# Ninein-like protein is overexpressed in head and neck squamous cell carcinoma and contributes to cancer growth and resistance to apoptosis

LINLIN YU[1,3], YONGMEI SONG[2], QIUHANG ZHANG[1] and QIMIN ZHAN[2]

[1]Department of Otorhinolaryngology Head and Neck Surgery, Xuanwu Hospital of Capital Medical University, Beijing 100053; [2]State Key Laboratory of Molecular Oncology, Cancer Institute (Hospital), Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, P.R. China

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**Abstract.** Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and survival rates are not improving. Better understanding of the molecular mechanisms of this disease becomes critical to develop more effective treatments. Ninein-like protein (Nlp), a recently identified centrosome-associated protein, is a key regulator in centrosome maturation, which contributes to chromosome segregation and cytokinesis. Recent studies have revealed overexpression of Nlp in several types of human tumors and suggested it was a potential oncogenic protein. To investigate the role of Nlp in the development of HNSCC, expression of Nlp in tumor tissues of 76 HNSCC patients were analyzed, and the correlations of Nlp expression with the clinicopathological characteristics were evaluated. Our data showed overexpression of Nlp in tumor tissues compared with their normal counterparts. Moreover, overexpression of Nlp correlated with tumor differentiation and immunohistochemistry analysis of preinvasive dysplasia and squamous cell carcinoma showed that overexpression of Nlp occurred in premalignant lesions. Biological studies with human HNSCC cell lines indicated that overexpression of Nlp promoted cell proliferation and inhibited cisplatin-induced apoptosis. Taken together, these results suggest a novel mechanism that is closely related to malignant phenotype and anti-cancer drugs resistance of HNSCC and support the notion that Nlp overexpression might contribute to the development of HNSCC.

*Correspondence to:* Professor Qimin Zhan, State Key Laboratory of Molecular Oncology, Cancer Institute (Hospital), Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, P.R. China
E-mail: zhanqimin@pumc.edu.cn

[3]*Present address*: Department of Otorhinolaryngology, Meitan General Hospital, Beijing 100028, P.R. China

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## Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for most cancers of the mouth, pharynx and larynx (1). It represents the sixth most frequent cancer worldwide, with an estimated 900,000 cases diagnosed each year (2). Despite advances in surgical and other treatments, HNSCC is still a devastating disease as the survival rates virtually have not changed for the past 30 years (3). While the relatively favorable prognosis for patients diagnosed with early stage HNSCC, >60% of patients apply to hospitals are of advanced stages and the 5-year survival rate for HNSCC patients in stages III and IV (Union International Contre Cancer stages) is <40% (4). Currently, much effort is focused on developing novel strategies for early detection, classification, prevention and treatment of the disease. To fulfill this purpose, a better understanding of the molecular mechanism of HNSCC is underscored.

Previously, centrosome amplification have been implicated in the pathogenesis of many types of solid tumors, including HNSCC (5-7). Because centrosomes direct the formation of spindles for the correct separation of chromosomes during mitosis, the presence of more than two centrosomes in a cell can result in lost or fragmented chromosomes after cell division and generation of aneuploidy, which are closely associated with cell transformation and tumorigenesis (7). Ninein-like protein (Nlp), a recently identified centrosomal protein, locates at chromosome 20. During interphase, Nlp is involved in microtubule organization. Two centrosomal kinases, Plk1 and Nek2, coordinately regulate Nlp at the $G_2$/M transition in order to displace it from centrosome and thus establish a condition for assembly of the mitotic spindle (8,9). Overexpression of Nlp caused aberrant spindle formation, suggesting that Nlp is a key regulator in centrosome maturation, which contributes to chromosome segregation and cytokinesis (8-11). Recent observations indicate that Nlp is deregulated in several types of human solid tumors (11,12), suggesting a role of this novel centrosome protein in the process of tumorigenesis.

In the current study, we analyzed the expression of Nlp in 76 primary HNSCC and associated non-neoplastic squamous epithelium. The correlations of Nlp overexpression with

patients' clinicopathologic characteristics were also evaluated. To further clarify the role of Nlp in HNSCC, we tested the effect of Nlp overexpression on tumor cell proliferation and apoptosis by using human head and neck cancer cell lines. To our knowledge, this is the first study addressing the expression of Nlp in HNSCC and may have implications for better understanding the molecular mechanism of HNSCC.

## Materials and methods

*Patients and tissue specimens*. Tissue specimens were obtained from patients who were diagnosed with primary HNSCC and underwent a surgical resection in Cancer Hospital of Chinese Academy of Medical Sciences (Beijing, China) and Xuanwu Hospital of Capital Medical University (Beijing, China). None of the patients had received radiotherapy or chemotherapy before surgery. In total, 76 formalin-fixed and paraffin-embedded HNSCC including 21 oral squamous cell carcinomas, 8 pharyngeal squamous cell carcinomas and 47 laryngeal squamous cell carcinomas were obtained from the pathological files of the hospitals. In 20 cases, a portion of the surgical tumor tissue specimen was also obtained in the operating room: the tumor was sharply excised, snap-frozen in liquid nitrogen and stored at -80˚C for Western blotting/real-time RT-PCR analysis. Non-neoplastic squamous epithelium from the resection margins was used as reference. In addition, three benign hyperplasias/hyperkeratosis and six premalignant lesions (two mild dysplasia, two moderate dysplasias and two severe dysplasias/carcinomas *in situ*) were also obtained from archival, paraffin-embedded blocks. Both tumor and normal tissues for subsequent studies were confirmed by pathologists. Clinicopathologic data of the patients were retrieved from medical records and the Institutional Review Board approved use of the tumor specimens in this study. The stage of disease was determined according to the tumor-node-metastasis staging system of the International Union Against Cancer, sixth edition (13). The histologic grade was determined according to the degree of differentiation of the tumor (Broder's classification).

*Cell culture*. Human HNSCC cell lines Tca8113 (squamous cell carcinoma from the tongue), FaDu (squamous cell carcinoma from the hypopharynx) and HEp-2 cells (squamous cell carcinoma from the larynx) were purchased from the China Center for Type Culture Collection (Shanghai, China). All the cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin in a humidified incubator containing 5% $CO_2$ at 37˚C.

