or PBS and were blocked with albumin (2%, overnight, 4℃, Serva, Germany), respectively. After trypsinization, 4×10^4 cells were seeded per 96-well and allowed to attach for 45 min. Thereafter, the medium and non-attached cells were removed. Each

well was washed twice with 100 μL pure RPMI-1640 cell culture medium. The number of attached cells per well was determined by luminescence assay (Celltiter-Glo Cell Viability assay; Promega, USA). Emitted luminescence was quantified with a luminometer. Each condition was performed in quadruplicate. For dose-dependent quantification of adhesion (0, 40, 80 or 160 nmol/L *mi*R-*196a*) non-modified 96-well plates were used.

Migration and invasion assays

For migration and invasion assays SW480 cells were used 48 h after transient transfection (mock or 160 nmol/L *mi*R-*196a*). Migration and invasion were assayed with 24-well HTS FluoroBlock Inserts in triplet approaches (8 μmol/L pore size; Becton Dickinson, USA). For invasion assays, membranes were covered with fibronectin in advance (10 μg/mL, 30 min, room temperature, Sigma) and blocked with albumin (2%, overnight, 4℃, Serva).

In brief, 4×10^4 cells were re-suspended in serum-free RPMI-1640 medium and added to the upper chamber. Consecutively, RPMI-1640 medium with 20% FCS and 100 ng/mL CXCL12 was added to the lower chamber. Chambers were incubated for 24 h at 37℃ in a humid atmosphere of 5% CO_2. After incubation, the amount of cell invasion and migration into the lower chamber was determined by luminescence assay (Celltiter-Glo, Cell Viability assay; Promega) according to the recommendations of the manufacturers. Emitted luminescence was quantified with a luminometer. Each condition was performed in triplicate.

Chemosensitivity

3×10^5 SW480 cells (mock or 160 nmol/L *mi*R-*196a*) were seeded per six-well plate. Twenty-four hours after plating, 5-fluorouracil (5-FU) (10 μg/mL), irinotecan (40 μg/mL), oxaliplatin (10 μg/mL), cisplatin (20 μg/mL) or placebo (1 × PBS) were added to the medium. The number of apoptotic cells was determined after 48 h by apoptosis assay. In brief, suspension cells were collected and adherent cells were trypsinized prior to fixation with 100% ethanol, stained with propidium iodide and analyzed by FACS without gating. Each condition was performed in quadruplicate.

Western blotting analysis

SW480 cells were harvested 2 d after transient transfection (mock or 160 nmol/L *mi*R-*196a*), washed twice with PBS (1 ×) and lysed in 2 × RIPA solution. For Western blotting analysis, 100 μg of protein was loaded on a 13% SDS-PAGE gel. After separation, the gel was transferred to a PVDF membrane (Roth, Karlsruhe, Germany). AKT protein was detected with a rabbit-anti-human antibody (1:1000, overnight, 4℃, rabbit-anti-human monoclonal antibody, pan AKT, 4685; Cell Signaling, Danvers, MA, USA). Phosphorylated AKT (pAKT) protein was detected with a rabbit-anti-human antibody (1:1000, overnight, 4℃, rabbit-anti-human monoclonal antibody, Phospho-AKT, 9267, Cell Signaling). MEK1/2 was detected with a monoclonal rabbit-anti-human antibody (1:1000, overnight, 4℃, rabbit-anti-human monoclonal antibody, 9122; Cell Signaling). pMEK1/2 was detected with a monoclonal rabbit-anti-human antibody (1:1000, overnight, 4℃; rabbit-anti-human monoclonal antibody, 9121; Cell Signaling). Alpha-tubulin was analyzed with a monoclonal mouse-anti-human antibody (T5168, 1:2000, overnight, 4℃, Sigma). The secondary antibodies used were goat-anti-rabbit (1:10 000, 1 h, RT, SC-2033, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat-anti-mouse (1:10 000, 1 h, RT, SC-2031, Santa Cruz Biotechnology). For visualisation the Roti Lumin systems 1 and 2 were applied (P79 and P80; Roth).

