Original Research

MicroRNA-30a promotes invasiveness and metastasis *in vitro* and *in vivo* through epithelial–mesenchymal transition and results in poor survival of nasopharyngeal carcinoma patients

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Abstract

Although microRNA-30a (miR-30a) has been shown to regulate cancer metastasis, the molecular mechanism has not yet been clearly elucidated in nasopharyngeal carcinoma (NPC). The present study was to investigate the miR-30a expression pattern and its potential functions and further to identify its target gene and corresponding clinical applications in NPC. MiR-30a was identified to be down-regulated in NPC primary tumors compared with metastatic tumors using quantitative real-time PCR. Furthermore, over-expression of miR-30a transfected with precursor increased the ability of metastasis and invasion of NPC tumor cells *in vivo* and *in vitro*. E-cadherin was screened as a putative target gene of miR-30a by computational algorithms. Luciferase reporter assays showed that over-expression of miR-30a directly reduced the activity of a luciferase transcript combined with the 3′-untranslated region (3′-UTR) of E-cadherin. Kaplan–Meier survival analysis and log-rank test were analyzed for 1077 NPC patients for overall survival, indicating that a high expression of E-cadherin was beneficial for NPC prognosis ($P = 0.001$). Importantly, NPC patients with high expression of E-cadherin had much lower risk of poor prognosis (hazard ratio = 0.757, $P = 0.017$) using multivariate analysis. In conclusion, miR-30a could play an important role in regulating NPC metastasis and potentially provide useful guidelines for individualized therapy.

Keywords: hsa-miR-30a, nasopharyngeal carcinoma, metastasis, E-cadherin

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Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that negatively regulate gene expression at the transcriptional level by binding to the 3′-untranslated region (3′-UTR) of target mRNAs.[1] During the past 5 years, the importance of miRNAs in cell biology has been confirmed, and particularly, the role in cancer is compelling. It is well established that some miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may function as either tumor suppressors or oncogenes.[2] MiRNAs have also been linked to cell metastasis in different cancer cells. For instance, the enhanced abundance of miR-21 contributes to the growth and metastasis in breast cancer[3] and colon cancer;[4] miR-625 plays an important role in the metastasis of gastric cancer.[5] Recently, it was found that miR-30a was down-regulated and inhibited invasion and metastasis in non–small-cell lung cancer.[6] However, the role of miR-30a still remains unclear in NPC. More extensive investigation is needed to elucidate the role of miR-30a in the development of NPC.

Nasopharyngeal carcinoma (NPC) is a malignancy arising from the mucosal epithelium of the nasopharynx, which is extremely common in Southern China and Southeast Asia. Most patients present with advanced-stage disease, having metastases to regional lymph nodes (LN) or even distant organs at the time of diagnosis.[7] As advanced-stage NPC may exhibit resistance to both chemotherapy and radio therapy, an improved understanding of the mechanism, which is related to metastasis of NPC, is essential for exploring new therapeutic regimes.

In this study, we examined and compared the level of miR-30a in seven NPC patients with primary tumors and corresponding metastasis tumors. In particular, the functions of miR-30a were thoroughly investigated in the malignant progression of NPC *in vitro* and *in vivo*. We performed a luciferase reporter assay to determine whether miR-30a directly binds to E-cadherin. We further analyzed the overall survival for NPC patients with a high expression of E-cadherin.

Materials and methods

Ethics statement

All the human tissue samples for routine pathology use were obtained from the Department of Pathology, Sun Yat-sen University Cancer Center (SYSUCC), with approval of the Institutional Clinical Ethics Review Board at SYSUCC. No informed consent (written or verbal) was obtained for the use of retrospective tissue samples from the patients in the study, since most of the patients were deceased, and informed consent was not deemed necessary and waived by the Ethics Committee.

Patients and clinical tissue samples

All tissue samples were formalin-fixed paraffin-embedded (FFPE) according to the standard procedures. To investigate the miR-30a level in NPC patients, paired primary and metastatic tumors from seven patients aged from 17 to 68 years old were enrolled in the study. Three of the patients were diagnosed with neck lymph-node metastasis, one with axilla lymph-node metastasis, one with lung metastasis, one with bone marrow metastasis, and one with recurrence. Paraffin-embedded specimens were constructed into tissue microarrays from 1077 patients at SYSUCC between January 1, 1991 and December 31, 2004. Protocols and instruments for the tissue array construction were described previously.[8] The clinicopathological characteristics and follow-up data of the patients are summarized in Table 1.