*Immunohistochemical staining*. Tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was then quenched by 3% $H_2O_2$ treatment. After antigen retrieval with sodium citrate, sections were blocked with 1.5% normal blocking serum in PBS for 1 h at room temperature and incubated with Nlp antibody (Immunohunt Corporation, Beijing, China) at a dilution of 1:100 at 4˚C overnight. Finally, sections were reacted with 3,3-diaminobenzidine at room temperature for 2 to 3 min or until desired stain intensity developed. All sections were counterstained with hematoxylin. Negative controls were obtained by omitting the primary antibody. In immunohistochemical analysis, visible brown granules in the cytoplasm were determined as positive staining. Specimens were reviewed with staining intensity and staining extent. Staining intensity was rated as follows: negative (0), weak (1), moderate (2), strong (3). Staining extent was rated according to the percentage of positive cells in the field. Samples with 0 to 4% of cells stained were rated as 0 and those with 5 to 24% of cells stained were rated as 1, those with 25 to 49% of cells stained were rated as 2, 50 to 100% of cells stained were rated as 3. The results of staining intensity and staining extent gave an overall staining score. The samples of staining where the score was 0 were marked as (-), those scored with 1 marked as (±), 2 to 3 marked as (+) and 4 to 6 marked as (++).

*RNA extraction and quantitative real-time PCR*. RNA extraction was performed as described (14). Real-time PCR was performed on ABI Prism 7000 Sequence detection System using the SYBR Premix Ex Taq™ kit (Takara, Dalian, China). The primers used for real-time RT-PCR were designed as follows: Nlp: 5'-ACCTGGGATTCTGAGGACTTTG-3' and 5'-ACTTTGCCGTCTCCGTCTTGAT-3'. GAPDH: 5'-CAT CAAGAAGGTGGTGAAGC-3' and 5'-GGAAATTGTGAG GGAGATGC-3'. The PCR reaction consisted of 2 $\mu$l 1:10 diluted cDNA, 0.2 $\mu$M of both forward and reverse primers and 10 $\mu$l 2X SYBR Premix Ex Taq™ in a total volume of 20 $\mu$l. PCR conditions were 95˚C for 10 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. The house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for normalization of the mRNA expression data of Nlp. Each sample was run in triplicate to ensure quantitative accuracy and the threshold cycle numbers ($C_T$) were averaged for both Nlp and GAPDH. The results were reported as T:N ratios and calculated using $2^{-\Delta\Delta CT}$ method (15).

*Protein preparation from tissue samples, cell extraction and Western blot analysis*. Protein preparation from tissue samples, cell extraction and Western blot analysis was performed, as described (14). Antibodies against Nlp (1:500 dilution) was obtained from Immunohunt Corporation, Beijing, China. Antibodies against ß-actin (1:3,000 dilution), GFP (1:500 dilution), caspase-3 (1:500 dilution), PARP (1:500 dilution) were obtained from Santa Cruz, CA.

*Stable transfections and establishment of stable cell lines*. HNSCC cells (Tca8113, $1x10^6$) were transfected with 8 $\mu$l of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 2 $\mu$g of pEGFP-Nlp [pEGFP-Nlp was constructed by inserting human Nlp cDNA into *Sal*I and *Sma*I sites of pEGFP-C3 vector (11)] or 2 $\mu$g of pEGFP empty vector. Cells were selected with 400 $\mu$g/ml G418 (Geneticin sulfate, Gibco). For each transfection, all of the colonies were trypsinized and collected to produce stable cell pools.

*Small interfering RNA studies*. For the RNA interference analysis, chemically-synthesized human Nlp-specific siRNA was designed as sense, 5'-(C AUG UAG AUU UGA GAG

AGA)dTdT-3' and antisense, 5'-(UCU CUC UCA AAU CUA CAU G)dTdT-3' (GeneChem, Shanghai, China). The non-specific siRNA (control) sequence was designed as 5'-(AUU GUA UGC GAU CGC AGA C)d(TT)-3' (GeneChem). These sequences had no significant homology to human coding cDNA. The FITC-conjugated control siRNA (GeneChem) was used to monitor the transfection rate. Transfection assays were conducted in FaDu cells. Transient transfection was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. For each transfection, cells were incubated with siRNA (for 24-72 h until they were ready to assay for gene knockdown analysis).

*Cell growth curve*. Transfected Tca8113 cells ($1x10^3$) were seeded onto 35-mm tissue culture dishes. The cells were trypsinized and manually counted in triplicate on days 1, 3, 5, 7 and 9 to draw the cell growth curve. Cell growth curve of FaDu cells after RNA interference was determined by MTT assay.

*Clonogenecity assay*. Transfected Tca8113 cells ($1x10^3$) were plated in 35-mm tissue culture dishes. When the colonies became visible (2-4 weeks) after incubation, the cells were fixed with methanol, stained with 0.1% crystal violet and colonies of >50 cells were counted.

*3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay*. To determine the cell growth after RNA interference, a total of $5x10^3$ FaDu cells in 100 $\mu$l of the medium were plated in 96-well plates and Nlp siRNA (150 nM) or control siRNA were transfected. Every 24 h up to 7 days, the medium was replaced with 100 $\mu$l of sterile MTT dye (0.5 mg/ml, Sigma) for 4 h at 37˚C, then culture medium was removed and 150 $\mu$l of DMSO was added and thoroughly mixed for 15 min. Spectrometric absorbance at wavelength of 570 nm was measured on a microplate reader (Bio-Rad). To assess the chemosensitivity to cisplatin, $1x10^4$ stable-transfected Tca8113 cells cultured for 24 h in 96-well plates were incubated with different concentrations of cisplatin (0.3, 1.0, 3.0 mg/l) for 72 h and then subjected to MTT assay as described above. For analysis of the siRNA-tansfected cells, $5x10^3$ FaDu cells were plated and siRNA (150 nM) were transfected. After 24 h, cells were subsequently treated with cisplatin (1.5 mg/l) for 72 h and then MTT assay was performed. The value of [A570 (cisplatin+)/A570 (cisplatin-)] x100% indicated the cell survival index.

