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ORIGINAL PAPER

The implications and mechanisms of the extra-nuclear nucleolin in the esophageal squamous cell carcinomas

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Abstract In recent decades, the multi-functional protein nucleolin (NCL) has been reported to express outside the nucleus of many cancer cells. However, the expression and role of the extra-nuclear NCL in esophageal squamous cell carcinoma (ESCC) were not well characterized. Here, NCL was detected by immunohistochemistry and Western blotting in 60 ESCC tissues. Further, the associations of NCL, EGFR, CXCR4 and Ki67 were analyzed by in vitro assays. Our results showed that NCL expression in all 40 cases of ESCC tissues with metastasis was extensively located in the nucleus, cytoplasm and cell membrane (extra-nucleus), while NCL expression in all 20 cases of ESCC without metastasis was merely limited into the nucleus (intra-nucleus).The extra-nuclear NCL expression was positively correlated with the expression of EGFR, CXCR4 and Ki67 and serves as an independent prognostic factor for ESCC patients. In vitro, NCL siRNA (si-NCL) efficaciously affected the expression of EGF or SDF-1-induced p-AKT, p-ERK and Ki67. Also, NCL siRNA inhibited the capacity of migration and invasion of ECA109 cells. In conclusions, our study suggests that NCL is implicated in the initiation and transduction of EGFR and CXCR4 signaling and further up-regulates Ki67 expression to modulate the biological behaviors of ESCC. Clinically, the extra-nuclear NCL expression can be used as an important indicator to determine metastasis and predict the prognosis, which help develop new therapeutic strategies against ESCC.

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Introduction

ESCC is the most common one of aggressive malignancies in the digestive tract [1, 2]. Surgical resection is routinely recommended as the mainstay strategy for the effective treatment of ESCC. Although the great advances have been made in multimodal therapies, 5-year survival rate of ESCC remains unsatisfactory [3–5]. It would be a key point to discover a useful biomarker to predict ESCC prognosis, or screen ESCC in high-risk population. So the understanding of molecular mechanisms of ESCC can benefit patients very well.

NCL is a nuclear protein highly expressing on the surface of proliferating cells [6, 7]. Although more than 90 % of NCL is found in the nucleolus, NCL has been divided into nuclear, cytoplasmic and cell surface NCL, depending on location in the cells. Reportedly cell surface NCL serves as a binding partner of several molecules and is implicated in cell differentiation, adhesion and leukocyte trafficking, inflammation, angiogenesis and tumor development [8–10]. Accumulating evidence validates cell surface NCL as an interesting target for treatment of cancer and is used for the targeted release of chemotherapeutic drugs due to its implications in the receptor–ligand internalization. Based on these reports, targeting cell surface NCL might trigger multi-inhibitory effects [11, 12]. However, the expressions and roles of NCL in ESCC have not been investigated till now.

In the present study, we analyzed and summarized the distribution properties and the biological functions of NCL using ESCC tissues and cells, with emphasis on the potential importance and advantages of developing efficient anti-cell surface NCL strategies.

Materials and methods

Patients and tissues

In this study, 60 ESCC tissues were selected and underwent paraffin sections, which had been histopathologically diagnosed and were retrieved from pathology archives in Nan Fang Hospital of Southern Medical University between October 2012 and October 2014. The histological grade and clinical stage of the tumors were defined according to the latest TNM classification of the International Union Against Cancer. The cases selected in this study fulfilled the following criteria: (a) newly diagnosed cancer of the esophagus without previous treatment and (b) histologically confirmed primary thoracic ESCC. The mean age of patients at diagnosis was 63 years (range from 46 to 78). Adjacent non-cancerous esophageal tissues were obtained at over 5 cm from the cancerous tissue. The procedures in the present study were subjected to the institutional ethical committees. All the samples included in this study were completely anonymized. Follow-up information from all the patients was obtained through visits or telephone. Mean follow-up period was 31 months, ranging from 5 to 100 months.

Cell culture and reagents

The human ESCC cell line ECA109 was obtained from American Type Culture Collection (ATCC, Manassas, Va.) and was cultured in DMEM (Gibco) supplemented with 10 % fetal bovine serum (FBS; Hyclone) and 100 U/ml penicillin/streptomycin (Gibco) and was maintained in a humidified atmosphere with 5 % CO_2 at 37 °C. Recombinant human EGF and SDF-1 were purchased from Sigma (St. Louis, USA). Antibodies were purchased from the same resources: anti-p-ERK, anti-t-ERK, anti-p-AKT, anti-t-AKT, anti-Ki67 and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, USA). All experiments were performed in the absence of FBS.