Table 1 Clinicopathological characteristics and follow-up data of 1077 NPC patients

Characteristics	Number of patients
Sex	
Male	803 (74.6)
Female	274 (25.4)
Age (years)	
Median (range)	47 (16–78)
Follow-up time (months)	
Median (range)	70 (1–120)
Clinical stage	
I–II	290 (27.2)
III–IV	775 (72.8)
Missing	12
Therapeutic modality	
Radiotherapy	768 (71.8)
Chemotherapy	12 (1.1)
Radiochemotherapy	290 (27.1)
Missing	7
WHO histological classification	
NKUC	837 (77.7)
NKDC	212 (19.7)
KSCC	28 (2.6)
OS rate (%)	
5 years	67.6

Abbreviations: NPC: nasopharyngeal carcinoma; WHO: World Health Organization; NKUC: non-keratinizing undifferentiated carcinoma; NKDC: non-keratinizing differentiated carcinoma; KSCC: keratinizing squamous cell carcinoma; OS: overall survival.

Cell culture

The human NPC cell lines CNE2 (poorly differentiated), SUNE1, 6-10B (low tumorigenic and metastatic),[9] and HONE1 (poorly differentiated) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, USA). Cell lines were maintained in a humidified incubator at 37°C with 5% CO_2.

Quantitative real-time RT–PCR

Total RNAs were isolated from cell lines and FFPE tissue using Trizol reagent (Invitrogen). We synthesized cDNA using One Step PrimeScript® miRNA cDNA Synthesis Kit (TaKaRa). The expression of mature miR-30a was determined by SYBR Green quantitative real-time PCR amplification on an ABI 7500HT instrument (Applied Biosystems). Each sample was analyzed in triplicate, and U6 was used for normalization. For data analysis, cycle threshold (C_t) for U6 (reference) and miR-30a (sample) was determined in triplicate (shown as arithmetical mean). The primers for qRT–PCR are shown in Supplementary Table 1. The relative expression was calculated using the relative quantification $= 2^{-\triangle\triangle Ct}$.[10]

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was performed following the protocol described, with some minor adjustments, on the website www.Perkinelmer.com. Digoxigenin-labeled locked nucleic acid-modified probes for hsa-miR-30a and negative control (scramble-miR) were purchased from Exiqon (Vedbek, Denmark). The detailed information is shown in the Supplementary text.

Western blot assay

The extracted proteins were separated in 10% SDS–PAGE gel and transferred to the nitrocellulose membranes (Amersham Bioscience). The membranes were hybridized with the indicated primary antibodies against E-cadherin (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA), N-cadherin (1:1000 dilution, R&D), and GAPDH (1:4000, Santa Cruz, USA) at 4°C overnight. In all cases, the corresponding horseradish peroxidase antibody was used (Amersham).

Cell proliferation and colony formation assays

The detailed methods are described in the Supplementary text.

In vitro migration and invasion assays

Scratch and transwell assays were used to determine the migration and invasion abilities of the tumor cells.[11] Details are given in the Supplementary text.

Computational prediction of miR-30a target gene and luciferase reporter assay

We searched for miR-30a targeting using miRNA target prediction algorithms such as TargetScan 6.2, miRBase, miRanda 3.0, and PicTar. Predicted miR-30a targets were identified using luciferase reporter assay. The 3′-UTR of the mRNA sequence of E-cadherin containing the predicted miR-30a-binding site was amplified by PCR. PCR primers are listed in Supplementary Table S1. Briefly, parts of 3′-UTR of wild type of E-cadherin were cloned into the pMIR-reporter vector (Applied Biosystems) downstream of luciferase. These vectors were then co-transfected with pre-miR-30a or miR-control in 293T cells, and luciferase activity was quantified. The graph shows the percentage of remaining luciferase activity calculated by normalizing the miR-30a expression values on the miR-control values.

Immunohistochemistry

The details of the method are shown in the Supplementary text.

Animals and spontaneous lymph-node metastasis assay

Five-week-old female BALB/c-nude mice (Slac Jingda Laboratory Animal Co., Ltd., China) were used for the analysis of tumor metastasis. The spontaneous LN metastasis model has been previously reported.[11] Briefly, a total of 1×10^6 cells were injected into the footpad of left hind limb of each nude mouse. After 30 days, the popliteal LN of the left hind limb was collected on the terminal day for routine tissue staining. The LN volumes were analyzed by volume = width × length × height/2. Mice LNs were routinely fixed and sectioned at 5 mm. To evaluate the micrometastases in mouse LNs, one section in every 10 sequential sections was selected for hematoxylin & eosin (H&E) staining. All experiments were approved by the Institutional Animal Care and Use Committee (reference number: S10211049).

