Id-1 and the p65 subunit of NF-κB promote migration of nasopharyngeal carcinoma cells and are correlated with poor prognosis

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Inhibitor of differentiation (Id)-1 and nuclear factor-kappa B (NF-κB) have been detected in many malignant tumors, and their presence has been correlated with the metastatic potential of these tumors. This study was undertaken to investigate the prognostic significance of the expression of Id-1 and the p65 subunit of NF-κB (NF-κB/p65) and the proteins’ roles in the invasion process of nasopharyngeal carcinoma (NPC) cells. The messenger RNA (mRNA) and protein levels of Id-1 and NF-κB/p65 in normal nasopharyngeal epithelial cells and NPC cell lines were examined using reverse transcription–PCR and western blot analysis, whereas the mRNA and protein levels of Id-1 and NF-κB/p65 in clinical NPC specimens were determined by reverse transcription–PCR and immunohistochemistry. Short hairpin RNA (shRNA) was used to silence Id-1 and NF-κB/p65 to allow for the examination of matrix metalloproteinase (MMP)-9 expression and migratory capacity changes in CNE-2 cells. Multivariate Cox analysis revealed that elevated Id-1 expression was a significant independent predictor of the 5 year overall survival rate (hazards ratio = 16.720, P = 0.005). Furthermore, elevated expression of both Id-1 and NF-κB/p65 was associated with poor clinical survival (P = 0.049). Targeting Id-1 and NF-κB/p65 mRNA with shRNA in CNE-2 cells inhibited MMP-9 expression and decreased the migratory capacity of CNE-2 cells. In conclusion, Id-1 expression is a novel independent prognostic marker molecule that helps identify NPC patients with a poor prognosis. Additionally, combined analysis of Id-1 and NF-κB/p65 can be useful for identifying patients at risk for unfavorable clinical outcomes. Id-1 or and NF-κB/p65 enhanced tumor cell migration, which is associated with the secretion of MMP-9.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant epithelial carcinoma of the head and neck area that occurs at a rate of 20–30 cases per 100 000 people in regions, such as Southeast Asia (population >100 million) and the Mediterranean basin (population >300 million) (1). Although NPC is radiosensitive, the survival rate for NPC has not been significantly improved even with the use of radiotherapy, radiochemotherapy or targeted radiotherapy (as adjuvant therapy) (2–5). Regional lymph node and distant metastases and locoregional recurrence are two major indications of NPC treatment failure (2–5). The evaluation of an NPC patient’s prognosis is primarily based on the clinical tumor-node-metastasis (TNM) staging. However, patients with NPC with similar clinical stage classifications often have different clinical outcomes, suggesting that TNM staging is insufficient for precisely determining an NPC prognosis. Therefore, identifying specific biomarkers that have diagnostic and prognostic value for NPC malignancies remains a priority.

Inhibitors of differentiation/DNA binding (Id) family proteins act as dominant-negative regulators of helix-loop-helix transcription factors and inhibit the expression of genes involved in cell differentiation (6). Recent studies suggest that Id proteins, especially Id-1, function as oncogenes. For example, Id-1 stimulates DNA synthesis, initiates the G1 to S phase transition (7–9) and extends the life span of primary human keratinocytes (10,11). In addition, upregulation of Id-1 has been found in many types of human malignancies, such as breast (12), pancreatic (13), prostate (14) and ovarian (15) cancers. Overexpression of Id-1 has recently been identified as a marker for unfavorable prognosis in breast and cervical cancers (15,16), and the protein expression level of Id-1 has been shown to correlate with the aggressiveness of prostate and ovarian carcinomas (14,17). Id-1/Id-3−/− double-knockout mice showed a significant reduction in the metastatic ability of tumor xenografts (18). These findings suggest that Id-1 contributes to the invasiveness of tumor cells. In a previous NPC study, Id-1 was identified as an activator of cell proliferation (19), but its role in invasion and its clinical significance to NPC have not been revealed.