Immunohistochemistry

Immunohistochemical staining was performed using antibodies as mentioned above (1:500 dilutions, Santa Cruz Biotechnology, USA). Paraffin-embedded sections (5 μm) were dewaxed in xylene and rehydrated using graded ethanol. Sections were incubated in 3 % hydrogen peroxide for 10 min to inactivate endogenous peroxides. Then, the sections were heated (at 100 °C) for 15 min in 0.01 mol/l citrate buffer and then cooled for 30 min at room temperature to expose antigenic epitopes. The sections were blocked with 2 % normal goat serum in PBS for 30 min and then incubated overnight at 4 °C with primary antibody against NCL. Using the EnVision System-labeled HRP antimouse (Dako, Denmark), the primary antibody was visualized with diaminobenzidine–H_2O_2 and counterstained with Mayer's hematoxylin. PBS was used instead of the primary antibodies for the negative controls. The immunostaining scoring was based on positive percentage and staining intensity. Positive percentage scores were assigned according to the following scale: 0, 0 % cells; 1, 0–25 % cells; 2, 25–50 % cells; and 3, >50 % cells. Staining intensity was also scored semiquantitatively as follows: 0, none; 1, mild; 2, moderate; and 3, intense. Among total scores, 0, 1–3, 4–6, and 7–9 were recorded as −, +, ++, and +++, respectively.

Nucleolin siRNA transfection

Cell lines were seeded in a 6-cm dish at density of 5×10^5 cells/dish and incubated overnight. Cells were prepared for transfection of si-nucleolin (sc-29230, Santa Cruz) or si-control (sc-37007, Santa Cruz). One milligram of si-nucleolin was added to Opti-MEM with Lipofectamine 2000 (Invitrogen) for transfection, according to the manufacturer's instructions. At 12 h following incubation, medium was changed into fresh DMEM containing 10 % FBS. Cells were harvested at 72 h following transfection of si-nucleolin. Then, cells were subjected to Western blot, transwell and wound-healing assay.

Western blotting

Total protein from tissues and cultured cells was extracted in cell lysis buffer (PIERCE, Rockford, IL) and quantified using the BSA method. A 10 % SDS–PAGE was performed, and 30 μg of protein of each sample was analyzed. Proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an electroblot apparatus. Membranes were incubated with primary antibodies: anti-p-ERK, anti-t-ERK, anti-p-AKT, anti-t-AKT, anti-Ki67 and anti-β-actin antibody (Santa Cruz Biotech). Antibody recognition was detected with either anti-mouse IgG or anti-rabbit IgG antibody linked to horseradish peroxidase (Sigma). Immunocomplexes were visualized by ECL (Amersham Pharmacia Biotech).

Invasion and wound-healing assays

The cell invasion assay was performed using transwell cell culture inserts (Invitrogen Life Technologies). The transfected cells were maintained for 48 h and allowed to migrate for an additional 24 h. The passaged cells were stained with crystal violet solution, and absorbance was measured at 590 nm. In the wound-healing assays, cell motility was assessed by measuring the movement of cells toward the scratch. The speed of wound closure was monitored after 24 h by measuring the ratio of the distance of the wound at 0 h. Each experiment was performed in triplicate.

Statistical analysis

Data were expressed as the mean ± standard error of three repeated assays. Differences between various groups were assessed using Student's t test. The p value <0.05 was considered to indicate statistical significance. The significant groups are marked with asterisks or pounds. Overall survival was defined as the time from the date of initial diagnosis to patient death or the date of the last available information on vital status. Correlations between categorical variables were performed using the Chi-square and Spearman's test with SPSS version 13.0 software.

