Pathology & Oncology Research (2020) 26:1639–1649
https://doi.org/10.1007/s12253-019-00733-x

ORIGINAL ARTICLE

Positive Correlative over-Expression between eIF4E and Snail in Nasopharyngeal Carcinoma Promotes its Metastasis and Resistance to Cisplatin

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Received: 29 November 2018 / Accepted: 27 August 2019 / Published online: 11 September 2019

Abstract

EIF4E is the rate-limiting factor in the mRNA translation of specific set of oncogenes. Snail is the core transcription factor of epithelial-mesenchymal transition (EMT), a key step of cancer metastasis. The connection between the two oncoproteins has not been well established in the human cancer tissues and in nasopharyngeal carcinoma (NPC). Here we showed that the positive correlative over-expression was seen between eIF4E and Snail in NPC tissues, and the expression was significantly higher in the metastatic NPC than in the un-metastatic NPC. In NPC cells, eIF4E knockdown significantly reduced Snail mRNA and protein levels, increased the mRNA level of E-cad (a direct downstream gene of Snail and a negative EMT marker), attenuated the invasive ability of the cells, and sensitized the cells to cisplatin in invasion. In contrast, enforced the expression of eIF4E significantly increased Snail mRNA and protein levels, and promoted the invasive ability in NPC cells. Under the condition of the high eIF4E expression, Snail knockdown significantly increased E-cad mRNA level and weaken the invasive ability of NPC cells. Finally, eIF4E directly bound Snail mRNA for translation initiation displayed by the RIP assay. Therefore, the results firstly suggested that eIF4E enhanced the Snail expression in both transcription and translation manner in human cancer tissues and targeting the eIF4E/Snail axis might intervene with the EMT and metastasis of NPC. This finding provided a new clue for further understanding the metastatic mechanism of human cancers and for preventing and treating NPC metastasis.

Keywords eIF4E · Snail · NPC · EMT · Invasion · Metastasis

Introduction

Nasopharyngeal carcinoma (NPC) is one of the common cancers in southern China, Southeast Asia, the Arctic, and the Middle East/North Africa [1]. One of the characteristics of NPC is the early metastasis that is the fundamental cause of the treatment failure [2]. Epithelial-mesenchymal transition (EMT) has been recognized as a critical step in the invasion and metastasis of cancers, including NPC [3, 4]. Snail is the core transcription factor of EMT, directly suppresses the transcription of E-cad, a negative marker of EMT [5, 6]. Studies showed that Snail over-expressed in NPC and promoted invasion and metastasis of NPC [7, 8].

Eukaryotic translation initiation factor 4E (eIF4E) is the rate limiting factor of the cap-dependent translation [9], and over-expresses in a variety of tumors [10–13]. Whenever over-expressed, eIF4E initiates the translation of specific set of oncogenes that impel the malignant transformation of normal cells and spur the proliferation, invasion, metastasis and anti-apoptosis of cancer cells. As it is well known, EBV infection is closely related to the occurrence and development of NPC. We recently found that the EBV oncogene LMP1 stimulates eIF4E expression in NPC and then promotes the proliferation and invasion of NPC cells [14, 15]. Downregulated β-catenin in the nucleus by targeting the MNK-eIF4E axis with CGP57380 decreased proliferation, cell cycle progression, migration, invasion, and metastasis of NPC [16]. Although the phosphorylated eIF4E was reported to be associated with lymph node metastasis and poor prognosis of NPC [17, 18], the high expression of eIF4E is not associated with lymph

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node metastasis and 3-year survival rate in NPC showed in some other studies [19].

The functional connection between the two important oncoproteins eIF4E and Snail was seen in the study that the phosphorylation of eIF4E could enhance the expression of Snail and MMP3 to promote the EMT and metastasis of mouse embryonic fibroblasts (MEFs) and mouse breast cancer [20]. In the lung epithelial cells, 4Ei-1, a specific chemical antagonist of the eIF4E-mRNA cap interaction, blocks ribosome recruitment to the Snail transcript accompanied by accumulation of Snail protein in the nucleus [21]. RACK1 promoted the phosphorylation of eIF4E and the translation of Snail in hepatic stellate cells [22]. The retinamide VNLG-152, gal and VNPT55 profoundly inhibit prostate cancer cells and xenograft cancer possibly by down-regulating protein expression of Snail, Slug, N-cadherin, vimentin, and MMP-2/−9 via antagonizing the Mnk-eIF4E axis [23, 24]. However, the connection between eIF4E and Snail has not been seen in human cancer tissues and in NPC.

Basing on the previous clues, we assumed that eIF4E could effect on Snail expression to promote EMT, invasion and metastasis of NPC in both tissue and cellular level. In order to test the hypothesis, we detected the protein expression of eIF4E and Snail in human NPC specimens, analyzed the expression relation between the two proteins, and knocked down eIF4E or transfected eIF4E to observe the change of the expression of Snail and E-cad and the invasive ability of NPC cells. Our results firstly showed that eIF4E promotes EMT, invasion and metastasis via enhancing the expression of Snail in NPC tissue and cellular level. The finding will elucidate the new molecular mechanisms of NPC metastasis, and lay a foundation for improving the prevention and treatment of NPC metastasis.

Material and Methods

Specimens

Total 124 cases of nasopharyngeal tissues from 2009 to 2012 were collected from the pathology department of Shunde Hospital of Chinese Medicine in south China. The Hospital Research Ethics Committee has approved the tissue work. The tissues contained 36 cases of chronic nasopharyngitis (NGI, as the control), 42 cases of un-metastatic NPC (un-NPC) when diagnosed, and 46 cases of metastatic NPC (met-NPC). The proportion of male to female was 22:9. The average age for the NPC patients was 46.0 years. All samples were fixed with Faure Marin and embedded in paraffin wax.

Immunohistochemical Staining

The staining procedure and the staining scoring system were described previously [14, 18]. Briefly, Paraffin embedded tissues were sectioned by 4~6 μm thick, the sections were added with the primary antibodies (1:200 mouse anti-human eIF4E monoclonal antibody, Cat:SC-9976, SantaCruz; and 1:100 Rabbit anti-human Snail polyclonal antibody, Cat:AP2054a, Abgent) and incubated at 4 °C overnight and then visualized with the SP-9000 immunohistochemistry kit (Zhongshanjinqiao Biological Technology Co., Ltd., Beijing) .