Nuclear factor-kappa B (NF-κB) is composed of p65 (also known as RelA), c-Rel and p50, which are sequestered in the cytoplasm by specific inhibitors, namely the inhibitor of κB proteins. Due to its strong transcriptional activity, the p65 subunit of NF-κB (NF-κB/p65) is responsible for most of NF-κB’s transcriptional activity. NF-κB/p65 also contributes to digestion of the extracellular matrix by triggering the production of matrix metalloproteinases (MMPs) in tumor cells and the surrounding mesenchymal cells (20–22), and its expression adversely impacts patient prognosis in human cancers, such as ovarian and breast cancers (23–25). Recent studies have showed that NF-κB might be regulated by Id-1 in several cancers (26–28). We have also recently shown that Id-1 contributes to head and neck squamous cell carcinoma survival via the NF-κB signaling pathways (29). The above evidence suggests that the co-expression of Id-1 and NF-κB proteins may be involved in NPC progression and act as activators of the invasion process in NPC.

To date, the expression profiles of Id-1 and NF-κB/p65 and their clinical significance in NPC have not been studied. The aim of this study was to investigate the expression patterns of Id-1 and NF-κB/p65 and their clinicopathological implications for NPC malignancy and to determine the underlying molecular mechanisms of cancer cell migration to better understand the processes underlying NPC malignancy.

Materials and methods

Cell lines and cell culture

Primary nasopharyngeal epithelial cell (NPEC) cultures and immortalized NPECs induced by Bmi-1 were established as described previously (30) and grown in keratinocyte/serum-free medium (Invitrogen corporation, Camarillo, CA). Four NPC cell lines (CNE-1, CNE-2, C666 and 5-8F cell line) were maintained in RPMI 1640 media (Sigma, St Louis, MO) supplemented with 2 mM l-glutamine and 10% fetal bovine serum (FBS). The cultures were grown for a maximum of 10 passages before retrieving fresh cells from frozen stock.
Patients and clinical tissue samples

A total of 131 patients with NPC were selected for this study. Six patients were subsequently excluded from this study due to incomplete follow-up data. In total, 125 subjects with NPC and 30 subjects with normal nasopharyngeal tissues were included in the study. Only the survival of NPC patients without distant metastases was considered (n = 110). These patients received standard curative chemoradiotherapy with or without consolidation therapy at the Sun Yat-Sen Memorial Hospital of Sun Yat-sen University and the Oncology Hospital of Guangzhou Medical University between January of 2000 and July of 2004 and clinical follow-up data were collected. The average observation length for overall survival was 55.5 months for patients who were still alive at the time this analysis was written. The survival time ranged from 3 to 83 months. Fifty-five patients (7%) died during the study period during which follow-up was completed. Tumor tissues were obtained from biopsy specimens, and normal tissues were used as a control. The tumor and normal tissues were confirmed by pathologic examination. The tissues used for immunohistochemistry were fixed in 10% buffered formalin and embedded in paraffin. Large pieces from the 155 biopsy specimens (five NPCs and two normal nasopharyngeal tissues) were divided into several pieces for western blot analysis. Patient consent and approval from the Institutional Research Ethics Committee were obtained to use these clinical materials for research purposes.

The principal inclusion criteria were the following: diagnosis of primary squamous cell carcinoma of only the NPC type, no history of previous malignancies and no history of previous radio or chemotherapeutic treatments. The main clinical and pathologic characteristics of the patients are presented in Supplemental Figure S1, available at CancerResearchOnline; 97 (77.6%) were male and 28 (22.4%) were female, and the median age was 46 years (age ranged from 15 to 70 years old). The clinical staging and the anatomic site of the tumors were assessed according to the sixth edition of the Union Internationale Contre le Cancer (UICC 2008) TNM classification of malignant tumors.

Immunohistochemical staining

All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin blocks. Sections (5 μm) were deparaffinized in xylene. Endogenous peroxidase was blocked with 3% hydrogen peroxide in deionized water for 10 min. The sections were incubated with normal rabbit serum (Zymed Laboratories, South San Francisco, CA) for 10 min to block non-specific binding sites. The sections were washed with PBS, and the cells were cultured for 72 h.