Results

Expression and distribution characteristics of NCL in ESCC tissues

To elucidate the expression status of NCL in ESCC tissues, we conducted the immunohistochemistry. In the present study, 11 females and 49 males with mean 63.0 years were included. Using the criteria described above, nuclear staining was seen in cancerous tissues of all ESCC sections (Fig. 1) and diffuse intra-nuclear staining was apparent in all cases. Of 60 cases, NCL extensively expressed in cell membrane and cytoplasm (extra-nucleus), as well as nucleus (intra-nucleus), including the extension score (++) in 29 cases and (+++) in 31 cases. However, nuclear staining was scarcely seen in adjacent non-cancerous tissues. Notably, NCL expression in ESCC tissues without metastasis was only located in the nucleus (Fig. 1a), while NCL expression in ESCC tissues with metastasis can be observed throughout cell membrane, cytoplasm and nucleus (Fig. 1b). Besides, EGFR, CXCR4 and Ki67 protein showed the same patterns of expression, which were located in the plasma and membrane of ESCC tissues (Fig. 1c–f). The expression rate of EGFR, CXCR4 and Ki67 was 76.7 % (46/60), 71.7 % (43/60) and 81.7 % (49/60), respectively. Immunohistochemical findings are summarized in Table 1 and illustrated in Fig. 1.

On the other hand, Western blotting results identified that EGFR, CXCR4 and Ki67 expression was different between the non-metastasis and metastasis group, whereas NCL showed no any statistical differences ($p = 0.367$). Together with IHC data, we found an interesting phenomenon that it is the distribution of NCL from intra-nucleus to extra-nucleus that can effectively indicated the metastasis status of ESCC, instead of staining intensity, indicating that the extra-nuclear NCL expression represents the occurrence of metastasis of ESCC.

Correlations of NCL, EGFR, CXCR4 and Ki67 with clinicopathological features

To further figure out the importance of extra-nuclear NCL expression, we analyzed the correlations. As shown in Table 1, there were no significant correlations of extra-nuclear NCL expression with clinicopathological parameters, such as age, gender or tumor size, respectively