NPC Cell Lines, Cell Culture and Treatment of the Cells with Cisplatin

CNE-2 is a cell line originally established from an NPC with poor differentiation. CNE2-EP is the CNE2 cells stably tranfected with the shRNA plasmids targeting eIF4E, and CNE2-EC is the negative control for CNE2-EP. CNE2-OE is the CNE2 cells transiently transfected with eIF4E expressing plasmids, and CNE2-OC is the blank control for CNE2-OE. CNE2-SE is the CNE2 cells stably transfected with eIF4E expressing plasmids, and CNE2-SC is the blank control for CNE-SE. CNE2-SES is the CNE2-SE cells transfected with Snail-siRNAs, and CNE2-SEC is the CNE2-SE cells transfected with the negative control siRNAs. The cell lines above all were established and maintained in our laboratory. The cells were cultured with the methods described previously [14]. For the chemical treatment of CNE2-EC and -EP cells for 48 h, cisplatin (DDP) was added in the final concentration of 2 μg/ml (IC50 for CNE2 cells).

Construction of Plasmids and Synthesis of shRNA Fragments

The primers 5'-CCGCTCGAGCGATGGCGACTGTCGAACCGG-3′ and 5'-CGGAATTCCAGAAGGTGTCTTCTTAAAC-3′ were used to amplify the full-length encoding sequence of eIF4E from the human cDNAs. The PCR products were cloned into pEGFP.C1 vector, which was then named as pEGFP.C1-eIF4E. The empty plasmid was called pEGFP.C1-C as the negative control.

The shRNA-pGCL-GFP-eIF4E plasmid targeting eIF4E was constructed by Shanghai Genechemical Technology Co. The functional targeted sequence was 5'-GAGGACGATGGCTAATTACAT-3′. The sequence of the negative control shRNA-pGCL-GFP-NC plasmid was not found in genomes of mice, rats, human, EBV or HPV.

Based on human genome sequence, three small RNA interference sequences specifically targeting Snail gene were designed by Shanghai Jierui Biotechnology Limited Company. The nomenclature and the small RNA interference sequences were: Snail-siRNA1, 5'CCUUCGUCCUUCUCCUCUAdTdT3' and 3'dTdTGGAAGCAGGAAGAGGAGAU5'; Snail-siRNA2, 5'AACUGCAAAUACUGCAACAdTdT3' and

3'dTdTUUGACGUUUAUGACGUUGU5'; and Snail-siRNA3, 5'ACUCAGAUGUCAAGAAGUAdTdT3' and 3'dTdTUGAGUCUACAGUUCUUCAU 5′. The NC-siRNA fragment was provided as the negative control by the company. According to our pre-experiment, Snail-siRNA3 was the most efficient one among the three fragments and thus was selected for the later experiments.

Transfection of Cells with Plasmids or siRNA Fragments

The transfection procedure was described previously [14]. Briefly, 4 μg plasmids or 100pM siRNAs was mixed with Lipofectamine 2000 (Invitrogen) in a proper proportion and added to the cells. After 6 h, the cells were cultured with the conventional medium. To establish stable cell lines, puromycin was added to screen (1 μg/ml) and maintain (0.5 μg/ml).

Measurement of mRNA Levels of eIF4E, snail and E-Cad with qRT- PCR

The measurement procedure was described previously [14]. Briefly, the sequences of the primers were as follows: 5'-CTGCGGCTGATCTCCAAG-3′ and 5'-TTCCCACA TAGGCTCAATACC-3′ for eIF4E; 5'-CGCGCTCT TTCCTCGTCAG-3′ and 5'-TCCCAGATGAGCAT TGGCAG-3′ for Snail; 5'-ACAGCCCCGCCTTATGATTC TC-3′ and 5'-AAGCGATTGCCCCATTCGTT-3′ for E-cad; and 5′- TGACTTCAACAGCGACACCCA-3′ and 5′-CACCCTGTTGCTGTAGCCAAA −3′ for GAPDH (as internal control). The $2^{-\Delta\Delta Ct}$ method was used to count the qRT-PCR value and the relative expression level of the target genes was calculated by the values of target genes/ GAPDH. The experiment was repeated three times.

Measurement of Protein Level of eIF4E and Snail with Western Blot

The measurement procedure was described previously [14]. Briefly, the cells were harvested and lysed with the RIPA buffer (Santa Cruz), after electrophoresis in SDS-PAGE gel the protein was transferred to the PVDF membranes, the proteins were blotted with the primary antibodies (1:500 eIF4E, 1:100 Snail and 1:500 β-actin, Santa Cruz) and the Luminol Reagent (Santa Cruz).

Cell Invasion Analysis

The used Transwell chamber was separated into upper and low parts with a polycarbonate membrane (Corning Costar Corp) coated with 0.1 ml Matrigel (50 mg/ml, BD Biosciences, USA). The procedure of Transwell analysis was described previously [15]. Briefly, 1×10^4 cells/well in 1640 containing 1% FBS were seeded onto the upper part of the chamber and 0.6 ml 1640 containing 10% FBS was placed in the low part. After cultured at 37 °C in 5% CO^2-in-air for 48 h, the membranes in the chamber were taken out and the non-invasive cells on the upper surface of the membrane were wiped off. Lately, the membranes were dyed by 0.1% crystal violet and the invasive cells were then recorded under microscope (100×). Each experiment was repeated three times.

RNA-Binding Protein Immunoprecipitation Assay

To determine if eIF4E can bind Snail mRNA, a RIP-assay [25] was performed with the RIP-Assay kit (Magna RIP™ Kit, Millipore, USA) by following the manufacture's steps. The cell lysates were derived from CNE2 cells with high expression of endogenous eIF4E and Snail. Four groups of the reaction were done with the cell lysate and beads labeled antibodies, the rabbit anti-IgG added in the negative control group (NC group), the anti-U1-SNRNP70 antibody added in the positive internal control group (PC group), the anti-eIF4E antibody added in the eIF4E group and no antibody added in the input group. The rabbit anti-IgG and the anti-U1-SNRNP70 antibody were included in the Magna RIP™ Kit. The anti-eIF4E antibody was supplied by Abcam as described previously. The target proteins and the non specific mRNAs bound to the beads were then isolated, and the mRNAs binding the target proteins and the non specific mRNAs were measured by qRT-PCR following the manufacturer's procedures. The primer sequences were 5'-GGGAGATACCATGATCACGA AGGT-3′ (FOR) and 5'-CCACAAATTATGCAGTCGAG TTTCCC-3′ (REV) for U1 cDNA, and 5'-CGCGCTCT TTCCTCGTCAG-3′ and 5'-TCCCAGATGAGCAT TGGCAG-3′ for Snail cDNA. The experiment was repeated three times and the average relative level of Snail mRNA was calculated.