PCR amplification programs consisted of an initial incubation at 50°C for 2 min followed by 40 cycles of the following steps: 95°C for 10 min, 95°C for 15 s and 60°C for 1 min.

Short hairpin RNA transfection

Four groups of Id-1-specific shRNA oligos and four pairs of NF-xB/p65-specific shRNA oligos were selected for use in this study; a scrambled shRNA was used as a negative control (GenePharma, Shanghai, People’s Republic of China). The four pairs of Id-1-specific shRNA were shRNA1, which targeted GGATTC- CACTCGTGTTGTTCC; shRNA2, which targeted GCCCATCTTGTTGCTTCTCA and shRNA3, which targeted GGTCACGTTTGGTGCTTCTCA and shRNA4, which targeted GCCCATCTTGTTGCTTCTC.

Prognostic significance of Id-1 and p65 subunit of NF-xB

CNE-2 cells were transfected with scrambled shRNA, Id-1 shRNA, NF-xB/p65 shRNA or both Id-1 and NF-xB/p65 shRNA in 2 ml of serum-free OptiMEM. Five hours after transfection, the medium was replaced with RPMI 1640 medium supplemented with 10% FBS at 37°C with 5% CO2. For transfection, 2 ml of CNE-2 cells were seeded at a concentration of 0.75 × 104 cells/ml per well and cultured in a six-well plate for 24–48 h. When the CNE-2 cells were between 30 and 50% confluent, each well of cells was transfected with a mixture containing either 10 μl of 50 nM specific shRNA (Id-1 shRNA, NF-xB/p65 shRNA or Id-1/NF-xB/p65 shRNA) or 10 μl of 50 nM scrambled shRNA and 10 μl of lipofectamine™ 2000 (Invitrogen Corporation) in 2 ml of serum-free OptiMEM. Five hours after transfection, the medium was replaced with RPMI 1640 medium supplemented with 10% FBS, and the cells were cultured for 72 h.

Fluorescent immunohistochemistry

CNE-2 cells transfected with scrambled shRNA, Id-1 shRNA and NF-xB/p65 shRNA were fixed in 70% methanol, incubated with MMP-9 antibody (1:1000; Santa Cruz Biotechnology) for 60 min and incubated with fluorescent isothiocyanate- or Rhodamin B isothiocyanate-conjugated secondary antibodies (Zymed Laboratories) using previously described protocols (32). To better identify the location of Id-1 and NF-xB/p65 in NPC tissues, the expression of these two factors was also detected using fluorescent immunohistochemistry. 4’-Diamidino-2-phenylindole dye was used to counterstain tissue sections to indicate the location of the nucleus. Tissue sections incubated with non-specific antibodies (mouse or rabbit IgG isotype from Zymed) served as negative controls.

Fluorescence-activated cell sorting

CNE-2 cells cultured with 60% confluence were transfected in transfection media with no shRNA. Id-1 shRNA, NF-xB/p65 shRNA or both Id-1 and NF-xB/p65 shRNA at 1.4 μg/ml for 16 h. allowed to recover in cell culture media for 72 h and then harvested for the evaluation of positive cells. Briefly, cells were washed in PBS, harvested by trypsinization and pre-incubated with 0.3% saponin in PBS for 10 min. They were then sequentially incubated with primary and secondary antibodies labeled with Alexa Fluor 488 (Molecular Probes) or with Alexa Fluor 594 (Molecular Probes). The cells were then washed with 0.3% saponin in PBS, incubated with a fluorescent isothiocyanate-conjugated secondary antibody on ice for 20 min, resuspended in PBS and analyzed with fluorescent-activated cell sorting (FACS) calibration using CellQuest-Pro software (BD Biosciences, San Jose, CA). Non-specific IgG was used as a control. Cells without any staining were served as blank controls.