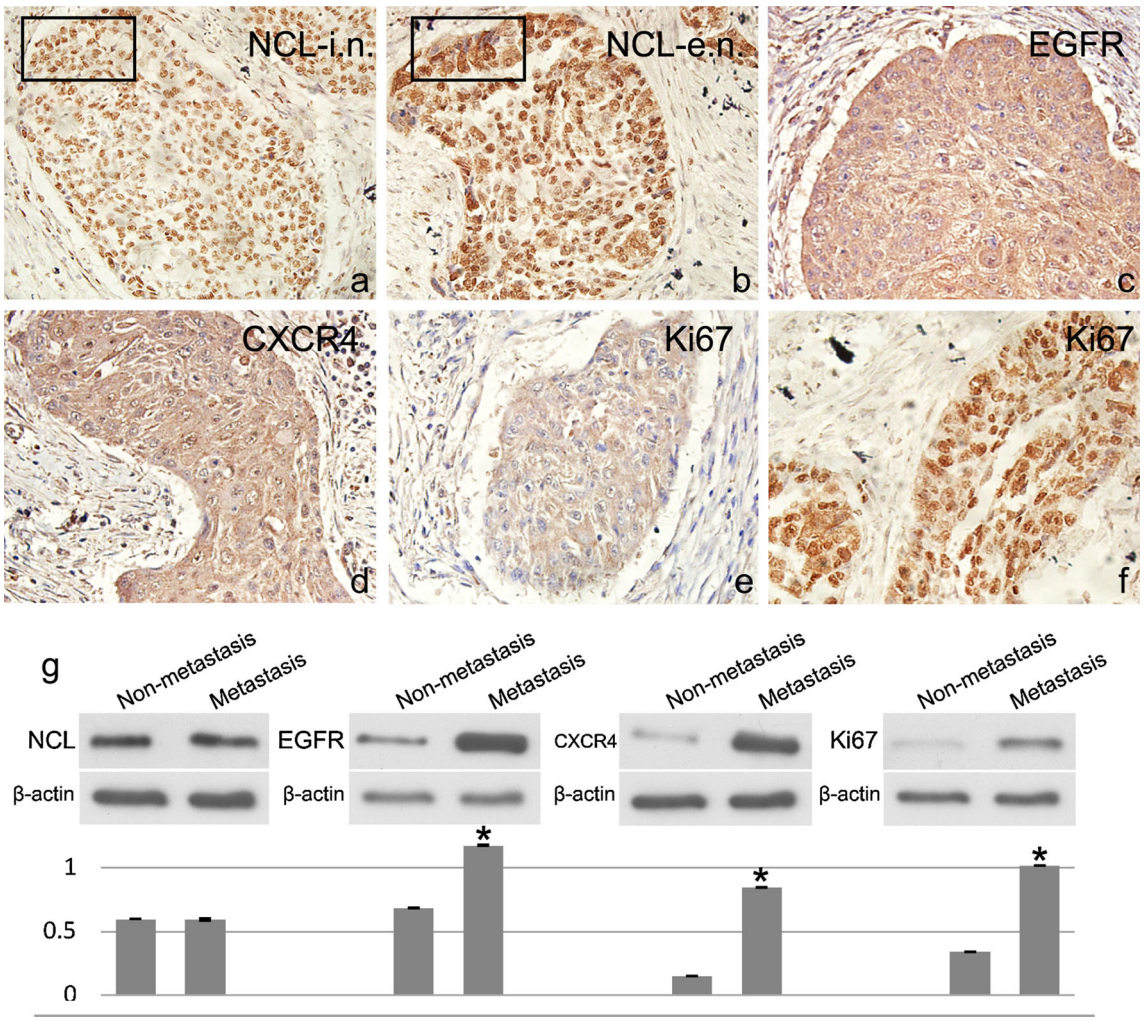

Fig. 1 Expression of NCL, EGFR, CXCR4 and Ki67 in ESCC tissues. **a** Normal esophageal mucosa demonstrated that low expression of NCL protein in all esophageal squamous cells. Conversely, cancer tissues showed that high NCL staining was only limited into the nucleus of all non-metastatic ESCC tissues (*Black rectangle*; Magnification: ×200); **b** a high expression level of NCL was observed extensively in the membrane, plasma and nucleus of all metastatic ESCC tissues (*Black rectangle*; Magnification: ×200); **c** high expression level of EGFR detected in ESCC (Magnification: ×200); **d** high expression level of CXCR4 detected in ESCC (Magnification: ×200); **e** moderate expression level of Ki67 detected in ESCC (Magnification: ×200); **f** The highest expression level of CXCR4 detected in ESCC (Magnification: ×200). **g** Western blot analysis was conducted to validate the expression of NCL, EGFR, CXCR4 and Ki67 in ESCC tissues with or without metastasis. Results shown are representative of three independent experiments; data were expressed as mean ± SEM. *$p < 0.001$, compared with si-control, Student's *t* test, *e.n* extra-nucleus, *i.n.* intra-nucleus

($p > 0.05$). Conversely, a significant correlation was observed between extra-nuclear NCL expression and metastasis of ESCC. These differences were significant at the $p < 0.001$ level. In another way, the expression of Ki67, EGFR and CXCR4 was also higher in ESCC with metastasis compared with those without metastasis ($p < 0.001$). Besides, we also observed correlations of extra-nuclear NCL, Ki67, EGFR and CXCR4 with depth of invasion ($p < 0.01$), indicating that all these proteins were involved in ESCC progression. Spearman's test showed significant correlations between the extra-nuclear NCL expression and Ki67, EGFR, CXCR4 ($p < 0.001$, $p = 0.002$, $p < 0.001$, respectively).

The extra-nuclear NCL expression and prognosis

As shown in Fig. 2, the patients were divided into the low (intra-nucleus) expression group and the high (extra-nucleus) expression group. Kaplan–Meier analysis with log-rank test was carried out to assess the prognostic significances. First, Kaplan–Meier survival curves showed that the extra-nuclear NCL expression was most associated with poor overall survival ($p < 0.000$; Fig. 2a). Second, a significant difference of overall survival was found between patients with high EGFR, CXCR4 or Ki67 expression and patients with low EGFR, CXCR4 or Ki67 expression, respectively. Kaplan–Meier survival curves

Table 1 Relationships between NCL, Ki67, EGFR, CXCR4 expression and clinicopathological variables

Variables	*N*	NCL			Ki67			EGFR			CXCR4		
		Intra-nucleus	Extra-nucleus	*p*	Low	High	*p*	Low	High	*p*	Low	High	*p*
Gender													
Male	49	17	32	0.736	9	40	0.679	12	37	0.498	11	38	0.059
Female	11	3	8		2	9		2	9		6	5	
Age													
≥63	35	10	25	0.412	4	31	0.174	11	24	0.122	12	23	0.260
<63	25	10	15		7	18		3	22		5	20	
Tumor size													
≥5	21	8	13	0.579	5	16	0.493	6	15	0.532	8	13	0.243
<5	39	12	27		6	33		8	31		9	30	
Metastasis													
Yes	40	0	40	0.000	4	36	0.031	3	37	0.000	3	37	0.000
No	20	20	0		7	13		11	9		14	6	
Depth of invasion													
T1	13	9	4	0.000	5	8	0.002	7	6	0.000	8	5	0.001
T2	18	8	10		2	16		5	13		8	10	
T3	29	3	26		4	25		2	27		1	28	