Lung metastases xenograft biosystem

Transient transfection (mock or 160 nmol/L *mi*R-*196a*) of SW480 was performed 48 h prior to assay start. 4×10^4 tumor cells were re-suspended in 0.2 mL pure RPMI-1640 medium and applied for induction of lung metastases in 7-8-wk-old nod-Scid mice. Nod-Scid mice were radiated with 1.8 Gy 1 d prior to intravenous injection (tail vein) of tumor cells. Lung tumors grew for 7 wk before the animals were sacrificed. Thereafter, lungs were resected and tumor nodules quantified manually using surgical magnifying glasses.

RNA isolation and semiquantitative RT-PCR

RNA isolation was performed using the Qiagen RNeasy Kit according to the manufacturers recommendations (Qiagen, Hilden, Germany). Gene transcription of *β-actin*, *HoxA7*, *HoxB8*, *HoxC8*, *HoxD8* was analyzed by a two-step RT-PCR: reverse transcription was performed with 2 μg of RNA (20 μL total volume; Ominscript RT Kit; Qiagen) according to the recommendations of the manufacturer. One microliter of cDNA was used as a template for the specific PCR reactions. Primers applied were *β-actin*-forward: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3', *β-actin*-reverse: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' (661 bp fragment), *HoxA7*-forward: 5'-CCGCATGAAGTGGAAGAAAG-3', *HoxA7*-reverse: 5'-CAGTCCACAAAAGTTGGGAG-3' (347 bp fragment), *HoxB8*-forward: 5'-GCAATTTCTACGGCTACGAC-3' and *HoxB8*-reverse: 5'-GAAACAGAAGCTGGAGCGG-3' (434 bp fragment), *HoxC8*-forward: 5'-CACGTTCAAGACTTCTTCCACCACG-3' and *HoxC8*-reverse: 5'-GGTTCCAGAACCGAAGGATGAAGTG-3' (449 bp fragment), *HoxD8*-forward: 5'-ACAGCCGATTTTTACGACCC-3' and *HoxD8*-reverse: 5'-GCTTCCTTTTTCGTTTCCCC-3' (399 bp fragment).

For amplification, a DNA Engine PTC200 (MJ Research, Watertown, USA) thermocycler was used. Cycling conditions of the respective PCR were as follows: initial denaturation (4 min at 95℃), followed by the respective number of cycles (*β-actin*: 20; *HoxA7*: 29, *HoxB8*: 29, *HoxC8*: 29, *HoxD8*: 29) of denaturation (1 min at 94℃), annealing (1 min; *β-actin*: 57℃; *HoxA7*:

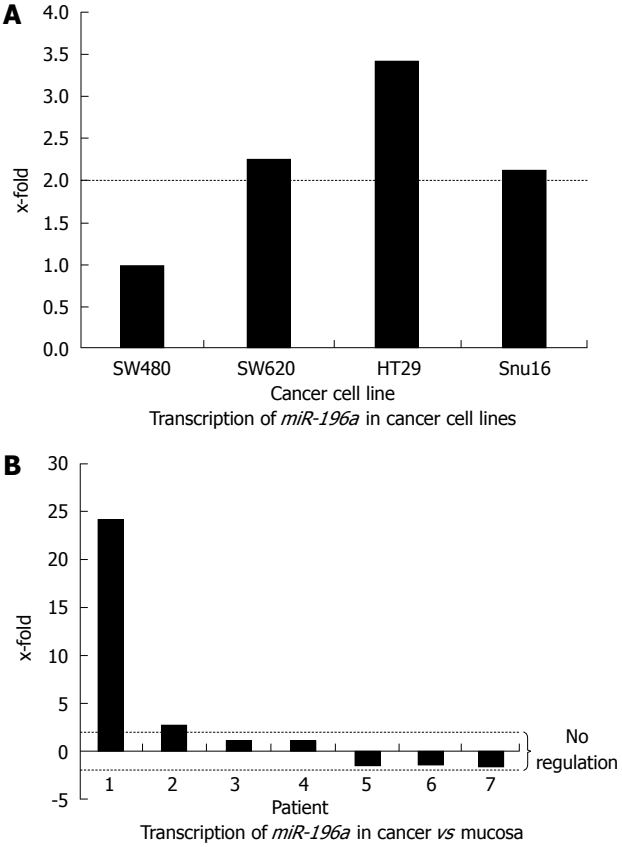

Figure 1 Transcription levels of *miR-196a* in cancer cell lines and human CRC. A: Cancer cell lines SW620, HT29 and Snu16 reveal increased *miR-196a* levels as compared to the primary colon cancer cell line SW480; B: *miR-196a* transcription is up-regulated in two of seven cancer samples in comparison to the matching mucosa sample. In contrast, no down-regulation in the respective tumor samples was observed.