Western blot analysis

Cells were harvested and washed with cold PBS solution, and the total protein was extracted using lysis buffer. Immunoblot experiments were performed according to standard procedures. The protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of...
protein were separated by electrophoresis on 12% sodium dodecyl sulfate/polyacrylamide gels and transferred onto Bio-dyne A Membrane (Gelman Laboratory, Pall Corp., Ann Arbor, MI). The membrane was probed with two primary antibodies, rabbit polyclonal anti-Id-1 (1: 2000, SC-488; Santa Cruz Biotechnology) and rabbit polyclonal anti-NF-κB/p65 (1: 2000, SC-109; Santa Cruz Biotechnology). The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:2000; Santa Cruz Biotechnology). An anti-glyceraldehyde 3-phosphate dehydrogenase mouse monoclonal antibody (1:4000; Santa Cruz Biotechnology) was used as an internal loading control.

Gelatin zymography
Gelatin zymography assays were performed as described previously (33). Briefly, culture media (supernatants) from scrambled and Id-1 shRNA-transfected CNE-2 cells were collected 72 h after transfection and concentrated seven times using a freeze-dry system. Wells of a sodium dodecyl sulfate-polyacrylamide gel containing 0.1% gelatin were loaded with the samples (~20 μg of protein from 1.2 million cells) and run for 100 min at 125 mV in a tris-glycine sodium dodecyl sulfate running buffer. The gels were renatured in zymogram renaturing buffer for 60 min and incubated overnight at 37°C in zymogram developing buffer. After staining with 0.5% Coomassie Blue G-250 and destaining with a 50% methanol and 10% acetic acid solution, clear bands of gelatinolytic activity were revealed. The gels were digitally photographed, and the band densitometry was assessed using a computer image analysis system (ImagePro plus 6.0) to obtain a semiquantitative measure of enzymatic activity.

Transwell migration assays
The assay was performed using chambers with an 8 micron pore size polyethylene terephthalate membrane and a thin layer of matrigel basement membrane matrix (BD BioCoat™ Matrigel™ Invasion Chamber). Forty-eight hours after transfection with Id-1 shRNA and/or NF-κB/p65 shRNA, the CNE-2 cells and culture medium were harvested, and 2.5 × 10⁴ cells in 0.5 ml harvest medium were placed in the upper chamber. The scrambled shRNA-transfected cells were used as a negative control. The lower chamber was filled with 10% FBS medium (0.75 ml). After incubating the cells for 22 h, the cells on the upper chamber of filter were fixed, stained and counted.

Statistical analyses
Statistical analysis was performed with the SPSS software (SPSS Standard version 13.0, SPSS). The association of Id-1 and NF-κB/p65 levels with NPC patients’ clinicopathological features and the correlation between the detected molecular features were analyzed by either the χ² test or the Fisher’s exact test. For survival analysis, we analyzed all of the NPC patients using a Kaplan–Meier analysis. The log-rank test was used to compare the different survival curves. Multivariate survival analysis was performed on all of the parameters from the Cox regression model. A P <0.05 was considered to be statistically significant.

Results

Id-1 and NF-κB/p65 expression in NPC cells
The mRNA and protein levels of Id-1 were high in CNE-1, CNE-2 and 5-8F cells, whereas the mRNA and protein levels of Id-1 in NPECs were the same as in the control. The mRNA and protein levels of NF-κB/p65 in the NPECs were low compared with the CNE-1, CNE-2, C666 and 5-8F cells (Figure 1).