Statistical Analysis

SPSS 17.0 statistical software was used to analyze the data. An alpha value of $P < 0.05$ was considered statistically significant and a value of $P < 0.01$ was considered highly significant.

Results

Protein Expression of eIF4E and Snail in the NGI and NPC Tissues

In order to confirm whether the protein expression of eIF4E and Snail is associated with the occurrence and metastasis of NPC, the immunohistochemical staining of NGI, un-NPC and

met-NPC was conducted. As shown in Fig. 1, the cells with the positive expression of eIF4E or Snail appear yellow and/or brown color in cytoplasm and/or nucleus. Otherwise, the cells with the negative expression appear no yellow and/or brown color. There were positive and negative cases for the two proteins in NGI, un-NPC and met-NPC tissues. As showed in Table 1, the positive rates were 11.8%, 71.9% and 93.9% for eIF4E, and 15.2%, 58.8% and 84.8% for Snail, in the NGI, un-NPC and met-NPC cases, respectively. The expression rates of both eIF4E and Snail were significantly higher in NPC than in NGI and in met-NPC than in un-NPC. The pathological results confirmed that eIF4E and Snail overexpressed in NPC and pushed the initiation and metastasis of NPC.

Correlation of Expressions of eIF4E and Snail in NPC

To understand whether the expression of eIF4E and Snail was correlated in NPC, the correlation of the expressions of the two proteins was analyzed. As showed in Table 2, the expression correlation coefficients (r) between eIF4E and Snail were 0.87 *(P < 0.05)* in un-NPC and 0.66 *(P < 0.05)* in met-NPC, respectively. The two rs showed that the expressions of eIF4E and Snail were closely positively correlated in both un-NPC and met-NPC, indicating that there is the cooperation between the two proteins.

Table 1 EIF4E and Snail expression in nasopharyngeal tissues

groups	eIF4E % (P/I)	Snail % (P/I)
NGI	11.8 (4/34)	15.2 (5/33)
un-NPC	71.9 (23/32)**	58.8 (20/34)**
met-NPC	93.9 (31/33)**/◆	84.8 (28/33)**/◆◆

Footnotes: NGI, nasopharyngitis; un-NPC, the un-metastatic NPC; met-NPC, the metastatic NPC; P, the positive cases; I, the informative cases; $^{**}p < 0.01$, compared with NGI group. $^{◆}p < 0.05$ and $^{◆◆}p < 0.01$, compared with un-NPC group

eIF4E Regulated Snail Expression in NPC Cells

To know whether eIF4E regulates the expression of Snail in NPC cells, the eIF4E expressing plasmids or shRNA plasmids targeting eIF4E were transfected into NPC cells to observe the expression change of Snail.

Effect of eIF4E knockdown on the Expressions of Snail and E-Cad (a Direct Downstream Gene of Snail) in CNE2 Cells

As shown in Fig. 2, compared with the control CNE2-EC cells, the mRNA and protein levels in stable eIF4E knockdown CNE2-EP cells were decreased by 80.9% and 73.7% for eIF4E, and by 60.4% and 48.0% for Snail, $P < 0.05$, respectively. Following the drop of eIF4E and Snail, the mRNA

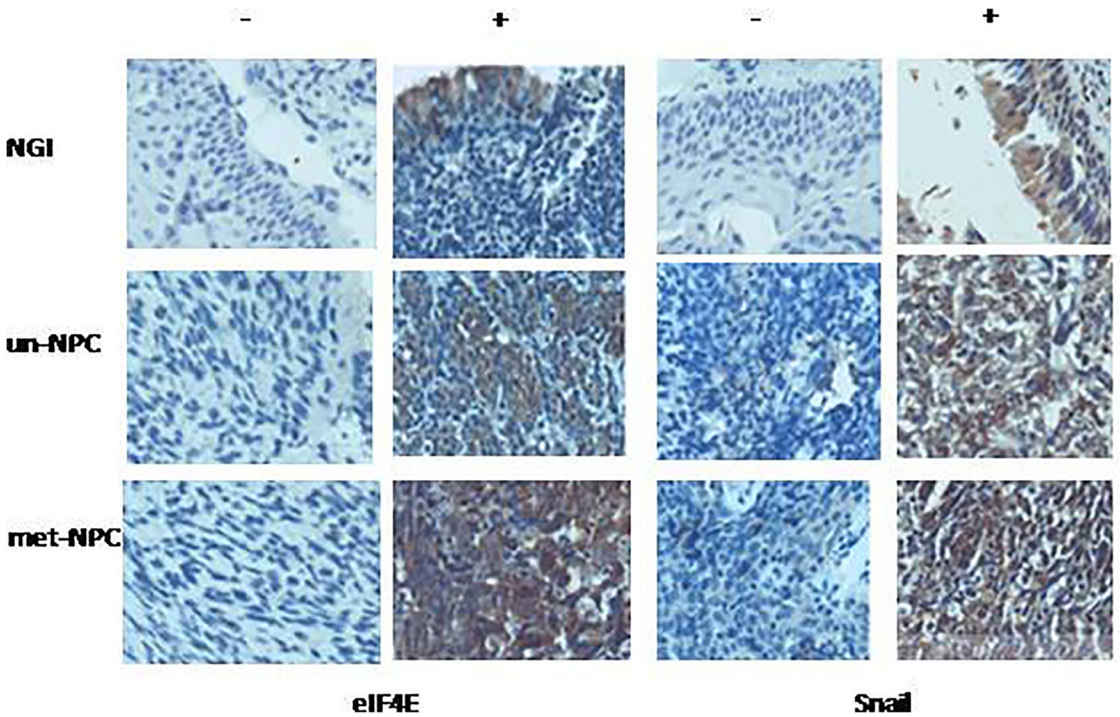

Fig. 1 Representatives of immunohistochemical staining of eIF4E and Snail in nasopharyngeal tissues. NGI, nasopharyngitis; un-NPC, the un-metastatic NPC; met-NPC, the metastatic NPC. -, negative staining, with no yellow/brown color in cytoplasm/nucleus; +, positive staining, with yellow/brown color in cytoplasm/nucleus. (200×)

Table 2 Spearman analysis of eIF4E and Snail expression in un-metastatic and metastatic NPC

NPC	eIF4E	Snail				*r*	*p*
		–	+	++	+++		
un-NPC (24 ICs)	–	8	0	0	0	0.87	<0.05
	+	1	3	1	0		
	++	0	2	5	1		
	+++	0	0	3	0		
20 ICs)	–	2	0	0	0	0.66	<0.05
	+	0	2	1	0		
	++	0	1	8	1		
	+++	0	1	1	3		

Footnotes: un-NPC, the un-metastatic NPC; met-NPC, the metastatic NPC; IC, the informative cases; − and + ~+++, the negative and positive degrees; r, the correlation co-efficient

level of E-cad was increased by 92.0%, $P < 0.05$. The result displayed that eIF4E knockdown impaired the expression of Snail and enhanced the expression of E-cad.