Statistical analysis

Statistics were assessed using SPSS16.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. Unless otherwise noted, the Student's *t*-test was used for comparison between two groups. Spearman's correlation was used to analyze the correlation between clinical features and E-cadherin expression. Receive-operating characteristic (ROC) curves were used to determine the optimal cut-off value for high- and low levels of E-cadherin. The Kaplan–Meier and log-rank test were used to estimate and compare the probability of overall survival. The Cox-regression model was used for assessing the significance of variables for survival. The significance level was defined at $P < 0.05$.

Results

MiR-30a is down-regulated in NPC primary tumors and up-regulated in NPC metastatic tumors

To better understand the functions of miR-30a in NPC patients, seven patients with a primary tumor and a metastatic tumor were first observed using qRT–PCR. Interestingly, a higher level of miR-30a was found in metastatic NPC than in primary NPC. Statistical analysis of the miR-30a expression suggested that the high expression of miR-30a was observed in the metastatic tumor (Figure 1(a), $P < 0.001$). qRT–PCR and FISH were also conducted to detect the miR-30a expression in four NPC cell lines (Figure 1(b) and (c)). The results demonstrating the higher level of miR-30a in the three cell lines such as CNE2, SUNE1, and HONE1 characterized by poor differentiation than in the cell line 6-10B characterized by low metastasis were shown in Figure 1(b) and 1(c). This finding gives a hint concerning the possible metastatic capability of miR-30a.

MiR-30a regulates NPC cell line invasion and migration *in vitro*

To elucidate the functional significance of miR-30a in NPC, we performed wound scratch and transwell assays *in vitro* based on 6-10B and HONE1 cell lines transfected with precursor- and control-miR-30a. The levels of miR-30a were elevated in both 6-10B and HONE1 cell lines transfected with precursor miR-30a (pre-miR-30a) (Figure 2(a), $P < 0.001$), respectively. Over-expression of miR-30a clearly promoted cell invasion in 6-10B and HONE1, compared with control-miR-30a in a time-dependent manner (Figure 2(b), $P < 0.001$). The transwell assay showed that the number of cells transfected with miR-30a precursor passing through the 8-μm pore insert to the underneath well were significantly more than those of cells transfected with control-miR-30a. The assay was performed in triplicate (Figure 2(c), $P < 0.001$). An MTT assay and cloning-formation assay were also conducted to determine whether miR-30a could affect the growth of 6-10B and HONE1 cells. There was no significantly increased viability of 6-10B and HONE1 cells between pre-miR-30a and pre-control (Figure S1(a) and (b), $p > 0.05$). These findings indicate that miR-30a is an important regulator for migration and invasion *in vitro*.

MiR-30a promotes tumor metastasis *in vivo*

To consolidate the results above, the functional assay was further performed *in vivo*. HONE1 cells transfected with pre-miR-30a and miR-30a-control were injected to the left hind foot of mice. As shown in Figure 3(a) and (b), detectable tumor volume and tumor weight of popliteal LN were seen in the HONE1/pre-miR-30a group of mice, while much smaller tumors were detected in the HONE1/miR-30a-control group mice ($P < 0.001$). Consistently, we took

