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

Wei Sun1,2,†, Ming-Ming Guo3,†, Ping Han1,2,†, Ji-Zhen Lin4,5, Fu-Ya Liang1,2, Guang-Mou Tan6, Hua-bin Li2,7, Musheng Zeng8,9 and Xiao-ming Huang1,2,*

1Department of Otorhinolaryngology-Head and Neck Surgery, Sun Yat-sen Memorial Hospital and 2Research Institute of Otorhinolaryngology-Head and Neck Surgery, Sun Yat-sen University, Guangzhou, Public Republic of China
3Department of Otorhinolaryngology-Head and Neck Surgery, Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Public Republic of China
4Department of Otorhinolaryngology-Head and Neck Surgery, Cancer Center, University of Minnesota, Minneapolis, MN, USA
5Department of Head and Neck Surgery, Oncology Hospital of Guangzhou Medical University, Guangzhou, Public Republic of China
6Department of Otorhinolaryngology-Head and Neck Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Public Republic of China
7State Key Laboratory of Oncology in South China and 8Department of Experimental Research Sun Yat-sen University Cancer Center, Guangzhou, Public Republic of China

*To whom correspondence should be addressed. West Yanjiang Road 107#, Guangzhou, Guangdong, Public Republic of China. Tel: +86 20 81332431; Fax: +86 20 81332655; Email: xiaomingh@hotmail.com

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 (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 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 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.

Abbreviations: FBS, fetal bovine serum; Id-1, inhibitor of differentiation-1; MMP, matrix metalloproteinase; mRNA, messenger RNA; NF-κB, nuclear factor-kappa B; NPC, nasopharyngeal carcinoma; NPEC, nasopharyngeal epithelial cell; PBS, phosphate-buffered saline; shRNA, short hairpin RNA; TNM, tumor-node-metastasis.

†These authors contributed equally to this work.

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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 radiotherapy with or without chemotherapy 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 completed. The average observation length for overall survival was 55.5 months for patients who were still alive at the time that this analysis was written. The survival time ranged from 3 to 83 months. Fifty-five patients (50%) died during the period during which follow-ups were 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 Review 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 the only NPC type, no history of previous malignancies and no history of previous radio or chemotherapy treatments. The main clinical and pathologic characteristics of the patients are presented in Supplementary Table S1, available at CancerGeneticsOnline.com; 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, Invitrogen) for 60 min at 4°C to block non-specific binding sites. The sections were immunostained with rabbit polyclonal anti-Id-1 (1:50, SC-488; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-NF-B/p65 (1:100, SC-109; Santa Cruz Biotechnology) antibodies over-night at 4°C, followed by an incubation with a biotinylated goat anti-rabbit IgG secondary antibody (Zymed Laboratories) for 30 min and a reaction with H2O2/horseradish peroxidase (Zymed Laboratories) for 30 min. Hydrogen peroxide-activated diaminobenzidine was applied for visualization. Three minute washes in phosphate-buffered saline (PBS) were carried out between each step. The tissue sections were lightly counterstained with hematoxylin, dehydrated with 10% FBS, and the cells were cultured for 72 h.