Effect of the Transient Transfection of eIF4E on Snail Expression in CNE2 Cells

As shown in Fig. 3, compared with the control CNE2-OC cells, the mRNA and protein levels in CNE2-OE (transiently transfected by eIF4E expressing plasmids) cells were increased by 191.0% and 184.0% for eIF4E, and by 99.3% and 106.0% for Snail, $P < 0.05$, respectively. It was seen that the amount of the endogenous eIF4E protein changed a little but the remarkable amount of the exogenous eIF4E protein (fused with GFP protein)

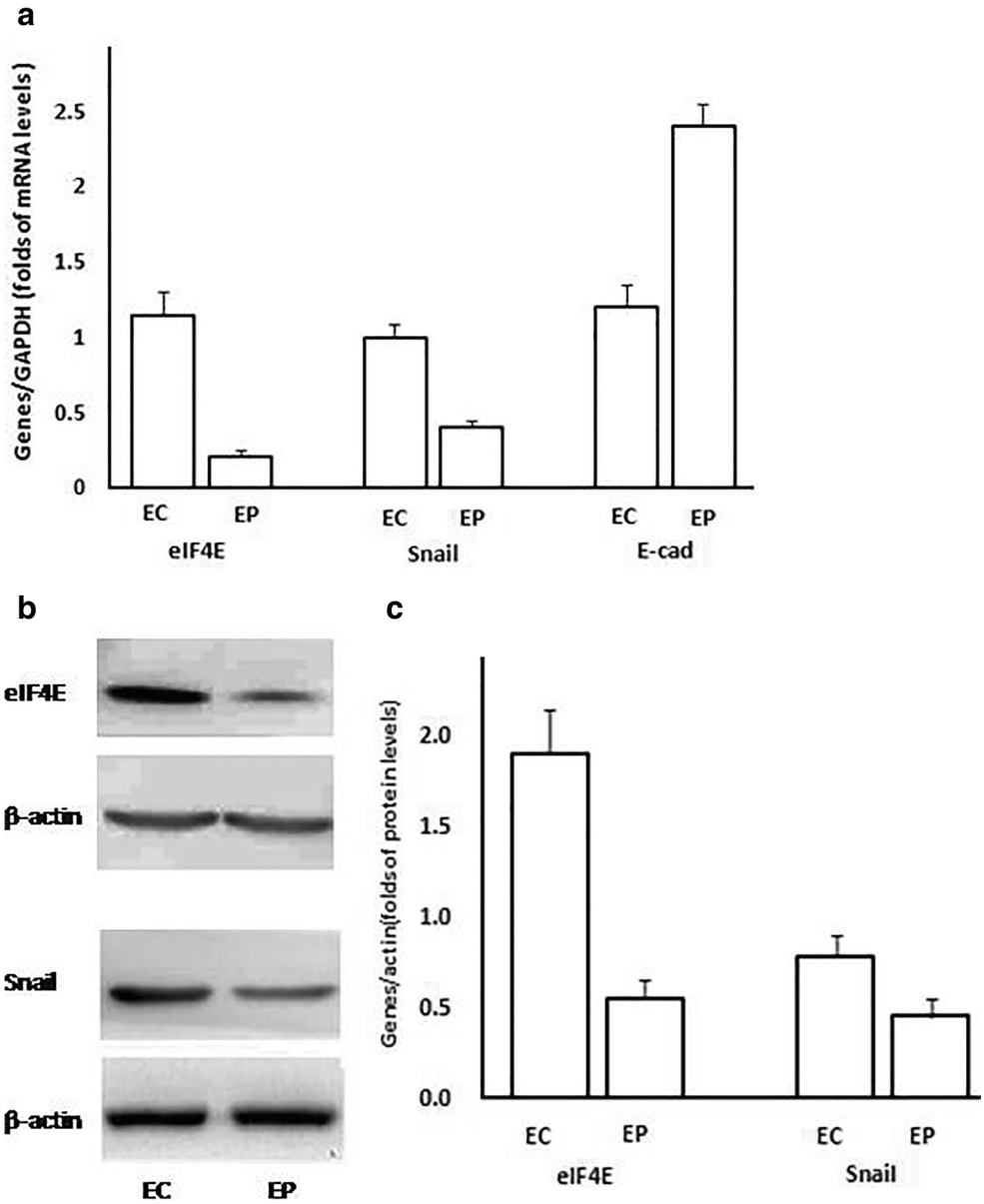

Fig. 2 The expression change of eIF4E, Snail and E-cad (the direct downstream gene of Snail) in CNE2 cells with the stable eIF4E knockdown. **a** The relative mRNA levels of eIF4E, Snail and E-cad (qRT-PCR). The height of the bar diagrams represented the qRT-PCR value of the target genes/GAPDH. **b&c** The relative protein levels of eIF4E and Snail (WB). The height of the bar diagrams represented the band gray value of the target genes/actin. EC, the control cells stably transfected with the negative shRNA plasmids; EP, the CNE2 cells stably transfected with shRNAs targeting eIF4E