showed high EGFR, CXCR4 or Ki67 expression was associated with poor overall survival ($p = 0.001$, $p = 0.002$, $p = 0.023$, respectively; Fig. 2b–d). In addition, univariate analysis demonstrated that patients with the extra-nuclear NCL expression tended to have a higher risk of death ($p < 0.001$). Meanwhile, other indicators including metastasis, EGFR, CXCR4 and Ki67 were also proved to be associated with overall survival through univariate analysis. However, age, gender and depth of invasion had no prognostic significances. Multivariate analysis indeed showed the extra-nuclear NCL expression was an independent prognostic factor for ESCC patients ($p = 0.005$).

Effects of cell surface NCL on the activation of EGFR signaling pathway in ECA109 cells

According to reports, the extra-nuclear NCL expression was abundantly situated on the cell surface. Thus, cell surface NCL may be associated with EGFR pathway on the cell membrane. In this study, we investigated the effects of NCL on EGF/EGFR pathways. Western blot showed that the silencing of NCL abrogated the protein levels of EGF-induced p-AKT and p-ERK in ECA109 cells after stimulation with 30 ng/ml EGF for 12 h. Importantly, Ki67 expression was also decreased obviously compared with control (Fig. 3). These findings indicated that cell surface NCL affected the initiation of EGF signaling.

Effects of cell surface NCL on the activation of CXCR4 signaling pathway in ECA109 cells

Based on the results above, subsequently we investigated the effects of NCL on CXCR4 signaling. As expected, Western blot showed that the silencing of NCL affected the protein expression of SDF-1-induced p-AKT and p-ERK in ECA109 cells after stimulation with 50 ng/ml of SDF-1 for 12 h (Fig. 3). Similarly, Ki67 expression was also decreased obviously compared with control (Fig. 3). These findings indicated that cell surface NCL was also involved in the activation of CXCR4 signaling.

NCL promoted invasive and migratory capacity of ECA109 cells

To elucidate whether NCL had influence on migratory and invasive capabilities of ECA109 cells, in vitro invasion and wound-healing assays were performed. As shown in Fig. 3, the transwell assay identified that the invasive potential of the ECA109 cells with NCL siRNA transfectants was obviously inhibited compared with non-transfected cells ($p < 0.001$, Student's t test). Similarly, the wound-healing assay demonstrated that the ECA109 cells with si-NCL transfectants exhibited reduced migration and invasion compared to the control group. On the contrary, the control group was not affected. Differences were significant ($p < 0.001$, Student's t test).