![Figure 1](https://academic.oup.com/carcin/article-abstract/33/4/810/2463814)

Fig. 1. The mRNA and protein levels of Id-1 and NF-κB/p65 in immortalized NPECs and NPC cell lines were analyzed by real-time PCR and western blot analysis, respectively. (A) Relative Id-1 mRNA expression levels were compared among the NPECs and the four cultured NPC cell lines, including CNE-1, CNE-2, C666 and 5-8F cell lines. (B) Relative NF-κB/p65 mRNA expression levels were compared among the NPECs and the four cultured NPC cell lines, including CNE-1, CNE-2, C666 and 5-8F cell lines. (C) Id-1 and NF-κB/p65 protein levels in the NPECs and four cultured NPC cell lines. The protein levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (D) Densitometry analysis revealed that the Id-1 protein levels in CNE-1, CNE-2 and 5-8F cells were higher than in NPECs. (E) Densitometry analysis revealed that the amount of NF-κB/p65 protein in the NPECs was lower than in CNE-1, CNE-2, C666 and 5-8F cells. Error bars: standard deviation calculated from three parallel experiments.
Univariate analysis showed that the prognostic predictors of overall survival in patients with NPC include the expression of Id-1 alone (P = 0.003), the combined expression of Id-1 and NF-κB/p65 (P = 0.049), the T stage (P = 0.000), the N stage (P = 0.002) and the clinical stage (P = 0.001) (Supplementary Figure S3 is available at Carcinogenesis Online).

Multivariate survival analysis. The Cox regression model was used to test the influence of each parameter on overall survival. We included Id-1 and NF-κB/p65 expression (low versus high), the clinical stage (I–II versus III–IV), T-stage (T1–2 versus T3–4) and the N-stage (N0 versus N1–3) in our analysis. We tested the impact of Id-1 and NF-κB/p65 expression on the overall survival rate. The results showed that the overall survival time significantly depends on the Id-1 expression level, the N stage and the clinical stage (P = 0.005, P = 0.035 and P = 0.021, respectively) (Table II).

Id-1 and NF-κB/p65 increase the expression of MMPs at the level of mRNA in CNE-2 cells

Accumulating evidence suggests that Id-1 and NF-κB/p65 are related to the aggressiveness of cancer cells and that the proteins may be related to MMPs in breast cancer cells, prostate cancer cells and glioblastomas (20–22,28,34). To investigate if either Id-1 or NF-κB/p65 regulates the expression of MMPs, CNE-2 cells were transfected with Id-1 shRNA, NF-κB/p65 shRNA or scrambled shRNA. The amount of Id-1 protein in Id-1 shRNA4-transfected cells and the amount of NF-κB/p65 protein in NF-κB/p65 shRNA3-transfected cells were significantly lower than in scrambled shRNA-transfected cells (data not shown). The CNE-2 cells were then transfected with Id-1 shRNA4 or and/or NF-κB/p65 shRNA3 or scrambled shRNA and were collected to examine mRNA by reverse transcription–PCR. We found that Id-1 and NF-κB/p65 silencing significantly decreased the mRNA expression level of MMP-9 and MMP-2 compared with controls 2 days after transfection (Supplementary Figure S4A is available at Carcinogenesis Online).

Id-1 and NF-κB/p65 increase the amount of MMP-9 and proteinase activity in CNE-2 cells

To determine if Id-1 or NF-κB/p65 contribute to the amount of MMP-9 protein and the proteinase activity of secreted MMP-9, Id-1 shRNA4 and/or NF-κB/p65 shRNA3-transfected cells and cultured medium (serum-free) were harvested. The effect of Id-1 and NF-κB/p65 on MMP-9 protein expression and proteinase activity was assessed using FACs and gelatin zymography assays. The FACs results showed that 3 days after Id-1 shRNA4 and/or NF-κB/p65 shRNA3 transfection, the level of MMP-9 protein in CNE-2 cells decreased by 26, 27 and 30%, respectively compared with cells transfected with scrambled shRNA (Supplementary Figure S4B is available at Carcinogenesis Online). To confirm the amount of MMP-9 and its cellular localization, we performed immunofluorescence analysis using Id-1 shRNA4 or NF-κB/p65 shRNA3-transfected and scrambled shRNA-transfected cells. MMP-9 protein was mainly localized in the cytosol in scrambled shRNA-transfected cells. As expected, Id-1 shRNA4- or NF-κB/p65 shRNA3-transfected cells yielded poor staining with the MMP-9 antibody (Supplementary Figure S4C is available at Carcinogenesis Online). To further estimate the activity of the secreted MMP-9 proteinase, samples of cell culture medium (serum-free) were collected 72 h after transfection. The activity of both secreted pro-MMP-9 and MMP-9 were strongly reduced in the culture medium from cells transfected with Id-1 shRNA4 or Id-1/NF-κB/p65 shRNA3 compared with the control cells (P < 0.05 and P < 0.01, respectively) (Supplementary Figure S4D is available at Carcinogenesis Online). These findings confirm the effects of Id-1 and NF-κB/p65 on MMP-9 activity and indicate that Id-1 and NF-κB/p65 are required for the upregulation of MMP-9 in CNE-2 cells.