58℃, *HoxB8*: 56℃, *HoxC8*: 62℃, *HoxD8*: 57℃) and elongation (2 min at 72℃). After the last cycle, a final extension (10 min at 72℃) was added and thereafter the samples were kept at 4℃. Seven microliters of the products were run on a 1.8% agarose gel, stained by ethidium bromide and analyzed under UV light.

Statistics analysis

The χ^2 test was used to compare all other patient and tumor characteristics by group. The *t* test was applied to compare results obtained from function assays. For all tests, $P < 0.05$ was considered significant.

RESULTS

miR-196a transcription in cancer cell lines

Real-time analyses of four cancer cell lines revealed U6 adjusted differences in regulation of *miR-196a* (Figure 1A). The SW480 cell line, which was initially isolated from a primary colon cancer, revealed the weakest transcription level. In contrast, SW620 cells, isolated from metastases of the same patient depicted a 2.25-fold up-regulation of *miR-196a*. HT-29, another colorectal cancer cell line revealed a 3.38-fold up-regulation of *miR-196a*. Similarly, SNU16 generated from metastases of a disseminated gastric cancer showed a 2.14-fold up-regulation of *miR-196a*.

miR-196a transcription in colon cancers versus mucosa

Real-time analyses of colon cancer and matching mucosa revealed an U6 adjusted up-regulation of *miR-196a* in two of seven colon cancers samples analyzed (24.3- and 2.5-fold, respectively; Figure 1B). In contrast, five of seven samples did not depict any transcription differences between tumor and mucosa (1.14-, 1.04-, -1.03-, -1.08- and -1.28-fold regulation, respectively).

Functional analysis using miR-196a transiently transfected SW480 cancer cells

Functional analyses did not depict any significant impact of *miR-196a* on proliferation (Figure 2A). Absorbance analyses after 4 d of cell culture revealed the following results: +m*iR-196a*: 1.506 ± 0.079, -m*iR-196a*: 1.533 ± 0.131; $P = 0.66$; (*vs* NS).

Interestingly, transfection with *miR-196a* decreased the adhesion of cancer cells to plastic and fibronectin but not to laminin (Figure 2B). Adhesion analyses revealed following results: for plastic surface: +m*iR-196a*: 10.2% ± 1.15%, -m*iR-196a*: 16.6% ± 1.73%; $P = 0.001$. For laminin coating: +m*iR-196a*: 3.86% ± 1.3%, -m*iR-196a*: 2.84% ± 0.95%; $P = 0.25$; (*vs* NS) and for fibronectin coating: +m*iR-196a*: 10.86% ± 1.64%, -m*iR-196a*: 13.8% ± 1.56%; $P = 0.08$; (NS).

In addition, *miR-196a* transfection resulted in a significant increase of migration and invasion (Figure 2C and D): Migration: +m*iR-196a*: 9.7% ± 3% *vs* -m*iR-196a*: 3.6% ± 2.4%; $P = 0.05$. Invasion: +m*iR-196a*: 12.6% ± 3% *vs* -*miR-196a*: 5.14% ± 3%; $P = 0.039$.

Influence of miR-196a on classical signal cascades

In order to analyze the relevance of *miR-196a* on activation of signal cascades we quantified phosphorylation of AKT and MEK (Figure 3A). Transient transfection with *miR-196a* resulted in an increased phosphorylation of (p)AKT but not of (p)MEK. These results imply that *miR-196a* increases activation of the *PI3K-AKT-mTor* signalling pathway.