RNA extraction, semiquantitative reverse transcription–PCR and real-time PCR

Total RNA from cell lines (NPECs and NPC cells) and tissues (12 normal nasopharyngeal tissues and 34 NPC tissues) was extracted using Trizol Reagent (Fermentas Life Sciences, Ontario, Canada) according to the manufacturer's instructions. In semiquantitative reverse transcription–PCR experiments, total RNA was isolated from cells using the Trizol reagent (Fermentas Life Sciences), incubated for 60 min at 60°C and then heated for 10 min at 70°C. The mixture was heat inactivated at 94°C for 5 min, and 1 μl of the inactivated reverse transcriptase reaction mixture containing 1× reverse transcriptase reaction buffer (Fermentas Life Sciences), 0.5 μg of oligo(dT)18 primer and 1 μl of RevertAidTM M-MuLV reverse transcriptase (Fermentas Life Sciences), incubated for 60 min at 42°C and then heated for 10 min at 70°C. The mixture was heat inactivated at 94°C for 5 min, and 1 μl of the inactivated reverse transcriptase reaction mixture containing 1× of the PCR mixture were combined and amplified according to a standard PCR reaction that is listed below. The sequences of the upstream and downstream primers used are as follows: MPP-9: 5'-GACACCTTGGGCTTACATG3' and 5'-CAAAAG-GGCTGTCATACATC3'; MMP-2: 5'-AGACTTTTCTTTCGAAGGACC GGTT3' and 5'-GGTGTCCTAGGTTGGGTA3'; β-actin: 5'-GCGTATCCAAAAGCAGTCTTTGGTTCAATCACC-3' and 5'-GCCAGAGGCATCTCACCTCGTGAGTA3'; PCR analysis was performed using the following conditions: the average temperature and cycles were 60°C for 30 cycles with the MPP-9 primers, 56.5°C for 35 cycles with the MMP-2 primers and 66.4°C for 30 cycles with the β-actin primers. The amplified products were applied to agarose gel (2%) and visualized under ultraviolet light for bromide staining. The integrated optical densities of each band were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

To determine the levels of NF-kB/p65 and Id-1 messenger RNA (mRNA), real-time PCR and data collection were carried out with an ABI PRISM 7900HT sequence detection system using SYBR® Green Real-Time PCR Master Mix (Invitrogen). The glyceraldehyde 3-phosphate dehydrogenase gene was used as an internal control to normalize the expression levels of NF-kB/p65 and Id-1. The following primers were used: glyceraldehyde 3-phosphate dehydrogenase - 5'-CTCTCTCTGTTGCGACATGC-3' and 5'-CCCCA-TACGAAAACTTCCTC-3'; Id-1: 5'-GTTTCAAGCCAGTCGAGA-3' and 5'-CAAGCCGTTCATGTGTCAGAAGCA-3'; and NF-kB/p65 - 5'-ATTGCC-GATGTAACCGGAAACT-3' and 5'-CCACCTGGTTCCGCTGAAAA-3'. The 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 1 s and 60°C for 1 s.

Short hairpin RNA transfection

Four pairs of Id-1-specific shRNA oligos and four pairs of NF-kB/p65-specific shRNA oligos were selected for use in this study; a scrambled shRNA was used as a negative control (GeneChem, Shanghai, Public Republic of China). The four pairs of Id-1-specific shRNA were shRNA1, which targeted GGATTC-CACCTGTTGTTTTC; shRNA2, which targeted GGACCTTCTGTTGACCA-3', shRNA3, which targeted GTCCAGCTGTTGTCTCTCA and shRNA4, which targeted GCCAGCTGTTGTTGTCTCA with chemically modified chemically modified 2′-O-methyl RNA. These four pairs of NF-kB/p65-specific shRNA were shRNA1, which targeted GGCACTATG-GACTCTCAAGA; shRNA2, which targeted GGACCCAGATACACCAA-GA; shRNA3, which targeted GTAATCCAGGTGGTGAAAGC and shRNA4, which targeted GAGTACCCGTACGTTA AACT. The scrambled shRNA control targeted UUCUCGGAGCUGGUCAGU.

CNE-2 cells were cultured in 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 × 105 cells/ml per dish 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-kB/p65 shRNA or Id-1/NF-kB/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-kB/p65 shRNA were fixed in 70% methanol, incubated with MMP-9 antibody (1:1000; Santa Cruz Biotechnology) for 90 min, washed and incubated with fluorescein 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-kB/p65 in NPC tissues, the expression of these two factors was also detected using fluorescein immunohistochemistry. 4′,6-Diamidino-2-phenylindole 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 (60% confluence) were transfected in transfection media with no shRNA, Id-1 shRNA, NF-kB/p65 shRNA or both Id-1 and NF-kB/p65 shRNA at 1 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 as described using RevertAidTM M-MuLV reverse transcriptase (Fermentas Life Sciences) on ice for 20 min, washed with 0.3% saponin in PBS, incubated with a fluorescein isothiocyanate-conjugated secondary antibody on ice for 20 min, resuspended in PBS and analyzed with fluorescence-activated cell sorting (FACS) caliber 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 Biodyne 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-kB/p65 (1: 2000, SC-109; Santa Cruz Biotechnology). The membrane was then incubated with horse-radish 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 BioCoatTM MatrigelTM Invasion Chamber). Forty-eight hours after transfection with Id-1 shRNA and/or NF-kB/p65 shRNA, the CNE-2 cells and culture medium were harvested, and 2.5 × 10^5 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-kB/p65 expression levels of both NF-kB/p65 and Id-1 were significantly upregulated in NPC tissues compared with normal nasopharyngeal tissues (P values of 0.03 and 0.04, respectively) (Figure 2C). In addition, five NPC tissues and two normal nasopharyngeal tissues were selected for analysis by western blot. The high protein levels of Id-1 and NF-kB/p65 in NPC tissues (Figure 2D) were consistent with the data obtained from Id-1 and NF-kB/p65 immunostaining (Figure 2A).