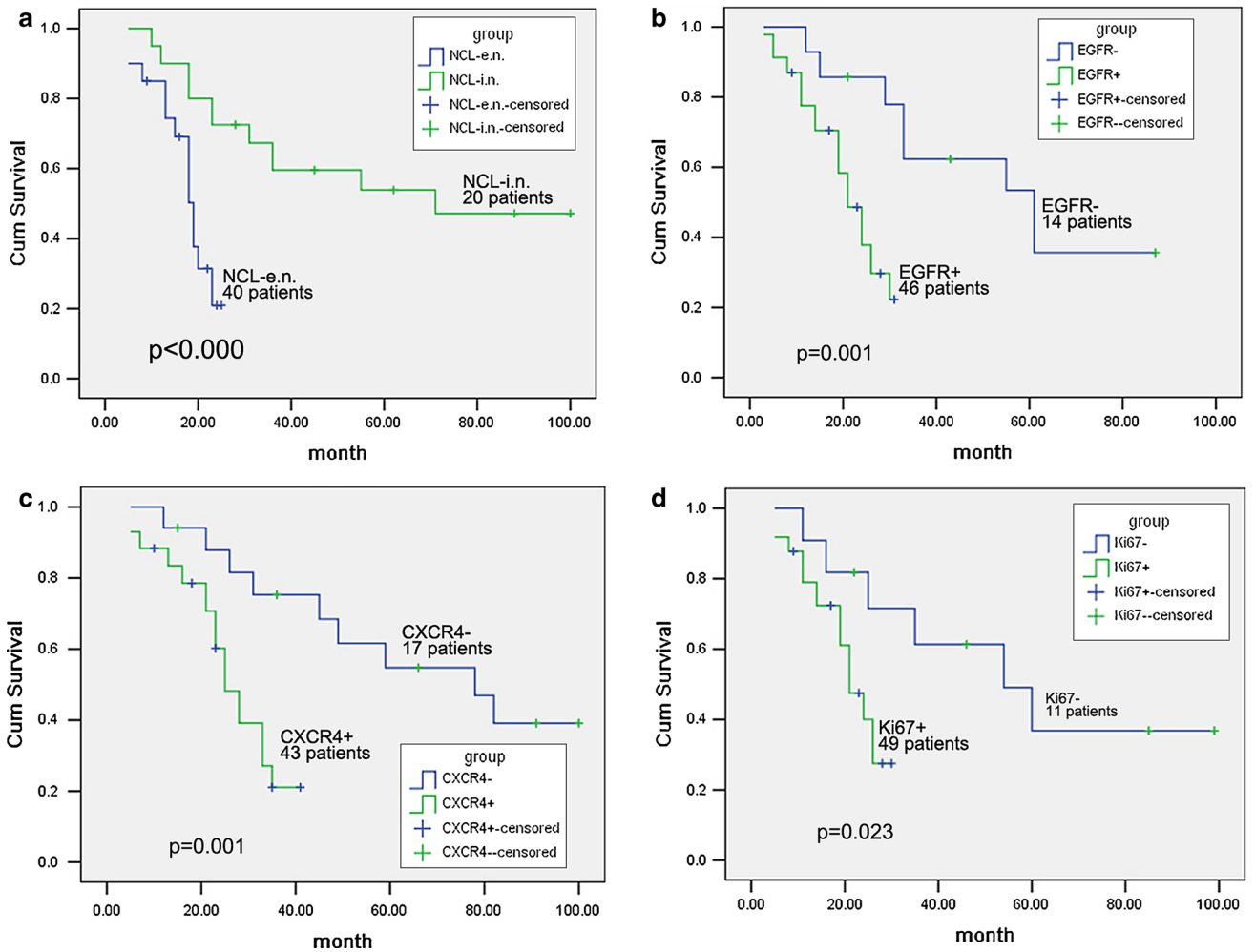

Fig. 2 The prognostic significance of NCL, EGFR, CXCR4 and Ki67 in ESCC patients. Kaplan–Meier analysis of overall survival based on the expression of NCL (**a**), EGFR (**b**), CXCR4 (**c**) and Ki67 (**d**) in all 60 patients. Kaplan–Meier survival *curves* showed that the high NCL, EGFR, CXCR4 and Ki67 expression was associated with poor overall survival. *e.n.* extra-nucleus, *i.n.* intra-nucleus

Discussion

As a kind of nucleoprotein, NCL has been reported to be associated with the tumor cell growth as well as angiogenesis [13]. The inhibition of cell surface NCL of the tumor will affect the proliferation and impair angiogenesis and tumorigenesis [13–15]. Therefore, it is essential to clarify the expression patterns and roles of NCL in ESCC. In this study, first we found that the distribution differences of NCL expression became evident between non-metastatic and metastatic ESCC tissues, that is, NCL expression without metastasis was only located in the nucleus of ESCC cells, while NCL expression with metastasis can be found throughout cell membrane, cytoplasm and nucleus. It should be pointed out that the intensity differences of NCL expression were not evident between non-metastatic and metastatic tissues, which were validated by Western blotting. Compared with EGFR, CXCR4 and Ki67, NCL can be predominately recommended as a useful biomarker, through which pathologists can easily know the status of ESCC metastasis; however, the real value of NCL is still demonstrated through the multicenter and large-scale studies.