Chemosensitivity analyses

Analyses of apoptosis did not reveal any significant impact of *miR-196a* (Figure 3B): +m*iR-196a*: 0.61% ± 0.08% *vs* -m*iR-196a*: 0.62% ± 0.07%, $P = 0.3$; (NS); nor in combination with 5-FU [+m*iR-196a*: 15.67% ± 1.45% *vs* -m*iR-196a*: 14.05% ± 0.74%, $P = 0.18$; (NS)] or irinotecan [+m*iR-196a*: 11.97% ± 0.51% *vs* -m*iR-196a*: 12.06% ± 1.36%, $P = 0.92$; (NS)]. However, *miR-196a* significantly increased chemosensitivity to oxaliplatin (+m*iR-196a*: 13.56% ± 2.08% *vs* -m*iR-196a*: 9.46% ± 1.19%, $P = 0.05$) and cisplatin (+m*iR-196a*: 23.11% ± 1.93% *vs* -m*iR-196a*: 18.42% ± 1.92%; $P = 0.04$). In summary, *miR-196a* increases chemosensitivity to platin derivates.

Lung metastases xenograft

Transient transfection of SW480 cancer cells with *miR-196a* resulted in a significant increase of pulmonary metastases growth after 7 wk of incubation: +m*iR-196a*: 7.5 ± 1.7 *vs* -*miR-196a*: 3.25 ± 0.96, $P = 0.009$ (Figure 3C).