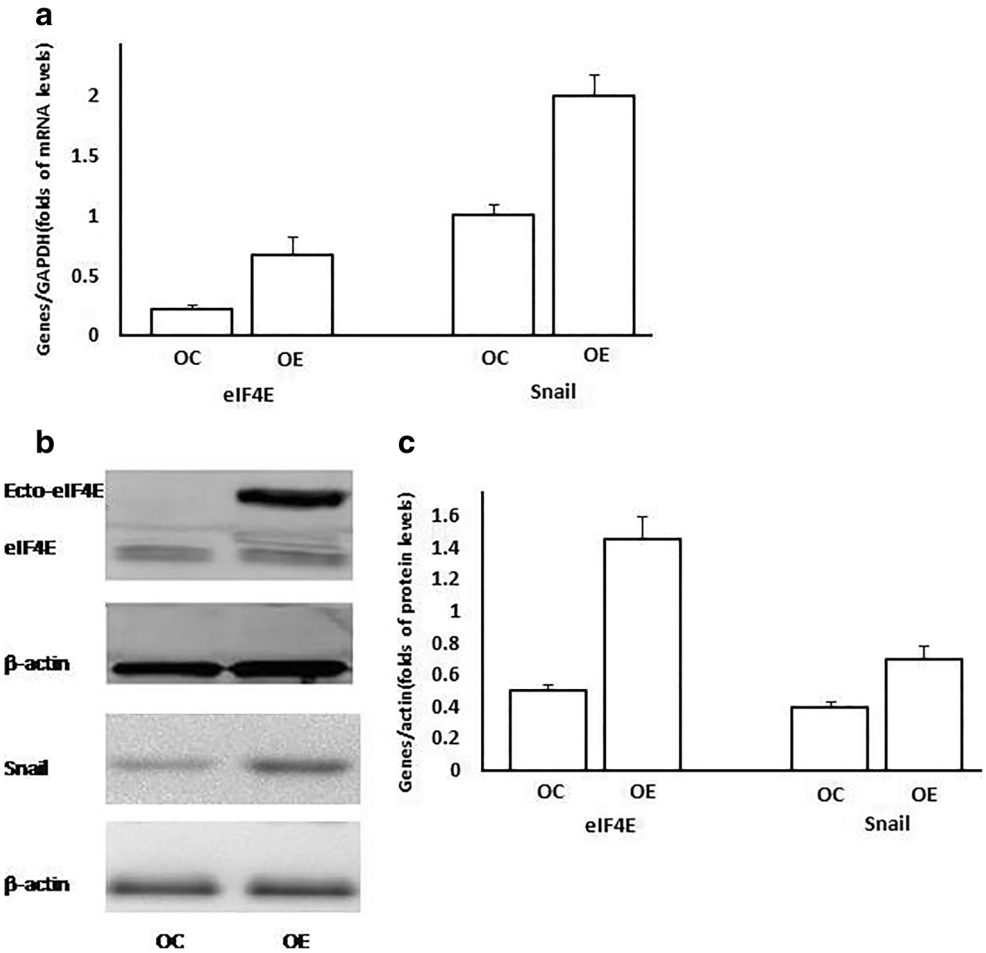

Fig. 3 The expression change of eIF4E and Snail in CNE2 cells with the transient transfection of eIF4E expressing plasmids. **a** The relative mRNA levels of eIF4E and Snail (qRT- PCR). The height of the bar diagrams represented the qRT-PCR value of the target genes/GAPDH. **b&c** The relative protein levels of the ecto-eIF4E and Snail (WB). The height of the bar diagrams represented the band gray value of the target genes/actin. OC, the blank control cells; OE, the CNE2 cells transiently transfected with eIF4E expressing plasmids

appeared in the transfected cells. The result displayed that eIF4E over-expression enhanced Snail expression at both mRNA and protein levels.

Establishment of CNE2-SE Cell Line with Stable Expression of Exogenous eIF4E

The CNE2-SE cells with stable expression of exogenous eIF4E and the negative control CNE2-SC cells were established through transfecting the pEGFP.C1-eIF4E expression plasmid and the pEGFP.C1 blank vector into CNE-2 cells, respectively. RT-PCR was used to detect the mRNA level of the CNE2-SE and CNE2-SC cells (Fig. 4c). Compared to CNE2-SC cells, the eIF4E mRNA level of CNE2-SE cells was increased by 958.0% ($P < 0.05$), indicating that CNE2-SE cell line with stable expression of exogenous eIF4E was successfully established.

Effect of eIF4E on Invasive Ability of NPC Cells

To determine whether eIF4E affected the invasive ability of NPC cells, we detected the change of the invasive ability of CNE2-SE cells that were steadily expressed exogenous eIF4E and of CNE2-EP cells that were stably knocked down with endogenous eIF4E. As shown in Fig. 4a, compared with the control CNE2-EC cells, the invasive ability of CNE2-EP cells was decreased by 41.3% ($P < 0.05$). Compared with the control CNE2-SC cells, the invasive ability of CNE2-SE cells was increased by 37.0% (Fig. 4d&e, $P < 0.05$). The results indicated that eIF4E promoted the invasive ability of NPC cells.

EIF4E Regulated the Invasive Ability of NPC Cells Via Snail

Since eIF4E promoted the invasion of NPC cells and enhanced the expression of Snail, eIF4E might regulate the invasion of NPC cells through Snail. Under the condition of the stable over-expression of eIF4E, CNE2-SE cells were transiently transfected with Snail-siRNA fragments specifically targeting Snail (afterward, called CNE2-SES cells) to detect the change of the invasive ability of the cells. As shown in Fig. 5, compared to the control CNE2-SEC cells that transfected with the negative siRNA fragments, the mRNA levels in CNE2-SES cells were decreased by 70.0% for Snail and increased by 88.5% for E-cad (Fig. 5a, $P < 0.05$),