Results

Id-1 and NF-kB/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-kB/p65 in the NPECs were low compared with the CNE-1, CNE-2, C666 and 5-8F cells (Figure 1).

Id-1 and NF-kB/p65 expression in normal nasopharyngeal epithelial tissues and NPC tissues

Id-1 and NF-kB/p65 were mainly detected in NPC tissues and were less frequently detected in normal nasopharyngeal epithelial tissues. Different patterns of Id-1 and NF-kB/p65 staining were observed within the cytoplasm of NPC tissues. Compared with NPC tissues, the Id-1 and NF-kB/p65 staining in normal nasopharyngeal epithelial tissues was generally weak and less frequent (Figure 2A). In addition, Id-1 was also expressed in the nuclei of some NPC cells (Figure 2A and B). Id-1 and NF-kB/p65 staining were quantitatively assessed and grouped into low or high categories. A summary of Id-1 and NF-kB/p65 expression in normal and NPC tissues is given in Supplementary Figure S2, available at Carcinogenesis Online. Id-1 expression was detected in 80.8% of the NPC tissues and 16.7% of the normal tissues examined. NF-kB/p65 expression was detected in 57.6% of NPC tissues and in 23.3% of normal tissues. A significant difference in Id-1 and NF-kB/p65 expression was demonstrated between NPC tissues and normal nasopharyngeal tissues (P = 0.000 and P = 0.001, respectively). Real-time quantitative PCR indicated that the mRNA expression levels of both NF-kB/p65 and Id-1 were significantly upregulated in NPC tissues compared with normal nasopharyngeal tissues (P values of 0.03 and 0.04, respectively) (Figure 2C).

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.

Fig. 1. The mRNA and protein levels of Id-1 and NF-kB/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-kB/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-kB/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-kB/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 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 cytosome 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.

Id-1 and NF-κB/p65 increase the invasive activity of CNE-2 cells

As MMP-9 plays a key role in the degradation of the basement membrane in many solid cancers (21,32), we investigated the migratory activity of Id-1 shRNA4- and/or NF-κB/p65 shRNA3-transfected cells...
Table I. Relationship between Id-1 and NF-κB/p65 expression and various clinicopathological factors of NPCs

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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).
using a basement membrane matrix, which serves as a reconstituted basement membrane in vitro. After 22 h incubation in Matrigel™ Invasion Chambers, significantly fewer cells migrated through the filter after transfection with Id-1 shRNA4, NF-κB/p65 shRNA3 or both (ratios of 0.068885 ± 0.01265, 0.038961 ± 0.012244 and 0.020408 ± 0.028862, respectively) compared with cells transfected with scrambled shRNA (ratio of 0.65962 ± 0.022706) (P < 0.05, P < 0.01 and P < 0.01, respectively) (Figure 4). These results demonstrate a potential relationship between the increased secretion of MMP-9 and the invasive capability of CNE-2 cells. These findings show that Id-1 and NF-κB/p65 are involved in the process of CNE-2 cell invasion in vitro.

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.

### 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

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<td>2.306–121.248</td>
<td>0.005</td>
</tr>
<tr>
<td>N stage</td>
<td>2.385</td>
<td>1.062–5.355</td>
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<td>UICC stage</td>
<td>2.999</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.

**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**

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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 Id-1 and NF-κB/p65.

Supplementary material

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

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Conflict of Interest Statement: None declared.

References

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