*DAPI analysis*. Cells grown on slides were treated with 1.0 mg/l cisplatin and incubated for 12 h. Then, the cells were fixed with methanol and stained with 0.1 $\mu$g/ml of 4',6'-diamidino-2-phenylindole hydrochloride (DAPI; Sigma, St. Louis, MO). Apoptotic cells were discriminated morphologically via fluorescence microscopy by the appearance of apoptotic bodies. About 300 cells per slide were investigated.

*Flow cytometry assay*. Flow cytometry was performed as described (16). Fluorescence-activated cell sorting (FACS) was performed with a Becton-Dickinson FACSort apparatus and used to quantitate the apoptotic population based on DNA levels.

*Statistical analysis*. Statistical analysis was done using the SPSS statistical software (SPSS, Inc., Chicago, IL). The relationship between Nlp expression and pathological characteristics or viariables was analyzed using the $\chi^2$ test. Student's t-test was done for comparison unless particular test was notified. $P<0.05$ was considered statistically significant. Results are presented as the mean ± SD of three independent experiments or triplicate samples.

## Results

*Overexpression of Nlp protein and mRNA in HNSCC*. To investigate whether Nlp abnormabilities are linked to human HNSCC, we first analyzed Nlp protein expression in tumor tissues. An immunohistochemical approach was used to examine 76 clinical tumor specimens and 68 normal adjacent tissue samples. As shown in Fig. 1, Nlp was found to be overexpressed in tumors compared with normal adjacent tissues. The results of immunohistochemistry are summarized in Table I. Among 76 human HNSCC samples, positive or strongly positive cytoplasmic staining (score between 2 and 6) of Nlp protein was detected in 50 tumors (65.8%). Weak or negative staining (score between 0 and 1) was detected in 26 tumors (34.2%). In contrast, of 68 normal adjacent tissues, only 3 sample exhibited positive staining for Nlp protein. The other 65 normal adjacent tissues presented weak or negative immunoreactions. In agreement with the observations in immunohistochemical assays, Nlp overexpression was detected by Western blot analysis in 14 of 20 tumors compared to their paired normal adjacent tissues (Fig. 2A). Consistently, using real-time RT-RCR, higher level of Nlp mRNA were observed in primary HNSCC specimens compared with that of the normal adjacent tissues. The fold changes in Nlp mRNA expression of the tumor relative to the non-tumor control were calculated for all cases (from 0.175- to 15.7-fold). Twelve of 20 HNSCC samples showed elevated Nlp mRNA level (fold >1.0) (Fig. 2B).

*Clinical significance of increased Nlp expression and its expression in premalignant lesions of HNSCC development*. The association between Nlp expression and the clinicopathologic characteristics of tumors was examined. As summarized in Table I, Nlp protein expression was significantly associated with the degree of tumor differentiation. Although higher expression of Nlp was seen in late stage HNSCC or HNSCC with lymph node metastasis, no statistically significant correlation was found between the Nlp protein expression and the tumor stage (P=0.087) or the nodal classification (P=0.078).

Most tissue sections of the primary carcinomas contained a combination of normal, hyperplastic, dysplastic and invasive tissue, providing the possibility to compare levels of Nlp expression in the different stages of epithelial transformation. Immunohistochemical analysis of Nlp protein expression was also done on 3 benign hyperplasias and 6 dysplasias. In contrast to the normal mucosa and the hyperplasias, we observed Nlp immunoreactive cells in the transformed but non-invasive epithelia adjacent to the tumor tissues, especially in those of moderate to severe dysplasias (Fig. 1B and C). Immunohistochemistry of isolated dysplastic lesions yielded similar pattern