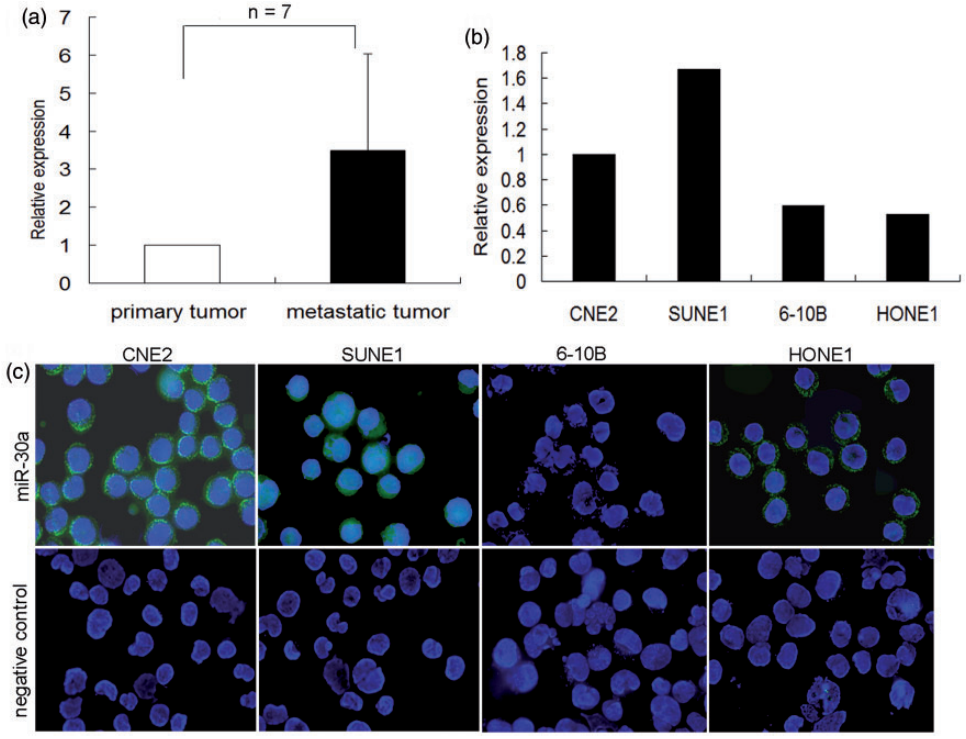

Figure 1 The expression of miR-30a in NPC tissues and cell lines. (a) Total RNAs were used to quantify miR-30a level by relative qRT–PCR in NPC primary tumours compared with corresponding metastatic tumors. Data indicate the mean (±standard deviation) of seven independent pair-matched samples ($P < 0.001$). (b) qRT–PCR assay showed the different level of miR-30a among four NPC cell lines. (c) FISH detection of miR-30a was applied in four NPC cell lines. Positive signal *in situ* hybridization is shown in green, whereas blue part depicts DAPI nuclear staining. (A color version of this figure is available in the online journal.)

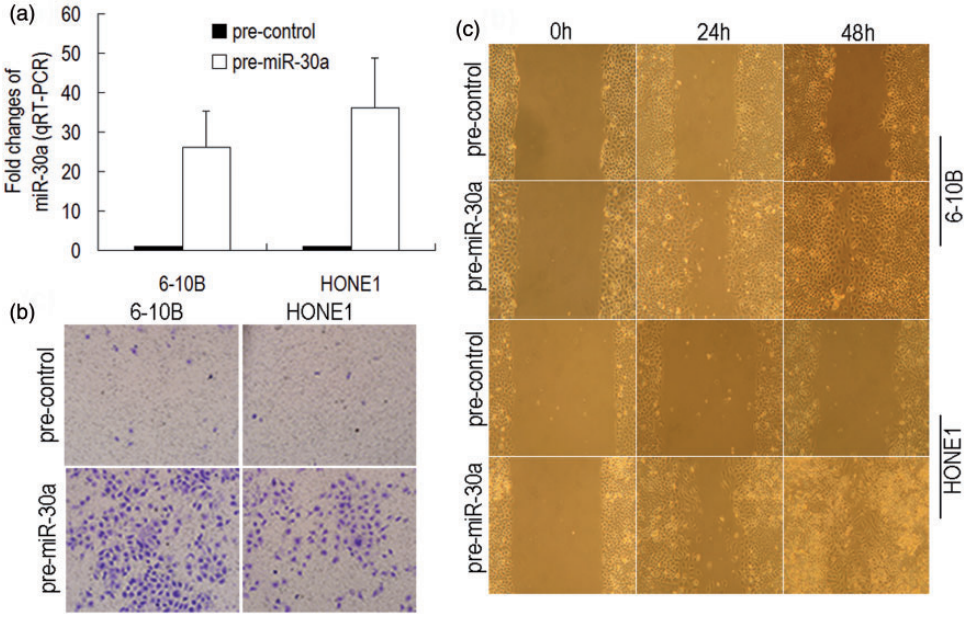

Figure 2 Over-expression of miR-30a promotes NPC cell invasion and metastasis *in vitro*. (a) The expression levels of miR-30a transfected with miR-30a precursor were significantly upregulated in 6-10B and HONE1 cell lines, relative to those of the control ($P < 0.001$). (b) MiR-30a promoted NPC cell invasion after wound scratch in a time-dependent manner ($P < 0.001$). (c) Total numbers of migration cells made by miR-30a were significantly more than control miR-30a ($P < 0.001$). (A color version of this figure is available in the online journal.)

seven metastatic tumors from the popliteal LN of the HONE1/pre-miR-30a group forming larger tumors more rapidly than the HONE1/miR-30a-control group (Figure 3(c)). Histological confirmation was made using H&E staining (Figure 3(d)). Notably, miR-30a expression of xenograft tumor detected by FISH was much higher in the HONE1/pre-miR-30a group than in the HONE1/miR-30a-control group (Figure 3(e)). Taken together, these