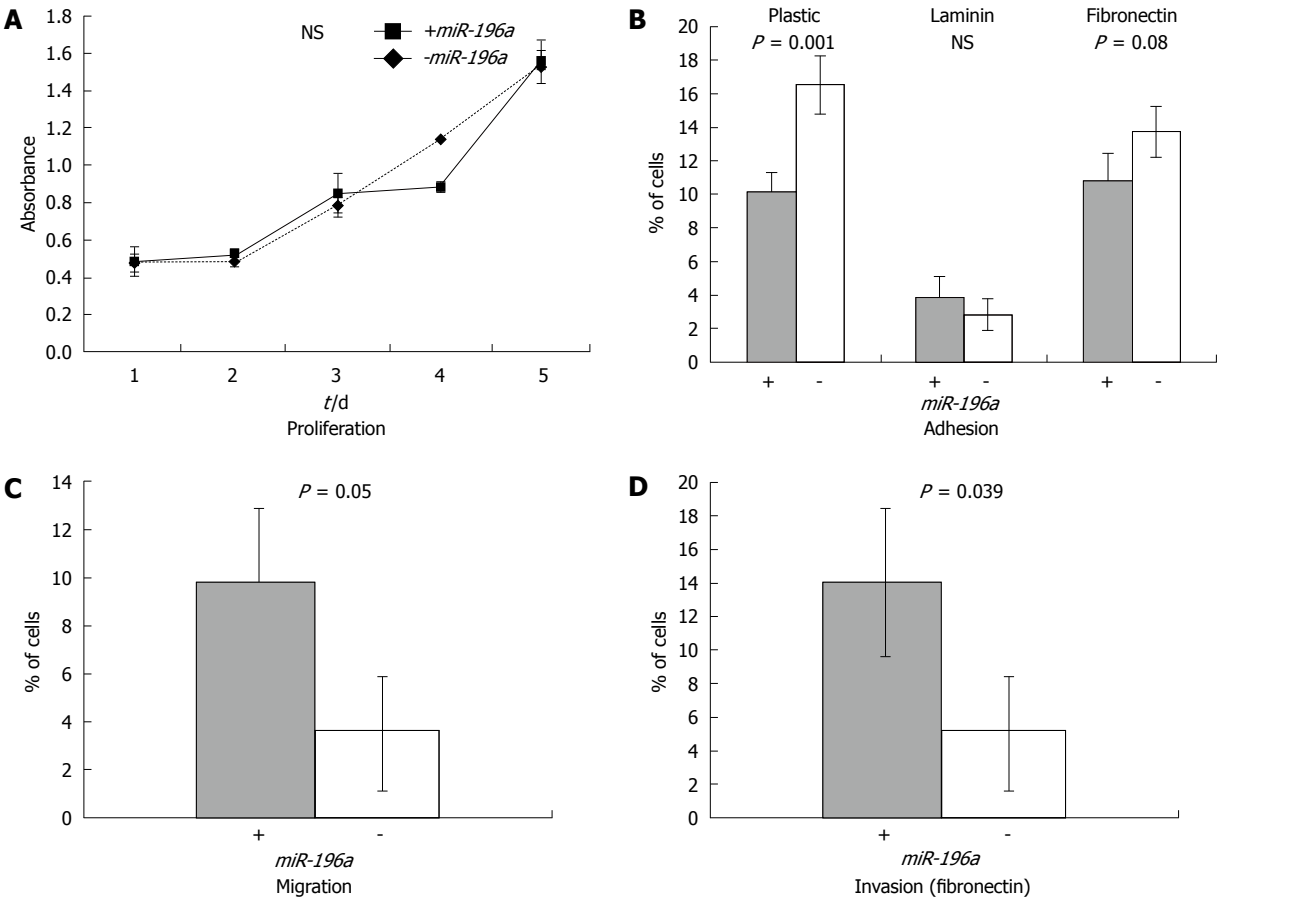

Figure 2 ***In vitro*** **effect of** ***miR-196a*** **in human CRC.** Transient *miR-196a* transfection significantly decreases adhesion, increases migration and invasion but does not impact on proliferation or apoptosis of SW480 colon cancer cells.

Verification of miR-196a target genes

Transient transfection of SW480 cells with *miR-196a* verified *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* as *miR-196a* targeted genes (Figure 4A). However, significant differences in target restriction were observed. While low *miR-196a* concentrations (20 nmol/L) sufficiently restricted *HoxB8* mRNA, higher concentrations were necessary to completely restrict *HoxC8* mRNA and to restrict a significant amount of *HoxD8* mRNA. However, the impact of *miR-196a* on *HoxD8* was weaker than on *HoxC8*. Only the highest *miR-196a* concentrations (240 nmol/L) decreased mRNA levels of *HoxA7*. These data verify the predicted *Hox* genes *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* as human targets of *miR-196a* but also reveal dose-dependent differences in restriction of target genes.

Dose-dependent inhibition of cellular adhesion

Transfection with *miR-196a* significantly decreased the adhesion of cancer cells to plastic in a dose-dependent manner. Numbers reflect the percentage of cells that adhered to the bottom of the well: 0 nmol/L *miR-196a*: 15.21% ± 0.47%; 40 nmol/L *miR-196a*: 14.27% ± 0.46%; $P = 0.07$; (NS); 80 nmol/L *miR-196a*: 12.43% ± 0.42%; $P = 0.002$ and 160 nmol/L *miR-196a*: 10.6% ± 0.3%; $P = 0.0003$ (Figure 4B).

DISCUSSION

Expression patterns of miRNAs are systematically altered in colon cancer as recently described by Schetter and colleagues[25]. In particular, Schetter *et al*[25] reported that at least 37 miRNAs are differentially expressed in colon cancer. Of those the expression profiles of *miR-20a*, *miR-21*, *miR-106a*, *miR-181b* and *miR-203* were validated. Interestingly, high *miR-21* expression was associated with poor survival.

We were interested in the relevance of *miR-196a* transcription for human colorectal cancer progression for specific reasons. Yekta and colleagues described *HoxB8* as a restriction target of *miR-196a* and predicted *HoxA7*, *HoxC8* and *HoxD8* as additional restriction targets in humans[22]. *Hox* genes are known to be master regulators of embryogenesis and oncogenesis[15]. We were able to confirm these data presented by Yekta and colleagues, as mRNA levels of those four *Hox* genes were reduced by *miR-196a*. However, dose-dependent differences in target restriction were observed. While low *miR-196a* concentrations resulted in a complete restriction of *HoxB8* mRNA, higher concentrations of *miR-196a* were mandatory to completely restrict *HoxC8* mRNA and to significantly decrease *HoxD8* mRNA levels. In contrast, even the highest *miR-196a* concentrations did not result in a complete restriction