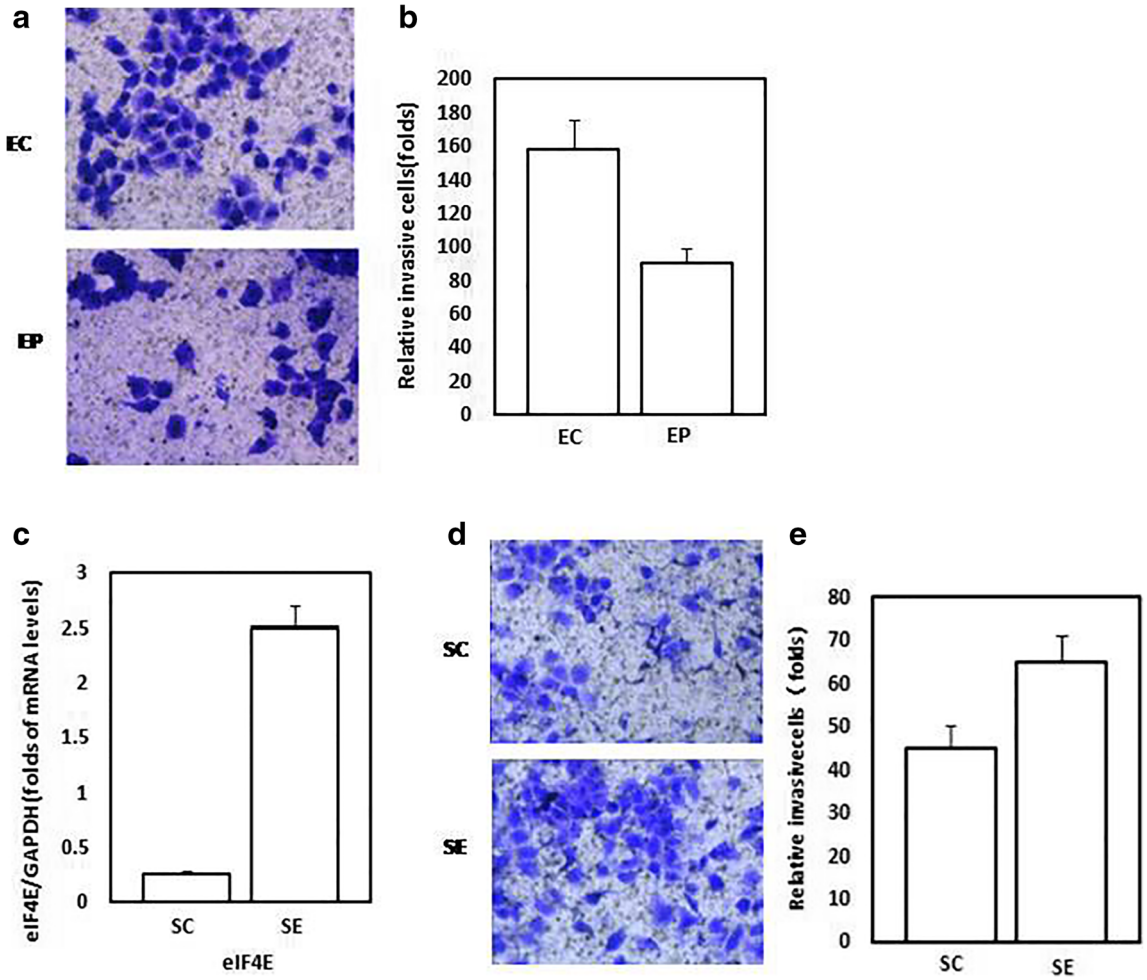

Fig. 4 eIF4E promoted the invasive ability of CNE2 cells (Transwell invasion analysis). **a&b** The invasive ability of the CNE2 cells was decreased by eIF4E knockdown. The cells on the Transwell membrane were dyed blue. The height of the bar diagrams represented the number of the invasive cells. **c** eIF4E mRNA level was enhanced in CNE2 cells stably transfected with eIF4E expressing plasmids (qRT- PCR). The height of the bar diagrams represented the qRT-PCR value of eIF4E/GAPDH. **d&e** The invasive ability was increased in CNE2 cells stably transfected with eIF4E expressing plasmids. The cells on the Transwell membrane were dyed blue. The height of the bar diagrams represented the number of the invasive cells. EC, the control cells stably transfected with the negative shRNA plasmids; EP, the CNE2 cells stably transfected with the shRNA plasmids targeting eIF4E; SC, the control cells transfected with the blank vectors; SE, the CNE2 cells stably transfected with the eIF4E expressing plasmids

and the invasive ability of the cells was decreased by 60.1% (Fig. 5c&c, $P < 0.05$). The results indicated that eIF4E regulated the invasive ability of NPC cells via Snail.

EIF4E Knockdown Sensitized CNE2 Cells to DDP in Invasive Ability

In order to determine whether eIF4E affected the sensitivity of CNE2 cells to DDP in invasive ability, CNE2-EC and CNE2-EP cells were treated with DDP (2 μg/ml) for 48 h to detect the change of invasive ability. As showed in Fig. 6, DDP decreased the invasive ability of CNE2-EC cells by about 50.0%. Compared with CNE2-EC cells, eIF4E knockdown reduced the invasive ability of CNE2-EP cells by about 40.0%, DDP furthered inhibiting the invasive ability of CNE2-EP cells by about 37.0%. The results indicated that eIF4E knockdown sensitized CNE2 cells to DDP in invasive ability.

EIF4E Bound Snail mRNA Determined by RIP Assay

Using the U1 primers, there were strong qRT-PCR signals in the PC group but no/faint signals in the NC, eIF4E and input groups, indicated that the RIP system worked specifically. Using the Snail primers, there were strong qRT-PCR signals in the eIF4E group, no signal in the NC group, and low signals (noise signal created by the non specific biding of Snail mRNAs to the beads) in the PC and input groups. The qRT-PCR signals in the eIF4E group was 165.227 ± 31.503 times ($n = 3$) as in the PC group, and 44.882 ± 0.019 times (n = 3) as in the input groups, suggesting that eIF4E specifically bound the Snail mRNAs.