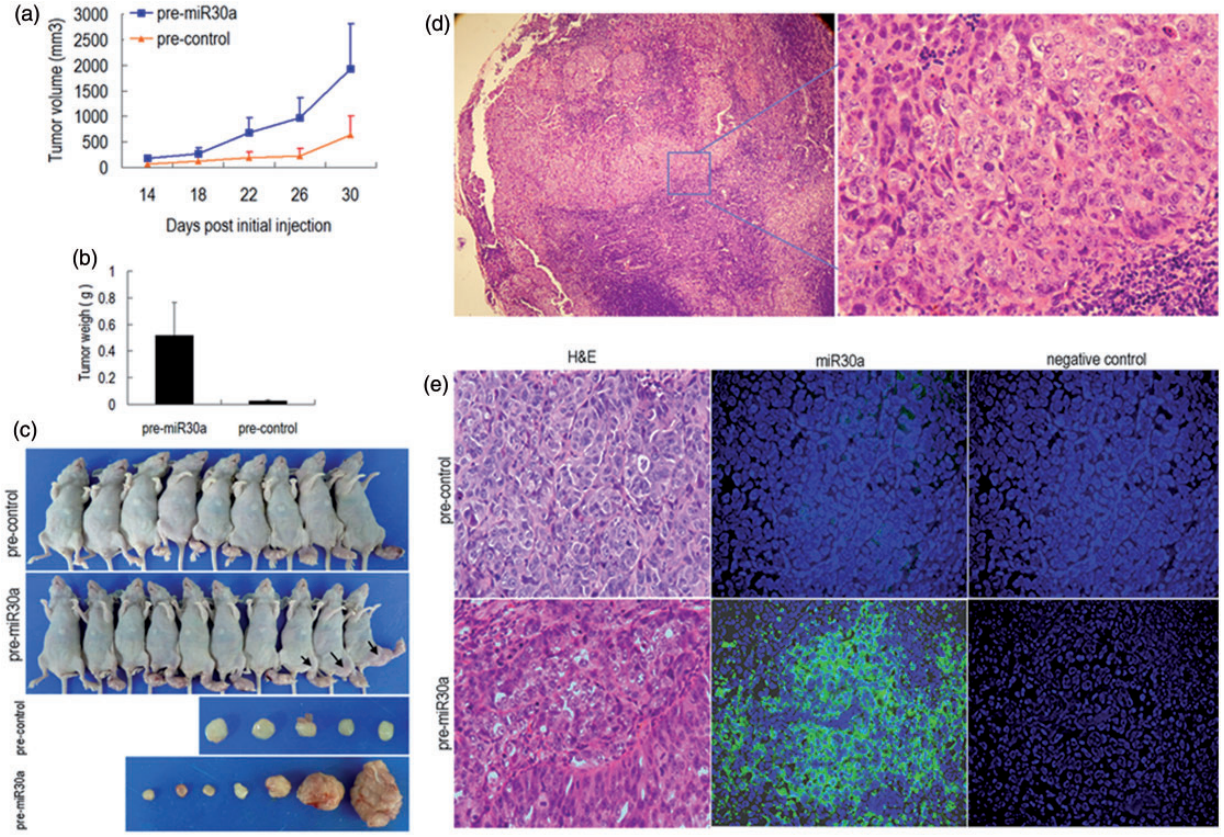

Figure 3 Effect of miR-30a on migration in nude mice. The popliteal lymph nodes (LN) of the left hind foot were observed *in vivo* migration assays. (a) Growth curves for HONE1/pre-miR-30a versus HONE1/pre-control cells in an *in vivo* migration assay. All *P* values were < 0.001. (b) Tumor weight was collected after nude mice were killed at 30 days post injection. Increasing weight of tumor from HONE1/pre-miR-30a was significantly compared with tumor from HONE1/pre-control ($P < 0.001$). (c) Mice and tumors extracted from HONE1/pre-miR-30a and HONE1/pre-control groups. The black arrows indicated the visible tumors. (d) Representative HE image of a popliteal LN after 30 days injection, enlarged image of the framed area in left picture. (e) Representative photomicrographs of FISH for miR-30a on xenograft tumor sections obtained from nude mice. (A color version of this figure is available in the online journal.)

findings demonstrate that miR-30a is involved with NPC metastasis in nude mice.