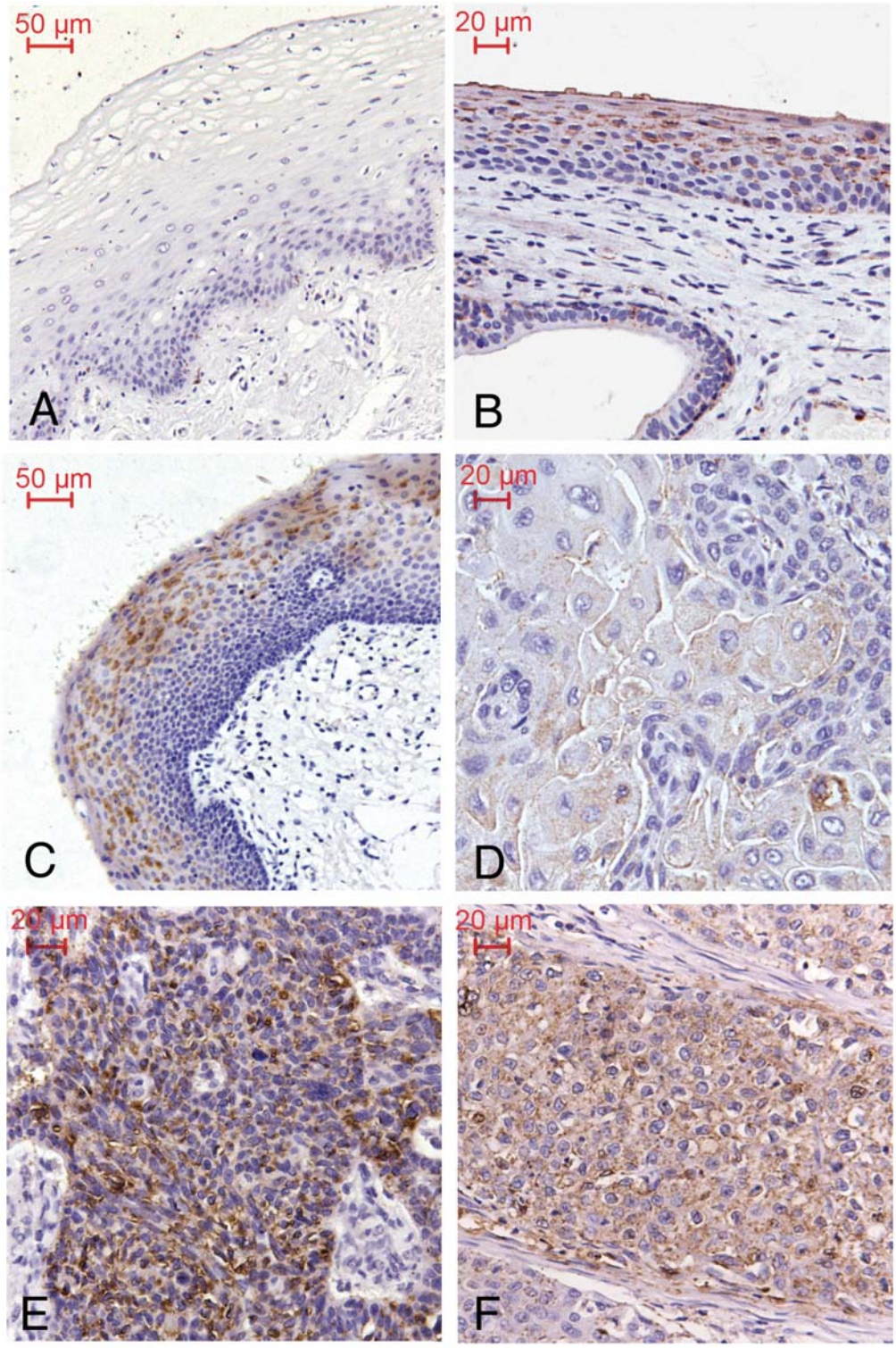


Figure 1. Immunohistochemical analysis of Nlp expression in normal adjacent epithelia, moderate dysplasia, severe dysplasia and tumor tissues of HNSCC patients. (A) Negative staining of Nlp in normal adjacent epithelia (x100). (B and C) Positive staining was seen in moderate dysplasia (B, x200) and severe dysplasia tissues (C, x100). (D, E and F) Representative cases for strongly positive staining with predominant cytoplasmatic staining pattern (D, x200), moderately positive staining (E, x200) and weakly positive staining (F, x200). All sections were counterstained with hematoxylin.

of immunostaining (data not shown). These findings indicate that overexpression of Nlp is an early event in HNSCC development.

*Nlp overexpression enhanced cell growth of cancer cells in vitro*. We detected Nlp expression in three HNSCC-derived cell lines at the protein level by Western blot analysis. As shown in Fig. 3A, Nlp was expressed at a lower level in Tca8113 compared with HEp2 and FaDu. To delineate the role of increased Nlp expression in tumorigenesis of HNSCC, Tca8113 cells was chosen for stable transfection with Nlp expression vector pEGFP-Nlp. Two independent clones (ENlp-2 and ENlp-9) were isolated for further studies. Western blot and RT-PCR analysis of these two populations of cells

Table I. Nlp expression in 76 HNSCC patients and their relation to clinicopathological factors.

| Variables | Nlp expression | | | Total no. | P-value[a] |
|---|---|---|---|---|---|
| | (-) or (±) | (+) | (++) | | |
| In general | | | | | |
| Normal adjacent tissue | 65 | 3 | | 68 | <0.001 |
| Tumor tissue | 26 | 22 | 28 | 76 | |
| Age (years) | | | | | |
| <50 | 9 | 5 | 11 | 25 | 0.453 |
| ≥50 | 17 | 17 | 17 | 51 | |
| Gender | | | | | |
| Male | 23 | 20 | 25 | 68 | 1.000 |
| Female | 3 | 2 | 3 | 8 | |
| T stage | | | | | |
| T1-2 | 17 | 12 | 10 | 39 | 0.087 |
| T3-4 | 9 | 10 | 18 | 37 | |
| N stage | | | | | |
| N0 | 19 | 19 | 16 | 54 | 0.078 |
| N1-3 | 7 | 3 | 12 | 22 | |
| Histological grade | | | | | |
| Well differentiated | 15 | 12 | 8 | 35 | 0.014 |
| Moderately differentiated | 9 | 8 | 8 | 25 | |
| Poorly differentiated | 2 | 2 | 12 | 16 | |

The correlation between Nlp protein expression and various clinicopathological factors was investigated. HNSCC were staged according to the criteria of the International Union Against Cancer (13). [a]Estimated by Pearson $\chi^2$ or Fisher's exact test (if expected count was <5).

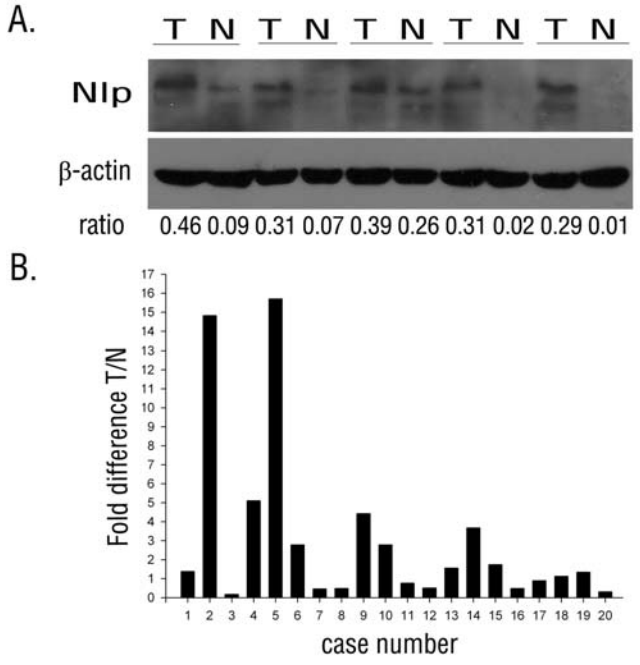


Figure 2. Increased Nlp protein levels and mRNA expression in HNSCC tumor tissues compared to the matched non-tumor tissues. (A) Western blot analysis of Nlp protein levels in representative pairs of HNSCC samples. ß-actin was used as a loading control. The ratio of Nlp/ß-actin was calculated by using densitometry. T, patient-matched tumor tissue; N, adjacent normal tissue. (B) Real-time RT-PCR analysis of Nlp mRNA level in 20 pairs of HNSCC samples. Fold difference indicates the ratio of mRNA expression level in tumor vs. normal tissue.

confirmed that they expressed higher levels of Nlp compared to their empty vector control (EGFP) (Fig. 3B and C). The results from growth curves demonstrated that growth capability of the ENlp cells was significantly stronger compared with the EGFP cells (Fig. 3D). In the colony formation assay, the colonies from ENlp cells were much larger than those from the EGFP cells and the numbers of colonies were also much more in ENlp cells compared with the EGFP cells (Fig. 3E and F).