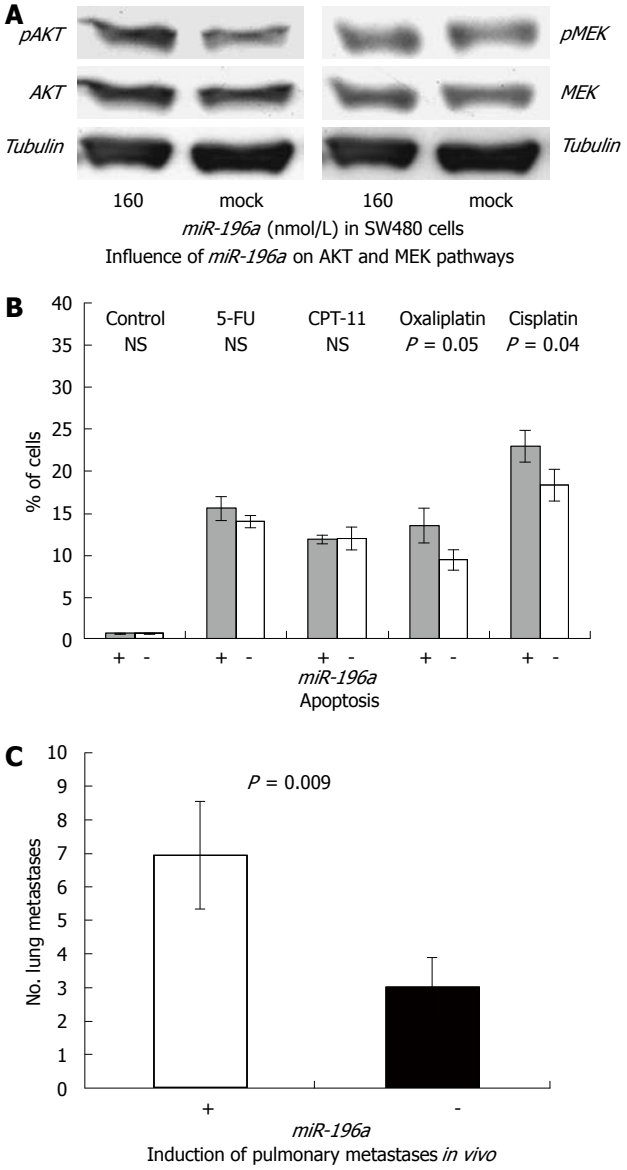

Figure 3 Impact of *miR-196a* on cellular signaling, *in vitro* chemosensitivity and *in vivo* induction of lung metastases. A: *miR-196a* transfection activates the AKT pathway but does not impact on the MEK pathway; B: *miR-196a* transfection significantly increases chemosensitivity towards oxaliplatin and cisplatin but not towards 5-FU or irinotecan; C: *miR-196a* significantly promoted growth of lung metastases in a xenograft biosystem after tail-vein injection and 7 wk of incubation.