MiR-30a directly inhibits E-cadherin expression through 3′-UTR

To gain an insight into the functional consequences of miR-30a, we aimed to identify its target genes. TargetScan 6.2, miRBase, miRanda 3.0, and PicTar were employed to search for a direct target of miR-30a. Of all of the hypothetical targets of miR-30a, E-cadherin aroused our interest (Figure 4(a)). Western blot analysis was performed to examine the protein levels of E- and N-cadherin between the groups of pre-miR-30a and pre-control, respectively. The decreased expression of E-cadherin was observed in the two cell lines transfected with pre-miR-30a (Figure 4(b)). The increased level of N-cadherin was shown in the cell lines transfected with pre-miR-30a (Figure 4(b)). To validate further E-cadherin directly binding to miR-30a, we cloned the 3′-UTR of E-cadherin into a reporter plasmid downstream of the luciferase gene to generate pMIR-reporter-E-cadherin and pMIR-reporter-vector. These plasmids including the miR-30a precursor, precursor control, and pCMV-Renilla (internal control) were transiently transfected into HEK 293T cells. At 48 h, after transfection, a luciferase reporter assay system was used to test luciferase activity. Overexpression of miR-30a using a miRNA precursor resulted in a significant decrease in luciferase activity in pMIR-reporter-E-cadherin 3′-UTR-transfected cells (Figure 4(c), right panel, $P = 0.039$), but not in pMIR-reporter-vector-transfected cells (Figure 4(c), left panel, $P = 0.42$). This finding confirmed that E-cadherin is a direct target of miR-30a in NPC.

E-cadherin is correlated to NPC prognosis

To further evaluate the clinical significance of E-cadherin, we performed IHC staining in a set of microarray tissues containing 1077 NPC samples. An optimal cut-off point for high and low levels of E-cadherin was applied using ROC curve. Representative IHC staining is shown in Figure 5(a). The 5-year overall survival (OS) rate of the 1077 NPC patients was 67.6% (Figure 5(b)). The 5-year OS rate of NPC patients with low-E-cadherin expression was 60.8% ($n = 235$), which was significantly lower than that in patients with high-E-cadherin expression (69.3%, $n = 842$; $P = 0.001$). Stratified by clinical stage, the high expression of E-cadherin still remained significantly of benefit to the patients in an advanced stage (Figure 5(b), $P = 0.044$). The different levels of E-cadherin with respect to clinical

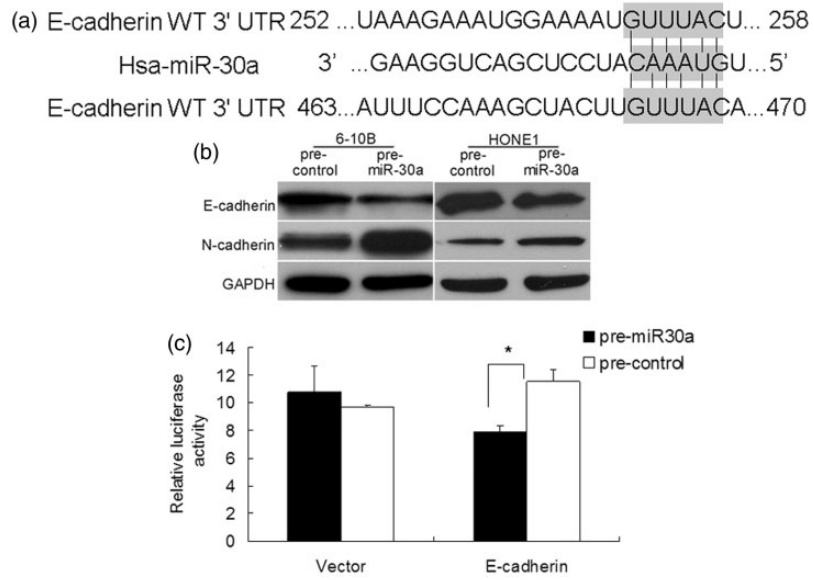

Figure 4 E-cadherin is a direct target of miR-30a. (a) Putative alignment of miR-30a with E-cadherin mRNA 3′-UTR jointly determined by the software TargetScan 6.2, miRBase, miRanda 3.0, and PicTar. Note that the seed matches at the 5' end of miR-30a (gray boxes) and the wild-type nucleotides at two sites of E-cadherin (gray boxes). (b) Western blot analysis was performed to investigate the expression of E-, N-cadherins after 24 h of pre-miR-30a transfection. (c) Luciferase reporter assay showed that miR-30a directly repress E-cadherin through 3′-UTR interactions ($P = 0.039$)

features are detailed in Supplementary Table 2. We found that high expression of E-cadherin was significantly negatively correlated with clinical stage, metastasis, and WHO classification ($P < 0.001$), but no significant association was observed between E-cadherin expression and other clinical features such as age and sex. Multivariate analysis revealed that the high level of E-cadherin expression was an independent, favorable prognostic indicator for OS (hazard ratio [HR] = 0.757, 95% CI = 0.602–0.952, $P = 0.017$) (Supplementary Table 3). Taken together, these results revealed that high-E-cadherin expression significantly correlated with good prognosis for NPC patients.