Univariate analysis showed that the expression of Id-1 in NPC clinical samples of a) was higher than that of NT (P = 0.03); id b) showed low expression of Id-1 in the nucleus (arrowhead) of NPC tumor cells, whereas NF-κB/p65 was observed in normal tissues (c), whereas high expression of NF-κB/p65 was observed predominantly in the cytoplasm of NPC tumor cells (d) (immunohistochemistry, ×400). (B) Fluorescent–immunohistochemistry clearly demonstrates that Id-1 is found in both the cytoplasm (asterisks) and the nucleus (arrowhead) of NPC tumor cells, whereas NF-κB/p65 staining was observed only in the cytoplasm of NPC tumor cells. Scale bar: 100 μm. (C). Real-time quantitative PCR indicated that both NF-κB/p65 and Id-1 were significantly upregulated in NPC tissues (NPCs) compared with normal nasopharyngeal tissues (NT), with P values of 0.03 and 0.04, respectively. (D) The protein levels of Id-1 and NF-κB/p65 in NPCs and NT were determined by western blot analysis (a). Densitometry analysis revealed that the Id-1 protein level in NPCs was higher than that of NT (P < 0.01) (b). Densitometry analysis revealed that the protein level of NF-κB/p65 in NPCs was higher than that of NT (P < 0.01) (c).

Id-1 and NF-κB/p65 expression and patient survival

Univariate survival analysis. In univariate survival analyses, the cumulative survival curves were calculated according to the Kaplan–Meier method (Figure 3). The differences in survival were assessed with the log-rank test. When the patient cohort was stratified according to tumor expression of Id-1, the 5 year overall survival rate in patients with low Id-1 expression and high Id-1 expression was 95.7 and 41.4%, respectively, and the difference between the two groups was significant (P = 0.000; Figure 3A). With respect to NF-κB/p65 expression, the 5 year overall survival rate in patients with low NF-κB/p65 expression and high NF-κB/p65 expression was 51.0 and 54.1%, respectively, and there was no significant difference between the two groups (P = 0.742; Figure 3B). The 5 year overall survival rates in patients with low expression of both Id-1 and NF-κB/p65, high expression of either Id-1 or NF-κB/p65 or high expression of both Id-1 and NF-κB/p65 were 90.0, 55.8 and 41.7%, respectively. There was a significant difference between the groups with low or high expression of both Id-1 and NF-κB/p65 (P = 0.014; Figure 3C), whereas no significant difference was found between the Id-1 and NF-κB/p65 lower expression group and the group where either Id-1 or NF-κB/p65 was highly expressed (P = 0.061; Figure 3C).
Table I. Relationship between Id-1 and NF-κB/p65 expression and various clinicopathological factors of NPCs