To further determine whether Nlp expression plays a role in HNSCC cell proliferation, the endogenous Nlp expression in FaDu cells, which was relatively high in the three HNSCC cell lines (Fig. 3A), was downregulated using siRNA strategy. Before making the RNAi analysis, the transfection rate was monitored by FITC-conjugated control fragment and it was up to >70% and was satisfying to perform the following Nlp RNAi analysis. Following introduction of siRNA into FaDu cells, cells were collected at indicated time points (24, 48, 72 h) and assayed for expression levels of both Nlp proteins and mRNA (Fig. 4A and B). Cells treated with Nlp siRNA exhibited substantially reduced levels for both Nlp proteins and mRNA. We then investigated effects of Nlp siRNA on the *in vitro* growth of HNSCC cells. Cellular proliferations were monitored by MTT assay daily for 7 days. As shown in Fig. 4C, cell proliferation was significantly suppressed by Nlp siRNA in parental cell line as compared with control-siRNA. These results suggest that a higher level of Nlp is associated with enhanced proliferation and oncogenicity in HNSCC.

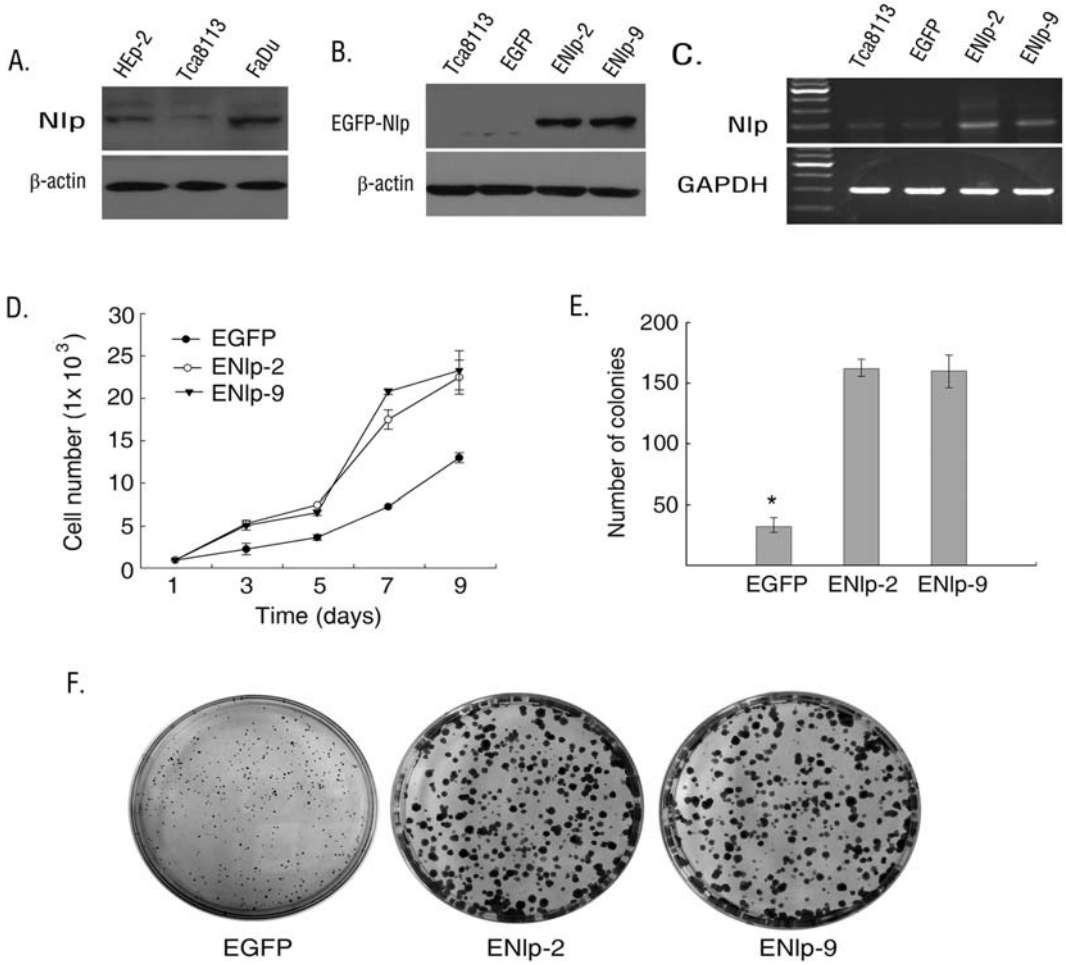


Figure 3. Expression of Nlp in HNSCC cell lines and the effects of Nlp overexpression on tumor growth. (A) Three HNSCC cell lines were screened with endogenous Nlp expression by Western blot analysis. (B and C) Western blot analysis and RT-PCR analysis show the levels of Nlp protein and mRNA in the parental Tca8113 cells, EGFP and two ENlps clones. EGFP was one representative of the empty vector transfectants and ENlp-2 and ENlp-9 were different isogenic cell lines transfected with pEGFP-Nlp. ß-Actin was used as a loading control for Western blot analysis and expression of GAPDH was provided to document equivalent loading for RT-PCR. (D) Growth curves reveal that ENlp cells proliferate more rapidly than EGFP cells. (E) Clonogenicity assay show that ENlp cells have higher clonogenecity compared with the control, EGFP cells. (F) A representative result of clonogenicity assays (EGFP vs. ENlp) using $1x10^3$ cells in each dish. All experiments were performed at least three times with consistent and repeatable results. *P<0.05 compared with ENlp-2 and ENlp-9 cells. Columns, mean of three individual experiments in each clone; bars, SD.