Discussion

In the present study, we reported for the first time that the level of miR-30a was found to be down-regulated in the NPC primary tumor while up-regulated in the NPC metastatic tumor in the small scale of participants. Furthermore, miR-30a has also been identified to regulate invasion and metastasis both *in vivo* and *in vitro*. The Luciferase reporter assay elucidated that miR-30a functions as a regulator for invasion and metastasis by directly targeting E-cadherin. The clinical data analysis showed that NPC patients with a high expression of E-cadherin had a favorable prognosis. It was concluded that miR-30a alterations may play an important role in NPC invasion and metastasis.

Invasion and metastasis are the main characteristics responsible for the majority of cancer mortality.[12] Intensity-modulated radiotherapy has been widely used in clinical practice and has improved the local control rates for NPC patients significantly. Thus, metastasis is currently the main cause for treatment failure. Recently, there has been increasing evidence of specific miRNAs being involved in the invasion and metastasis of cancer.[4–6,10]

MiR-30 has been frequently found to be one of the microRNAs that are down-regulated in different solid cancers including prostate, colon, thyroid, lung, and liver cancers as well as in leukemia.[10,13–18] A previous Taiwanese miRNAs profiling analysis study[19] demonstrated that miR-30a was decreased in NPC tissues. This is different from our study showing that the lower expression of miR-30a was observed in NPC primary tumors compared with NPC metastatic tumors. In addition, our result also showed that miR-30a level is a bit higher in NPC than in nasopharyngitis though remains no difference between them. There may be several explanations for this discrepancy. Selected patients derived from different areas and small sample size enrolled in the study could lead to this inconsistent result. Different methods used in the study might also yield the bias.

However, the biological function of miR-30a in NPC still remains unknown. Recently, the biological functions of miR-30a were the most reported to be involved in metastasis. A previous study has been identified that miR-30a is highly related to the metastasis of several cancers including breast, lung, bladder, and colon cancers.[20] MiR-30a has already been verified to be associated with metastasis in a large series of HCC.[18] At the same time, the different functions of miR-30 family members have been highlighted in several contexts. Previous investigations showed that there were different functions existing for miR-b/d and miR-30e, which shared a seed region. MiR-30b/d displayed an oncogenic role in melanoma cells, while miR-30e was anti-metastatic in breast cancer as the same as miR-30a. Consistent with the function of miR-30b/d, the cellular and mice experiments in the present study suggested that miR-30a may play a role in NPC metastasis. These different observations emphasize the target-dependence of miRNA function in cancers and alternatively rely upon cancer-specific