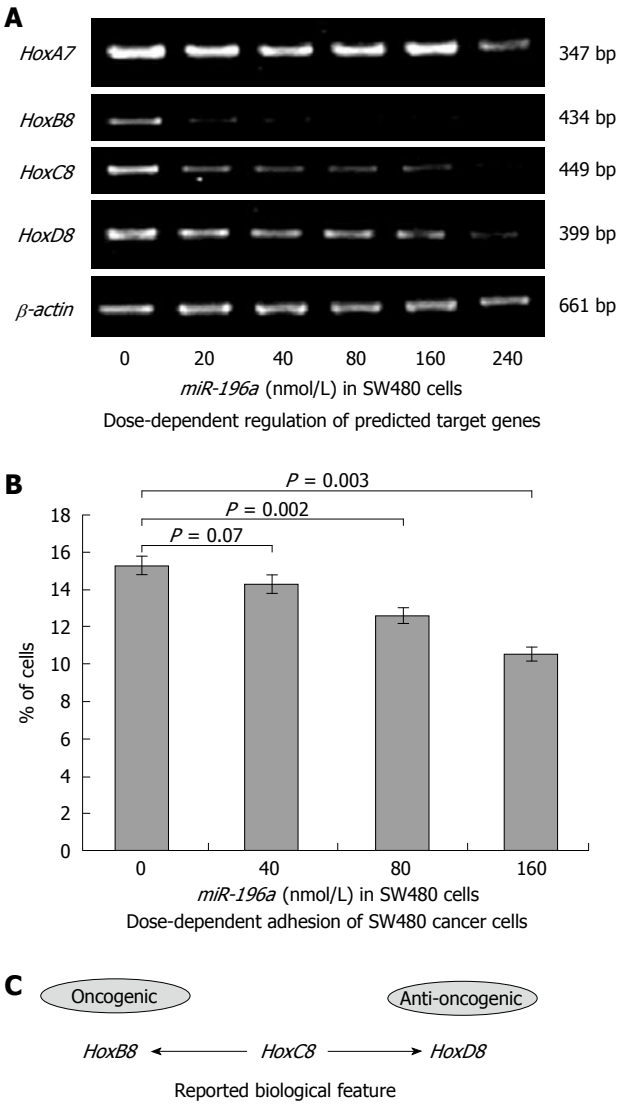

Figure 4 Dose-dependence of *miR-196a* promoted effects. A: *miR-196a* decreases *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* mRNA levels with a dose-dependent and gene-specific character; B: *miR-196a* inhibits cancer cell adhesion to plastic covers in a dose-dependent manner; C: Biological features of *HoxB8*, *HoxC8* and *HoxD8* as reported in literature. *HoxB8* exerts an oncogenic effect, while *HoxD8* might have tumor-suppressive relevance. For *HoxC8* both, pro-oncogenic and anti-oncogenic features, have been reported.

of *HoxA7*. These data clearly reveal mRNA specific and dose-dependent target restriction. To clarify the dose-dependence of *miR-196a* we performed adhesion assays after transfection with different concentrations of *miR-196a*. These assays revealed a dose-dependent inhibition of tumor cell adhesion.

To further analyze the impact of *miR-196a* on tumor cells, we then performed functional assays and found that high *miR-196a* concentrations increased migration and invasion of cancer cells in trans-well assays and inhibited adhesion to different surfaces and matrix proteins. Chemosensitivity assays with standard chemotherapeutics revealed that *miR-196a* does not sensitise against 5-FU nor irinotecan, but does sensitize against the platin derivatives oxaliplatin and cisplatin. However, *miR-196a* did not impact on proliferation or apoptosis of colon cancer cells.

Analyzing signaling cascades that are often altered in human cancer, we observed that induction of the pro-migratory phenotype is most likely linked to activation of the *PI3K-AKT-mTor* pathway, as *miR-196a* increased the level of pAKT. In contrast, no change in the pMEK/MEK ratio was observed. Our data are consistent with earlier reports showing that overexpressed miRNAs can act as oncogenes. A well known example is *miR-17-92*, which is significantly increased in small-cell lung cancer and correlates with a poor prognosis[20]. Interestingly, the known targets of the *miR-17-92* include the two tumor suppressor genes *PTEN* and *RB2*[21]. As a consequence, restriction of *PTEN* unleashes the *PI3K-AKT-mTor* pathway as also observed for *miR-196a*. However, the exact mode of action of *miR-196a* has still to be analyzed.

Quantitative real-time PCR of *miR-196a* in matching colon cancer and colon mucosa samples showed an up-regulation in 28% of samples. In contrast, all other cancer samples revealed no regulation at all. Most interestingly, the metastatic cancer cell lines SW620 and HT29 showed a significant up-regulation of *miR-196a* in contrast to SW480 cells isolated from a primary colon cancer. Therefore, *miR-196a* is up-regulated in a subset of colorectal cancers and might exert an oncogenic function, when transcribed at a high level. Matching these observations, Croce and colleagues recently found that 75% of pancreatic cancers expressed *miR-196a* at a high level, predicting poor patient survival (14.3 mo *vs* 26.5 mo) when investigating the miRNA transcription pattern in pancreatic adenocarcinoma with large scale miRNA chips[24]. Therefore, similar mechanisms seem possible for pancreatic and colorectal cancer.