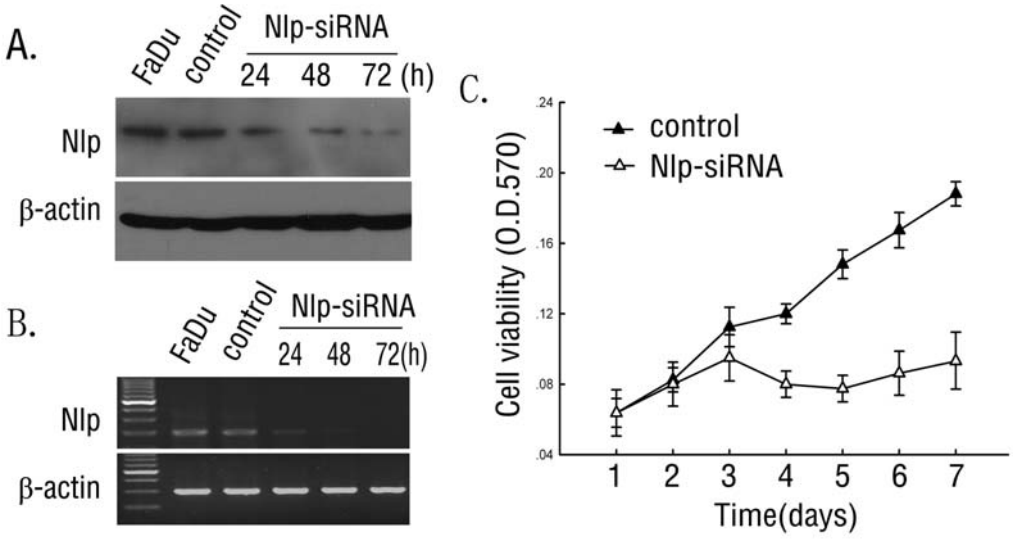


Figure 4. siRNA directed against Nlp suppresses *in vitro* growth of the HNSCC cells. (A and B) Expression of Nlp at indicated hours after the transfection of siRNA were detected by Western blot analysis and RT-PCR. (C) MTT assay of *in vitro* proliferation of the cells. These experiments were performed at least three times with consistent and repeatable results.