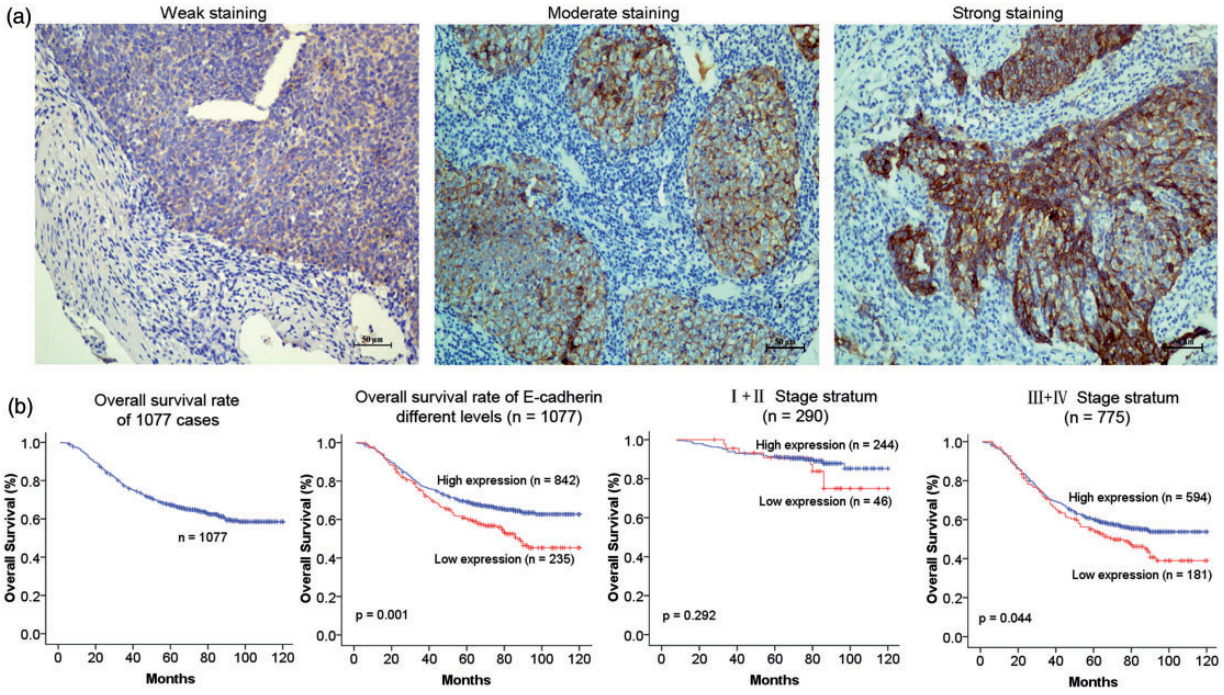

Figure 5 Low expression of E-cadherin has an implication for NPC poor prognosis. (a) Representative staining was shown for E-cadherin expression. (b) Kaplan–Meier survival curve analysis and log-rank test were analyzed for assessing 1077 NPC patients overall survival. High expression of E-cadherin had a significant association with good survival ($P = 0.001$). Stratified by clinical stage, high expression of E-cadherin remains still beneficial for NPC patients with advanced stage ($P = 0.044$). (A color version of this figure is available in the online journal.)

type. To validate the aberrant expression of miR-30a between primary and metastatic NPC, we performed *in vivo* and *in vitro* studies to address the capability of invasion and metastasis and identified that miR-30a is a metastasis-related miRNA in NPC patients. This result suggests that miR-30a may be directly involved in NPC metastasis and may represent a novel diagnostic tool and therapeutic strategies for NPC patients with advanced stage. Considering the importance of miR-30a in predicting prognosis and guiding a treatment in NPC, further studies are required to extend from a single miR-30a to multiple miR-30 family members to understand deeply the cross-talk in metastatic pathways.

Even though the molecular mechanism underlying metastasis is not fully understood, the epithelial–mesenchymal transition (EMT) is still a key regulator in cancer invasiveness and metastasis.[21] It is generally known that EMT is characterized by losing polarized, epithelial phenotype such as E-cadherin down-regulation and acquiring the mesenchymal-like phenotype-like N-cadherin up-regulation. Loss of E-cadherin expression has been implicated in cancer progression and metastasis.[22] Due to their ability to simultaneously target multiple transcripts, miR-30 family members are able to participate in many different pathological process. Several EMT genes for miR-30a have already been identified. MiR-30a has been shown to be involved in down-regulating vimentin during EMT of primary pancreatic epithelial cells in the model of human fetal pancreatic islets.[23] Previous studies have demonstrated miR-30a-targeting vimentin and Snail 3′-UTR functions as regulating migration and invasion in breast cancer[24] and non–small-cell lung cancer,[25] respectively. In particular, evidence is growing that EMT phenomena significantly contribute to NPC metastasis. Pathologists observed a kind of special morphology named ‘neoplastic spindle cells’ in NPC, which was identified as the up-regulation of the mesenchymal markers such as vimentin, Snail1, and predominantly at the leading edge or cancer-cell infiltrating stroma.[26] According to previous studies, various strategies can be applied to screen the putative targets of miRNAs. In the present study, we adopted computational predictions to screen and performed a luciferase reporter assay to validate the miR-30a-targeting 3′-UTR of E-cadherin directly. It was also shown that NPC patients with lower expression of E-cadherin had unfavorable survival. Therefore, miR-30a plays an important role in the metastasis of NPC by inhibiting E-cadherin expression. However, EMT phenomena involving with phenotypic markers like E-, N-cadherins, and transcriptional factors such as Snail and Twist and signaling pathways such as Wnt and Ras are complicated processes that are needed to clarify in the further investigation.

In conclusion, we identified deregulated expression of miR-30a that can distinguish primary NPC and corresponding metastasis tumor, confirming the direct involvement of miR-30a in cancer metastasis. The verification of the miR-30a target gene, E-cadherin, offers more strong evidence in the mechanisms of NPC metastasis. Taken together, our findings suggest that altered miR-30a may monitor metastasis occurrence and represent a promising therapeutic strategy for suppressing NPC metastasis.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; JYS and YXZ conceived and designed the experiments; HYW wrote the manuscript; H-YW, Y-YL, XPW, and SF conducted the experiments; MYH, XZ, QS, and LD contributed reagents/materials; H-YW, Y-YL, and SF contributed equally to this work.

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