To verify the oncogenic potential of high *miR-196a* concentrations, we further analyzed the impact of *miR-196a* in an *in vivo* lung metastases xenograft biosystem. After transient transfection of cells with high concentrations of *miR-196a* prior to tail-vein injection, mice developed significantly more pulmonary metastases within 7 wk as compared to mock-transfected cells.

In summary, we observed an oncogenic effect of high *miR-196a* concentrations. However, several data imply that *miR-196a* might function as a double-edged sword with opposing effects at different concentration for following reasons. (1) *miR-196a* is transcribed in colon mucosa at low levels, implying a role for the epithelial phenotype. (2) A hypothesized suppressive effect of low *miR-196a* transcription levels on tumor dissemination might be exerted through a dose-dependent restriction of *miR-196a* target genes *HoxB8*, *HoxC8* and *HoxD8*. Up-regulation of *HoxC8* and *HoxB8* in colorectal cancer was reported as early as 1997, however the relevance of those genes for carcinogenesis had not been analyzed[26]. A relevant leukemogenic property of *HoxB8* mediated through inhibition of differentiation has been described for acute myeloid leukemia[27,28]. These data are intriguing, as low concentrations of *miR-196a* completely restrict *HoxB8*, thus erasing the pro-oncogenic and leukemogenic effects of *HoxB8*. (3) Only very limited data concerning the relevance of *HoxD8* is available, indicating that *HoxD8* are up-regulated after chemical induced re-differentiation of neuroblastoma cells[29]. However, this observation is of particular interest, as high *miR-196a* concentrations are needed to significantly reduce *HoxD8* mRNA levels, which might result in an inhibition of differentiation, thus promoting oncogenic features as observed in our analyses. (4) The data concerning the relevance of *HoxC8* is unclear. Both pro- and anti-oncogenic influences have been discussed. In particular, *HoxC8* was reported to be a retinoic acid induced gene, rescuing *APC* mutants in zebrafish[30]. In contrast, studies on prostate cancer have reported a correlation with aberrant *HoxC8* expression and a malignant phenotype[31,32]. As *Hox* genes are master transcription factors, they might exert different functions at variable expression levels. However, the observation of Croce and colleagues that *miR-196a* predicts poor survival in pancreatic cancer might rather correlate with inhibition of *HoxD8* than *HoxB8* expression, as *HoxD8* has a suppressive and *HoxB8* a progressive character in the literature[24]. Further studies analyzing the clinical and biological impact of *miR-196a*, as well as additional large scale analyses of restriction targets, are warranted.

COMMENTS

Background

MicroRNAs (miRNAs) are small RNA molecules regulating gene expression in vertebrae and non-vertebrae. In humans, more than 50% of all known miRNA genes are located in cancer-associated regions, indicating that miRNAs might play an important role in oncogenesis. Some miRNAs are known to function as oncogenes, while others act as tumor suppressors inhibiting tumor growth.

Research frontiers

Hox proteins are major transcription factors that play a crucial role during embryogenesis, organogenesis and oncogenesis. The miRNA *miR-196a* depicts complementarity to the mRNA of *HoxB8*, *HoxC8* and *HoxD8*. Therefore, the relevance of *miR-196a* for human tumorigenesis has been discussed.

Innovations and breakthroughs

High levels of *miR-196a* activated oncogenic pathways inside the human tumor cells and induced tumor cell detachment, migration and invasion. In addition, *miR-196a* promoted growth of lung metastases in mice. However, *miR-196a* also increased the chemosensitivity towards platin derivatives such as cisplatin and oxaliplatin.

Applications

High levels of *miR-196a* might predict response of cisplatin- or oxaliplatin-containing chemotherapies. In future, suppression of *miR-196a* by anti-miR technologies might inhibit tumor progression and dissemination.

Terminology

miRNAs are endogenous small RNA molecules of 20-25 nucleotides length, regulating gene expression by inhibiting transcription, inducing direct cleavage of the targeted mRNAs or blocking translation through their complementarity *versus* targeted mRNAs at 3' untranslated regions.

Peer review

This is a very interesting study which contributes to our understanding of colorectal cancer, its development and prognosis. The paper is well written.

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