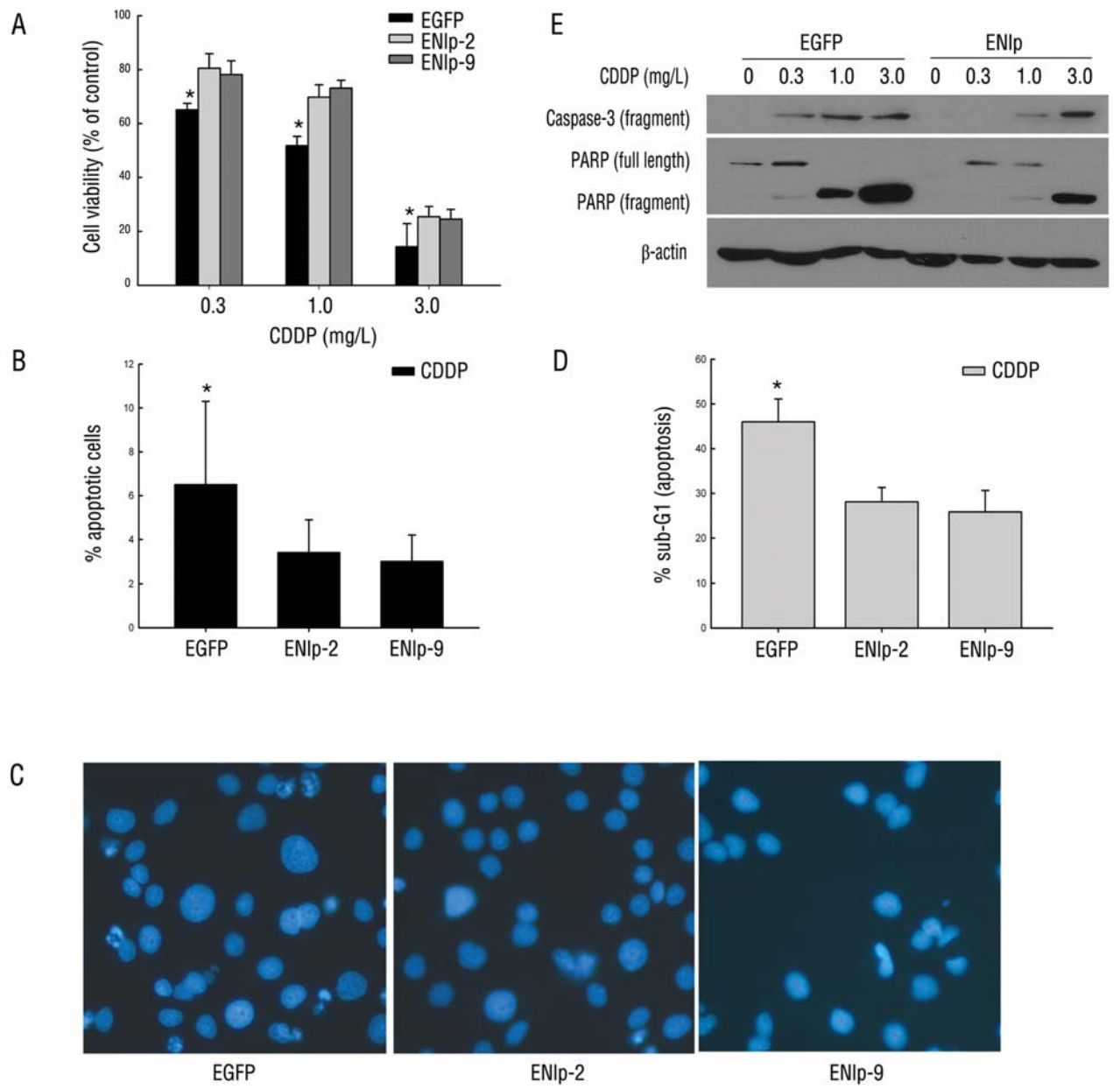


Figure 5. Nlp contributes to resistance to chemotherapy by cisplatin. (A) Effects of Nlp overexpression on the viability of the transfected Tca8113 cells. Cell viability relative to the untreated control were determined by MTT assay. (B) Percent of apoptotic cells after incubation with cisplatin (1 mg/l) for 12 h, determined via DAPI staining. (C) A representative photograph shows that the apoptotic cells were detected by DAPI staining in EGFP, ENlp-2 and ENlp-9 cells. (D) Effects of Nlp overexpression on sub-$G_1$ cell population. Cells were incubated in a culture medium for 72 h after cisplatin (1 mg/l) treatments and analyzed by a flow cytometry analysis. (E) Apoptotic response and cleavage of caspase-3 and its substrate PARP were examined by Western blot analysis in EGFP and ENlp cell lines after cisplatin treatment for 24 h. ß-actin was used as an equal loading control. All experiments were performed at least three times with consistent and repeatable results. *P<0.05 compared with ENlp-2 and ENlp-9 cells. Columns, mean of three individual experiments in each clone; bars, SD.

*Inhibitory effects of Nlp overexpression on cell death induced by cisplatin (cis-diamminedichloroplatinum).* To determine the effects of Nlp overexpression on sensitivity of HNSCC cells to cisplatin, ENlp cells and EGFP cells were treated with cisplatin and followed by MTT assay for survival rate. As shown in Fig. 5A, when cells were treated with escalating concentration of cisplatin, ENlp cells displayed a significantly increased survival as compared with EGFP cells.

We next examined whether increased survival of Nlp-overexpressing cells after cisplatin treatments was due to Nlp inhibition of apoptosis. The morphologic changes in cell nuclei were examined by a fluorescent microscope. As shown in Fig. 5B, after incubated with cisplatin for 12 h, DAPI-stained EGFP cells showed an increased number of condensed or fragmented nuclei, which was characteristic of apoptosis. In contrast, ENlp cells were resistant to cisplatin-induced cytoxicity, as manifested by little change in cell morphology. Examples of apoptotic cells in the individual cell lines are depicted in Fig. 5C. These results demonstrated that the EGFP cell death was probably due to apoptosis. Furthermore, using flow cytometry analysis, we evaluated the degree of apoptosis by percentage of hypodiploid cells. Cisplatin treatments resulted in higher percentage of EGFP cells in sub-$G_1$ phase as compared with ENlp cells (Fig. 5D), indicating that the degree of apoptosis in ENlp cells was lower than EGFP cells.

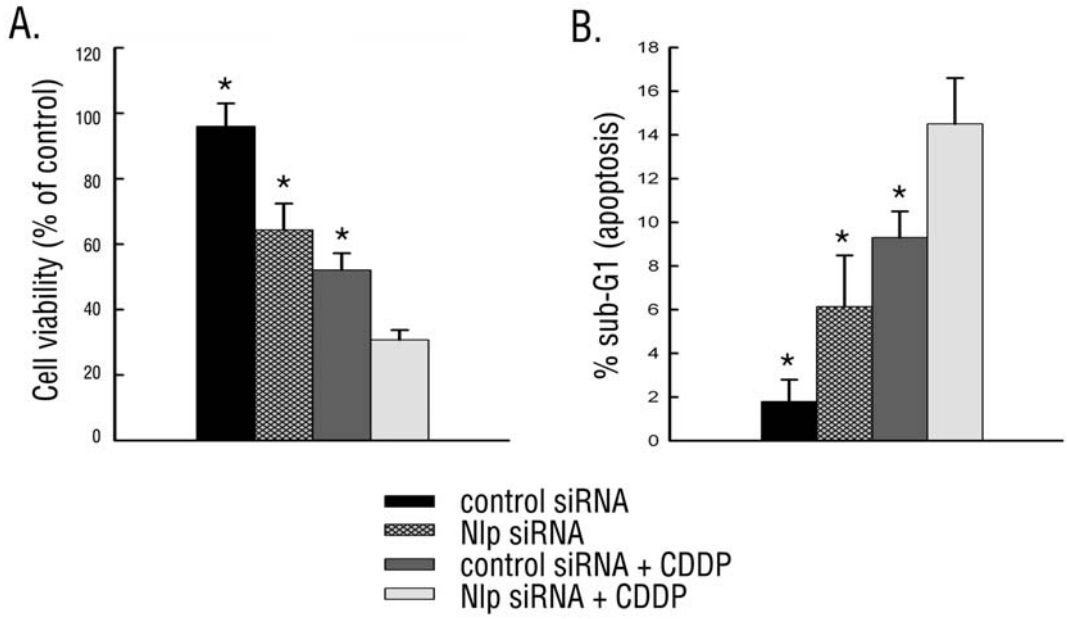


Figure 6. siRNA-directed suppression of Nlp enhances sensitivity to cisplatin-induced apoptosis. (A) Survival cells quantitated by MTT assay after transfection with Nlp siRNA or control siRNA at 150 nM only or subsequent addition of 1.5 mg/l CDDP. Cells treated with PBS were used as a control and their viability was set at 100%. (B) The sub-$G_1$ DNA content of cells treated with Nlp siRNA or control siRNA only or subsequent addition of cisplatin was analyzed by flow cytometric analysis. All experiments were performed at least three times with consistent and repeatable results. *P<0.05 vs. Nlp siRNA + CDDP group.

To provide further evidence that cisplatin-induced apoptosis was inhibited by the overexpression of Nlp, caspase-3 activation indicated by cleavage of caspase-3 and PARP (a substrate of caspase-3) was examined by Western blot analysis. As shown in Fig. 5E, cisplatin activated caspase-3 and induced cleavage of PARP in both EGFP cells and ENlp cells in a concentration-dependent manner (as evident by an increase in their content of the cleavage fragment). However, the cleavage was much weaker in ENlp cells than in EGFP cells. Taken together, these results indicated that cisplatin-induced apoptosis were significantly inhibited by Nlp overexpression.