Some reports showed that NCL existed in the nucleus and cytoplasm of B cell chronic lymphocytic leukemia; what is more, NCL is also involved in the stability of bcl2 mRNA in CLL cells and results in enhanced bcl2 mRNA expression [16]. When NCL siRNA suppressed the endogenous NCL expression of the glioblastoma cell line, glioblastoma cell proliferation was inhibited rapidly and cell cycle progression also arrested [17]. Based on this evidence, NCL may serve as a hallmarker in the proliferation of tumors. In this work, the extra-nuclear NCL expression was identified as an independent prognostic factor for ESCC patients. The extra-nuclear NCL expression was also confirmed to be associated with the

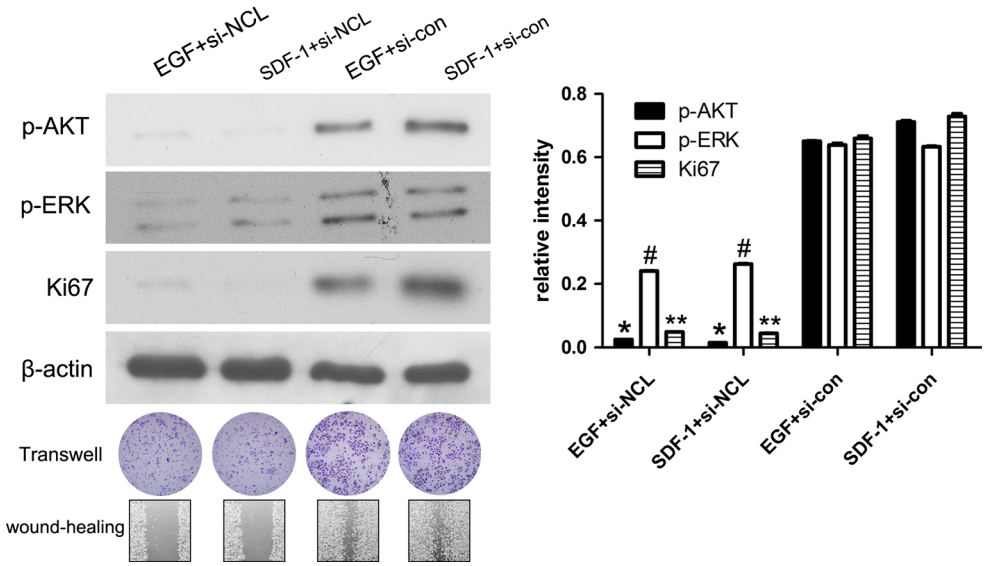

Fig. 3 The molecular and biological effects of NCL on EGFR and CXCR4 pathway in ECA109 cells. ECA109 cells were deprived of serum overnight and then stimulated with EGF (30 ng/ml) or SDF-1 (50 ng/ml) in the presence of si-NCL or si-control for 12 h. Cell lysates were obtained to probe for p-AKT, p-ERK and Ki67 by Western blotting analysis. β-actin was used for protein loading control. Transwell assay showed ECA109 cells that invaded through the micropore. Wound healing within the *scrape line* was recorded at 24 h. Representative *scrape lines* are shown at the first 24 h. Results shown are representative of three independent experiments; data were expressed as mean ± SEM. $*/^{\#}/**p < 0.001$, compared with si-control, Student's *t* test

expression EGFR, CXCR4 and Ki67. These findings suggested that NCL plays a vital role in the development of ESCC. So we assumed that NCL may be required for activation or biological effects of EGFR, CXCR4 and Ki67 in ESCC.

Next, we found that the blockade of cell surface NCL affected the initiation of EGF and CXCR4 signaling, leading to the decreased Ki67 expression. Based on the transwell and wound-healing assay, functional studies demonstrated that the invasive and migratory capacity of the ECA109 cells with NCL siRNA transfectants was obviously inhibited compared with non-transfected cells. Recent studies have identified the target roles of cell surface NCL in controlling tumor development, which may be attributed to three aspects: first, cell surface NCL can bind to some ligands to generate Ca^{2+} influxes and prime signal transduction pathways [18]. Second, the cell surface NCL acts as a receptor for various growth factors, cell adhesion molecules (integrins, selectins) or viruses. Third, HB-19 could bind the C-terminal RGG domain of cell surface NCL and abrogate biological activity of NCL though the competitive inhibition of various ligands [19, 20]. Therefore, we are convinced that developing NCL antagonists would benefit the ESCC patients in the near future.

In summary, in this study, we shed light on the expression and roles of the extra-nuclear NCL in ESCC tissues and cells. The distribution of NCL differs greatly between metastatic carcinoma tissues and non-metastatic carcinoma compared with EGFR, CXCR4 and Ki67. NCL can be used as an important indicator to distinguish cancer metastasis clinically, which greatly allows for diagnosis and treatment of ESCC and provides novel therapeutic opportunities for proliferative diseases.

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Conflict of interest None.

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