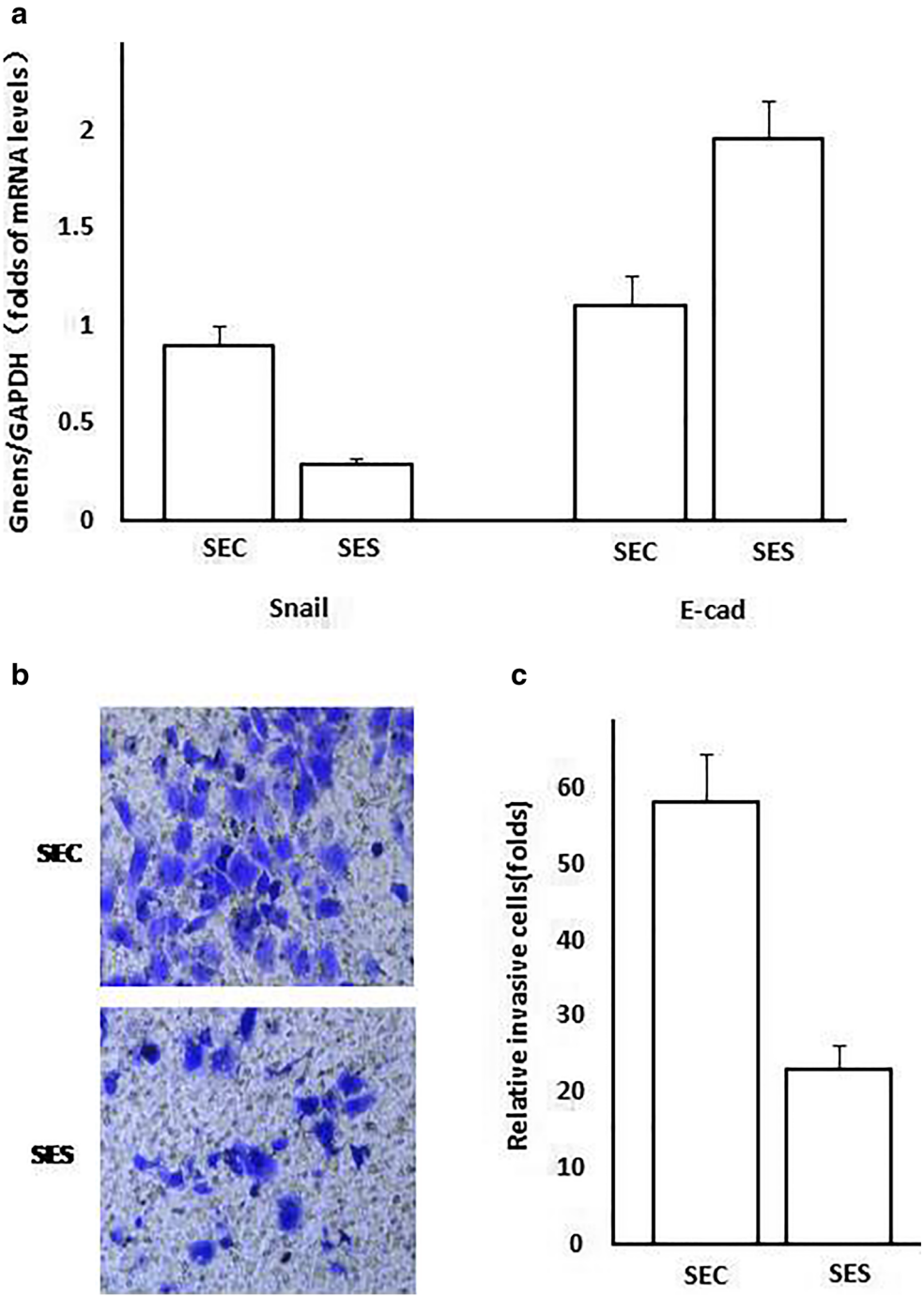

Fig. 5 Snail knockdown decreased the invasive ability of CNE2 cells (Transwell invasion analysis). **a** The change of mRNA levels of Snail and E-cad (qRT-PCR). The height of the bar diagrams represented the qRT-PCR value of the target genes/GAPDH. **b&c** Snail knockdown decreased the invasive ability of CNE2 cells. The cells on the Transwell membrane were dyed blue. The height of the bar diagrams represented the number of the invasive cells. SEC, the SE cells transfected with the negative siRNA fragments; SES cells, the SE cells transfected with the siRNA fragments targeting Snail; SE, the CNE2 cells stably transfected with the eIF4E expressing plasmids

Discussion

Metastasis at the early stage is the dominant characteristic of NPC [1, 2], in which EMT is a key step [3, 4]. EIF4E is the rate limiting factor of translational expression of growth factor genes and oncogenes [11, 12]. Snail is the core transcription factor and the direct suppressor of EMT related gene E-cad [5–8]. Both eIF4E and Snail strongly promote EMT, invasion and metastasis of cancer. Recently, a study [20] found that the phosphorylation of eIF4E could enhance Snail expression to promote EMT and metastasis in mouse embryonic fibroblasts (MEFs) and mouse breast cancer. Other studies showed that the treatments with some chemical antagonists might affect the expression of both eIF4E and Snail in the cellular level or xenograft tumor [23, 24]. However, whether eIF4E can enhance Snail expression to promote EMT and metastasis in human cancer tissues is not known.

This study explored the expression relationship between eIF4E and Snail in NPC and the effect of both proteins on NPC metastasis. We found that: ①the protein expression of

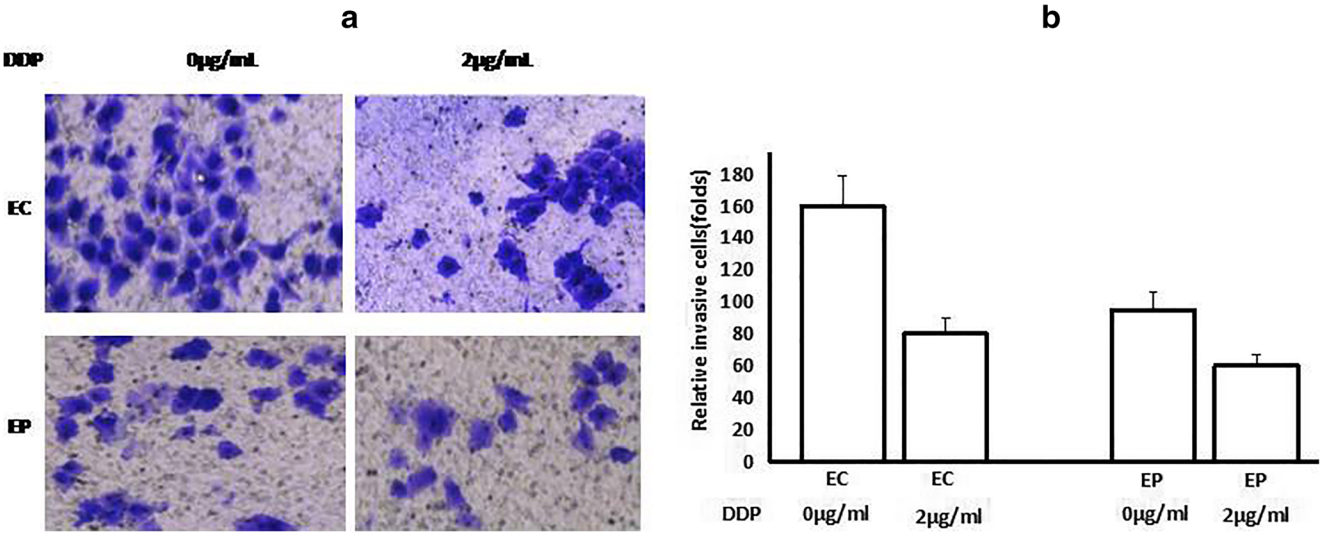

Fig. 6 eIF4E knockdown sensitized CNE2 cells to cisplatin (DDP) in invasive ability (Transwell invasion analysis). The cells on the Transwell membrane were dyed blue. The height of the bar diagrams represented the number of the invasive cells. EC, the control cells stably transfected with the negative shRNA plasmids; EP, the CNE2 cells stably transfected with the shRNA plasmids targeting eIF4E