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<th>NF-κB/p65 expression</th>
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Fig. 3. Kaplan–Meier curves of overall NPC patient survival. (A) The 5 year overall survival rates were 95.7 and 41.4% in patients with low (n = 23) and high (n = 87) Id-1 expression, respectively. There was a significant difference in the overall survival rate between the two groups (P = 0.000). (B) The 5 year overall survival rates were 51.0 and 54.1% in patients with low NF-κB/p65 expression (n = 49) and high NF-κB/p65 expression (n = 61), respectively. There was no significant difference in the overall survival rate between the two groups (P = 0.742). (C) The 5 year overall survival rates were 90.0, 55.8 and 41.7% in patients with low Id-1 and NF-κB/p65 expression (n = 10), high expression of either Id-1 or NF-κB/p65 (n = 52) and high expression of both Id-1 and NF-κB/p65 (n = 48), respectively. There was a significant difference between the high and low Id-1 and NF-κB/p65 groups (P = 0.014). There were no differences between the low Id-1 and NF-κB/p65 expression and the high expression of either Id-1 or NF-κB/p65 groups (P = 0.061).
Prognostic significance of Id-1 and p65 subunit of NF-κB

Table II. The results of the Cox regression model using conventional parameters and Id-1 expression, NF-κB/p65 expression and the combination of Id-1 and NF-κB/p65 expression

<table>
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<th>Parameter</th>
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<th>95% Confidence interval</th>
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<td>UICC stage</td>
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<td>1.176–7.649</td>
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Fig. 4. In vitro tumor cell invasion capability assay for cells transfected with Id-1 shRNA and NF-κB/p65 shRNA. (A) Representative photos showing the cell density on the filter in CNE-2 cells transfected with Id-1 shRNA and/or NF-κB/p65 shRNA. (B) Quantitative analyses for the cells migrating through the filter in three independent experiments (P < 0.05, P < 0.01 and P < 0.01, respectively). Columns, mean; bars, standard deviation.

Discussion

The current TNM staging and histopathological grading systems are useful prognostic indicators for NPC (35). However, they have limitations with regard to providing critical information regarding patient prognosis. Patients with the same clinical stage and/or pathological grade of NPC often display considerable variability in disease recurrence and survival (36). Therefore, new objective measures and biomarkers are necessary to effectively differentiating patients with favorable outcomes from those with less favorable outcomes. Molecular biomarkers in conjunction with standard TNM and histopathological strategies have the potential to predict prognoses more effectively.

Id-1 or NF-κB/p65 overexpression has been found in many types of human malignancies, such as breast, pancreatic, prostate and ovarian cancers and has been indicated to be a marker of unfavorable prognoses (12–15). Lines of evidence have also suggested that the overexpression of Id-1 is correlated with more aggressive clinical behaviors of prostate and ovarian carcinomas (14,17). However, in NPC studies, Id-1 has only been identified as an activator of cell proliferation (19), and its role in invasive behavior and its relationship with clinical significance in NPC have not been explored. Our results in this study suggest that NPC patients with high Id-1 expression in tumor cells have a significantly worse 5 year survival rate compared with NPC patients with low Id-1 expression both in univariate and in multivariate analysis (P = 0.003 and P = 0.005, respectively), whereas NPC patients with high NF-κB/p65 expression do not have a significantly different 5 year survival rate compared with NPC patients with low NF-κB/p65 expression (P = 0.744 by univariate analysis). These results are consistent with previous findings that the overexpression of Id-1 in malignant tumors is associated with a poor outcome (37–40). Furthermore, NPC patients which display high expression of both Id-1 and NF-κB/p65 in tumor cells had significantly worse prognoses and a significantly lower 5 year survival rate compared with NPC patients with low expression of both Id-1 and NF-κB/p65 (P = 0.049 by univariate analysis). To the best of our knowledge, this study demonstrates for the first time that overexpression of Id-1 and NF-κB/p65 predicts the poor prognosis of NPC.