*Downregulation of Nlp results in increased sensitivity to cisplatin-induced apoptosis*. To further confirm the role of Nlp in cell resistance to cisplatin, we examined whether down-regulation of Nlp correlated with enhanced susceptibility to cisplatin-induced apoptosis. FaDu cells were transfected with either Nlp siRNA or control siRNA at 150 nM in concentration only or followed by addition of 1.5 mg/l cisplatin 24 h later. The concentration for cisplatin was set by $IC_{50}$ previously determined by MTT assay (data not shown) for FaDu cells. Ninety-six hours after transfection, cell viabilities were analyzed with MTT assay. As shown in Fig. 6A, control siRNA-treated cells displayed higher percentage of viable cells upon cisplatin treatment compared with Nlp siRNA-treated cells, which is consistent with our observations that Nlp overexpressing cells exhibited enhanced cisplatin resistance. Furthermore, downregulation of Nlp by Nlp-specific siRNA exhibited higher levels apoptosis upon cisplatin treatments compared with control siRNA-transfected cells by percentage of hypodiploid cells (Fig. 6B). These results indicated that siRNA mediated knockdown of Nlp can enhance sensitivity of these HNSCC cells to anti-cancer drugs.

## Discussion

Recent studies have described overexpression of Nlp in several types of human tumors and suggested it was a potential oncogenic protein (11,12). In this study, we have shown for the first time that Nlp, a novel centrosome-associated protein, is overexpressed in human HNSCC. Using an immunohistochemical staining approach, we found that increased Nlp expression occurred in a significant proportion (65.8%) of HNSCC. The frequency was parallel to that of overexpression by Western blot analysis (70%) and real-time RT-PCR examination (60%), suggesting that Nlp overexpression is a common abnormality in this type of tumor.

Similar to other epithelial neoplasms, head and neck carcinogenesis appears to evolve through a multistep process involving biomolecular changes, ensuing premalignant lesions and consequent invasive cancer (17). The elucidation of mechanisms governing malignant changes from dysplasia to HNSCC may provide better understanding of tumorigenesis, as well as new approaches to early prevention and treatment. Our findings showed that Nlp overexpression occured in different stages of head and neck epithelial tumorigenesis, from normal tissues to premalignant and malignant lesions. Deregulated expression of Nlp in premalignant lesions of HNSCC was detected not only in non-invasive transformed epithelia adjacent to the tumor tissues but also in dysplastic lesions of the head and neck that had not yet acquired the fully malignant and invasive phenotypes. The finding that Nlp is overexpressed in the early stage of HNSCC suggests that Nlp may be a functionally significant event in the epithelial transformation of HNSCC and might serve as a marker for the early detection of HNSCC.

When performing immunohistochemistry analysis, we also found that higher levels of Nlp expression in HNSCC is

associated with poor differentiation of tumors. Although the statistical analysis revealed no significant correlation between Nlp expression and the tumor stage, as well as the lymph node stage, there was a clear trend towards correlation for these two clinicopathologic variables. Functional analysis in the current study showed that HNSCC cells expressing exogenous Nlp exhibited stronger proliferating ability compared to those expressing lower Nlp levels. Conversely, reduction of endogenous Nlp protein via siRNA knockdown technique was shown to suppress cell proliferation, suggesting an association of Nlp with a more aggressive phenotype of HNSCC cells. Taken together, the results suggest that Nlp upregulation may contribute to the malignancies of HNSCC.

With regard to the precise mechanism by which Nlp plays a role in HNSCC occurrence and progression, Nlp-induced chromosomal aberrations and genomic instability may mainly contribute to its oncogenic properities, given the preponderance of chromosomal abnormalities and aneuploidy in HNSCC (6,18). Previous studies have shown that overexpression of Nlp caused aberrant spindle formation and led to multinuclearity and chromosomal instability (9,11). Indeed, several centrosome-associated kinases and their target substrates in the regulation of the centrosome have been involved in HNSCC development and progression. For example, Aurora-A and Polo-like kinase (Plk), which are involved in the control of centrosome maturation, have been shown to be amplified and have prognosis significance in HNSCC (19,20). Tumor suppressor p53, which is thought to be required for the centrosome duplication, is inactivated in the majority of HNSCC (21,22). Therefore, it is also possible that Nlp acts synergistically with other centrosome-associated proteins in HNSCC development.

Previously, Qu *et al* examined the expression of Nlp in human ovarian cancer and found that overexpression of Nlp counteracted the apoptosis of ovarian cancer cells induced by paclitaxel (12). Similar findings have also been reported for other centrosome-associated proteins. For example, overexpression of Aurora-A, which is also involved in the control of centrosome maturation, led to a resistance to apoptosis induced by cisplatin (23). In the present study, we found that overexpression of Nlp in HNSCC cells not only conferred a proliferative advantage but also renders cell more resistant to the growth inhibitory and apoptotic effects of cisplatin. Conversely, siRNA-mediated reduction of Nlp expression results in increased sensitivity to cisplatin-induced apoptosis. Cisplatin is a DNA-damaging agent that has been commonly used in the chemotherapy of head and neck cancer, both alone and in combination with other chemotherapeutic agents or radiation therapy (24). However, development of cisplatin resistance is a major obstacle in the clinical treatment (25). Our findings suggest that Nlp induces cancer cell resistance to cisplatin and other agents of this kind and, if so, inhibitors of Nlp may be valuable adjuncts to these agents in the treatment of cancers that overexpressing Nlp. The results also suggest that Nlp inhibition of drug-induced cell death may not only account for the failure of standard chemotherapy but may also further help explain why Nlp overexpression contributes to malignant phenotype of HNSCC.

In summary, we report that overexpression of Nlp is a frequent and early event in HNSCC that is maintained during tumor progression. Nlp contributes to tumor aggressiveness through increasing the capacity of cell proliferation and apoptosis resistance. This study will add more information on understanding the molecular mechanisms of tumorigenesis in HNSCC and will shed light on the development of better treatment and better diagnostic and preventive approaches to HNSCC. The importance of Nlp in the pathogenesis of HNSCC, given its possible therapeutic value, warrants further investigation.

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