eIF4E and Snail in NPC tissues was significantly increased. The expression rates of both eIF4E and Snail in un-NPC were significantly higher than that in NGI, and the rates in met-NPC were significantly higher than that in NGI and un-NPC, which indicated that the over-expression of either protein played an important role in the occurrence and metastasis of NPC. In their relationship, the expressions of eIF4E and Snail were highly positively correlated in both un-NPC and met-NPC, suggesting that the two proteins synergized with each other. ②In NPC cells, the levels of Snail mRNA and protein were significantly increased after the transfection with eIF4E expressing plasmids, and significantly decreased after the knockdown of eIF4E, indicating that eIF4E regulated the expression of Snail at both mRNA and protein levels. ③In cellular behavior, the invasive ability of CNE2 cells was significantly enhanced after the transfection with eIF4E expressing plasmids, contrarily, significantly decreased after the knockdown of eIF4E accompanied by the raised mRNA level of E-cad (an inhibitor of EMT). Under the condition of the over expression of eIF4E, the invasive ability of the cells was significantly decreased after Snail knockdown, accompanied by up regulation of E-cad. ④eIF4E knockdown sensitized the NPC cells to cisplatin in invasion. The results above clearly showed that eIF4E enhanced the mRNA and protein expression of Snail in NPC and targeting the eIF4E/Snail axis might intervene with the EMT and metastasis of NPC.

In NPC, eIF4E may activate Snail transcription by enhancing the translational expression of the upstream regulatory

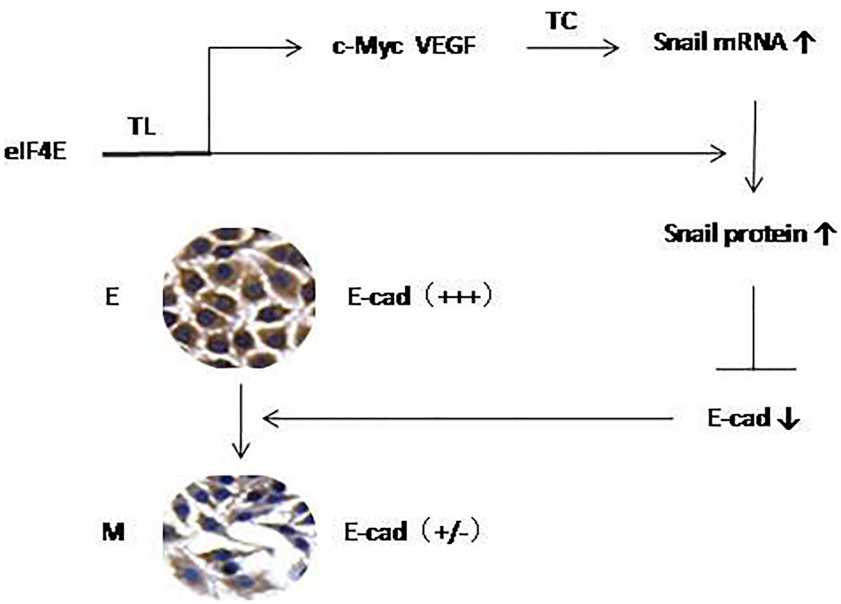

Fig. 7 eIF4E promotes the expression of Snail at both mRNA and protein levels and then reduces the expression of E-cad to push NPC EMT. Immunocytochemistry with E-cad antibody in CNE2 cells. TL, translation; TC, transcription; E, with more epithelial feature; M, with more mesynchymal feature

factors of Snail. The results of this paper showed that eIF4E improved the mRNA level of Snail. However, since eIF4E is a translation initiation factor rather than a transcription factor, it is unlikely for eIF4E to activate Snail transcription directly. According to the reports, eIF4E can directly initiate the mRNA translation of c-myc, TGF-β, VEGF and NF-kB [14, 26–28]. These four genes are all known to be important factors to activate Snail transcription [29–31]. Reports displayed that c-myc binds to the promoter of snail to promote EMT of colorectal cancer [32] and that NF-κB binds to the Snail promoter and triggers its transcription to drive hepatocellular carcinoma angiogenesis and metastasis [33]. The autocrine VEGF in breast tumor cells drives the expression of Snail and the breast tumor progression [34]. TGF-β activates the transcription of the Snail gene and TGF-β inhibition reduces the formation and growth of liver tumors in mice [35]. Furthermore, we found that eIF4E initiates the translational expression of c-myc and VEGF in NPC [14]. Thus, eIF4E could activate the transcription of Snail by enhancing the translational expression of at least c-myc and VEGF in NPC.

EIF4E may directly enhance the translational expression of Snail in NPC. The results of this paper showed that eIF4E increased the protein level of Snail. Since eIF4E indirectly increased Snail mRNA level, the increased Snail protein level could be interpreted as the result of the increased Snail mRNA level. However, a recent study [20] reported that the phosphorylated eIF4E in MEFs and mouse breast cancer could enhance the translational expression of Snail. In addition, our RIP assay showed that eIF4E directly bound to Snail mRNA, which is the initiated step for Snail translation. The result reconfirmed that eIF4E directly initiates the translational expression of Snail. In 2009, a study [36] showed that Yb-1 gene is able to up regulate the translational expression of Snail in mammary epithelial cells transfected with H-Ras, and concluded that the translational expression of Snail was mainly initiated by YB-1. The previous reports and our result evidenced that the translational expression of Snail can be initiated in both Yb-1 mediated cap-independent manner and eIF4E mediated cap-dependent manner. Therefore, eIF4E might up regulate Snail expression in a dual manner of both transcription and translation levels in NPC, highlighting the importance of eIF4E regulation of Snail.

To our best knowledge, this is the first report that eIF4E increased the mRNA and protein expression of Snail and then reduced the E-cad expression in human cancer tissues and that eIF4E promotes EMT, invasion and metastasis of NPC by eIF4E/Snail/E-cad pathway (Fig. 7). Since eIF4E and Snail are the key regulators of EMT, invasion and metastasis, the discovery of this article will be of great significance to understand the molecular mechanism of NPC invasion and metastasis, and to establish a new prevention and treatment method for NPC metastasis.

Acknowledgements This work was funded by the Natural Science Fund of Guangdong Province (2015A030313519), the National Natural Science Fund of China (81572566) and the Science and Technology Planning Project of Guangdong Province (2016A020215146).

Compliance with Ethical Standards

Conflict of Interest Authors Yunhong Yao, Tianyun Pang, Ying Cheng, Weiwei Yong, Haixian Kang, Yi Zhao, Sen Wang and Xinrong Hu declare that they have no conflict of interest.

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