In the immunohistochemistry-based study, we examined the expression of both Id-1 and NF-κB/p65 in NPC tissue versus normal nasopharyngeal epithelial tissue. Our results indicate that Id-1 and NF-κB/p65 are highly expressed in the cytoplasm of NPC cells compared with normal nasopharyngeal epithelial tissues (P = 0.000 and P = 0.001). In addition, Id-1 was also expressed in the nuclei of some NPC cells. Previous studies have shown that NF-κB/p65 is usually found in the nucleus and/or the cytoplasm of tumor cells (21–25). However, the results of this study show that NF-κB/p65 is exclusively localized to the cytoplasm in NPC cells, which is in line with recent NPC studies (41–43). The reason why NF-κB/p65 was localized to the cytoplasm in NPC cells is still not fully understood, however some data suggest that the translocation of NF-κB/p65 from the cytoplasm to the nucleus is inhibited by EBNA1 in NPC cells (43). The results also show that Id-1 and NF-κB/p65 are highly expressed in N1–3 NPC tissue compared with N0 NPC tissues (P = 0.008 and P = 0.002, respectively). The strong expression of Id-1 and NF-κB/p65 in NPC cell lines and tissues was confirmed by real-time PCR and western blot analysis. The upregulation of Id-1 and NF-κB/p65 proteins is related to NPC lymph node metastasis, as shown in this study. This scenario is similar to that observed in other type of human cancers, such as prostate (14), cervical (16), breast (44) and ovarian cancers (17).

To better understand how Id-1 and NF-κB/p65 exert their metastatic and invasive function, we examined the effect of Id-1 and NF-κB/p65 on CNE-2 cell invasive activity using Matrigel™ Invasion Chambers. As expected, the results showed that the number of cells migrating through the filter was significantly decreased after transfection with Id-1 shRNA4 and/or NF-κB/p65 shRNA3 compared with scrambled shRNA-transfected cells. These findings reveal that the loss of Id-1 and NF-κB/p65 expression inhibits the migratory ability of CNE-2 cells, whereas Id-1 and NF-κB/p65 overexpression promotes cell migration. These results are consistent with previous studies regarding the expression of Id-1 and NF-κB/p65 in other malignancies and indicate that these genes may play a key role in the invasion and metastasis of NPC and correlate with a poor prognosis in NPC.
During cancer progression, MMPs such as MMP-9 and MMP-2 are known to facilitate the breakdown of the basement membrane and the extracellular matrix to allow the infiltration of cancer cells and thus metastasis (28,34). Increasing amounts of evidence suggest that there is a close association between Id-1, NF-κB/p65 and the expression and secretion of MMPs. NF-κB/p65 has been shown to contribute to extracellular matrix destruction by triggering the production of MMP-9 (20,22). Id-1 could regulate MMP proteins as well as cell invasion (28,34,45), and the Id-1 mRNA and protein levels were also increased in metastatic breast cancer cells found in the lung (46,47). Our study demonstrates that transient transfection of CNE-2 cells with Id-1 shRNA4 and/or NF-κB/p65 shRNA3 significantly decreased the mRNA levels of MMP-9 and MMP-2. The results of FACS and gelatin zymography show that both MMP-9 protein and its proteinase activity were significantly reduced by Id-1 shRNA4 or Id-1 shRNA4/NF-κB/p65 shRNA3. Neither MMP-9 nor its proteinase activity was reduced by transfection with scrambled shRNA. These results indicate that Id-1 or Id-1/NF-κB/p65 expression in NPCs are essential for tumor cell migration by regulating MMP-9 secretion in NPC tumor cells.

In conclusion, we identify Id-1 as a novel independent prognostic maker molecule for reduced patient survival. Our results show that a combined analysis of Id-1 and NF-κB/p65 expression can help to identify patient subgroups that are at higher risk and have a poor prognosis and who therefore may need special therapeutic consideration. The results of our study suggest that Id-1 and NF-κB/p65 can enhance tumor cell migration, which is correlated with MMP-9 secretion in NPC cells. However, there are some limitations to this study. Although NF-κB/p65 is regulated by Id-1 in some cancers (26–29), the relationship between Id-1 and NF-κB/p65 has not been identified in this present study. We will focus on to identify this connection in future studies. Collectively, our findings might help clinicians to individualize the surveillance scheme and therapy, for example, favoring a more aggressive regimen in patients with tumors that have a high Id-1 expression level or high levels of both Id-1 and NF-κB/p65.

Supplementary material

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/.

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References


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