

Y-Box-binding protein-1 is a promising predictive marker of radioresistance and chemoradioresistance in nasopharyngeal cancer

Wei-Lin Tay¹, George Wai-Cheong Yip¹, Puay-Hoon Tan^{1,2}, Ken Matsumoto³, Richard Yeo⁴, Tze-Pin Ng⁵, Srinivasan Dinesh Kumar¹, Masafumi Tsujimoto³ and Boon-Huat Bay¹

¹Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; ²Department of Pathology, Singapore General Hospital, Singapore; ³Laboratory of Cellular Biochemistry, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan; ⁴Department of Radiation Oncology, National Cancer Centre, Singapore and ⁵Department of Psychological Medicine, National University Hospital, Singapore

The Y-Box-binding protein-1, a member of the cold-shock domain DNA- and RNA-binding protein superfamily, is known to mediate chemoresistance. The aim of this study was to determine the expression of Y-Box-binding protein-1 in nasopharyngeal cancer *in vitro* and in tumor tissue samples as well as analyze the clinicopathological significance of Y-Box-binding protein-1 expression in nasopharyngeal cancer, in particular as a predictor of outcome after treatment. The Y-Box-binding protein-1 expression profile was evaluated at the mRNA and protein levels in poorly differentiated CNE-2 nasopharyngeal cancer cells by real-time RT-PCR, western blot analysis and immunohistochemistry. Y-Box-binding protein-1 expression in 143 nasopharyngeal cancer specimens was examined by immunohistochemistry and correlated with clinicopathologic parameters. Y-Box-binding protein-1 mRNA and protein were found to be expressed in CNE-2 nasopharyngeal cancer cells *in vitro*. Of 143 patient tissue sections, 137 (96%) were stained positive for the Y-Box-binding protein-1 protein. Y-Box-binding protein-1 immunostaining was observed to be predominantly cytoplasmic. A higher recurrence of nasopharyngeal cancer was found in patients whose tissues had increased Y-Box-binding protein-1 expression ($P < 0.001$). The Cox proportionate hazard regression model also established that high Y-Box-binding protein-1 immunoreactivity was significantly correlated with increased risk (2.13 times) of recurrence as compared to low Y-Box-binding protein-1 immunoreactivity ($P = 0.01$). Within groups of patients treated by radiotherapy or chemoradiotherapy, recurrent cases had significantly higher Y-Box-binding protein-1 expression than nonrecurrent cases ($P < 0.001$ and $P = 0.0035$, respectively). These data suggest that Y-Box-binding protein-1 expression has clinicopathological significance with potential as a predictive marker of recurrence in nasopharyngeal cancer patients who undergo radiotherapy or chemoradiotherapy. *Modern Pathology* (2009) 22, 282–290; doi:10.1038/modpathol.2008.181; published online 31 October 2008

Keywords: nasopharyngeal carcinoma; Y-Box-binding protein-1; immunohistochemistry; radiotherapy; chemoradiotherapy

Nasopharyngeal cancer is a tumor of epithelial origin that typically originates in the pharyngeal recess of the nasopharynx. The multifactorial etiology

of nasopharyngeal cancer includes genetic predisposition, Epstein-Barr virus (EBV) infection, and exposure to environmental and dietary carcinogens. More than 80% of nasopharyngeal cancer patients are from China, Southeast Asia and other Asian countries whereas this cancer is rare in other parts of the world, with sporadic cases occurring in America and Europe.¹ Nasopharyngeal cancer was initially classified by the WHO (World Health Organization) in 1978 into three categories based on the degree of differentiation, viz., keratinizing squamous cell carcinoma (WHO Type 1), and nonkeratinizing

Correspondence: Dr B-H Bay, MBBS, PhD, Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, 4 Medical Drive, Blk MD10, Singapore 117597, Singapore or Dr P-H Tan, MBBS, FRCPA, Department of Pathology, Singapore General Hospital, Outram Road, Singapore 169608, Singapore.
E-mail: antbaybh@nus.edu.sg or tan.puay.hoon@sgh.com.sg
Received 30 June 2008; revised and accepted 08 October 2008; published online 31 October 2008

carcinoma which was further subdivided into the differentiated subtype (WHO Type 2) and undifferentiated subtype (WHO Type 3) before the recent addition of another category: basaloid squamous cell carcinoma.²

WHO Type 3 nasopharyngeal cancer is the most common histopathological type in endemic areas such as Southern China where it accounts for more than 97% of nasopharyngeal cancer cases. On the other hand, WHO Type 1 nasopharyngeal cancer is more prevalent in Western countries where it comprises up to 50% of nasopharyngeal cancer diagnosed but less than 5% in endemic areas.^{3,4} Radiation therapy is the mainstay of treatment for locally and regionally confined stages of nasopharyngeal cancer.⁵ Both WHO Type 2 and Type 3 nasopharyngeal cancers are generally more radiosensitive and have a higher local control rate after radiotherapy than WHO Type 1 but are also more likely to undergo distant metastasis.⁶ Although nasopharyngeal cancer has remarkable radiosensitivity and chemosensitivity in the early stages of treatment due to p53 functionality and retention of clonality, there is usually resistance to these treatment modalities in the more advanced stages.⁷ Radiotherapy has been integrated with chemotherapy in locally advanced nasopharyngeal cancer, using the chemotherapeutic drug cisplatin in combination with fluorouracil.⁷⁻⁹

The Y-box-binding protein-1 (YB-1) represents the most evolutionarily conserved nucleic-acid-binding protein currently known.¹⁰ It is a member of the cold-shock domain DNA- and RNA-binding protein superfamily which is involved in transcriptional and translational control.¹¹ YB-1 is a multifunctional protein involved in cell proliferation, apoptosis and DNA replication. YB-1 upregulates multidrug resistance 1 (*mdr1*) gene transcription which encodes for P-glycoprotein in response to genotoxic stress.¹¹⁻¹⁴ P-glycoprotein is known to be involved in the efflux of xenobiotics in cells, thus inducing chemoresistance to numerous anticancer agents.

Hence, the aim of this study was to evaluate the expression of YB-1 in CNE-2 poorly differentiated nasopharyngeal cancer cells *in vitro* at both mRNA and protein levels and nasopharyngeal cancer tissue samples by immunohistochemistry. We also correlated YB-1 protein expression with clinicopathological parameters and analyzed the expression of YB-1 expression with the outcome of radiotherapy and chemoradiotherapy in nasopharyngeal cancer patients.

Materials and methods

Cell Culture

Poorly differentiated CNE-2 nasopharyngeal cancer cells were routinely maintained in specialized nasopharyngeal cancer medium containing RPMI-1640 supplemented with 10% fetal bovine serum

(Hyclone), 2 mM sodium pyruvate, 2 mM penicillin-streptomycin L-glutamine (Invitrogen) and 2 mM minimum essential media nonessential amino acids solution (Invitrogen). MDA-MB-231 breast cancer cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Both cell lines were grown in a humidified atmosphere of 5% CO₂ at 37°C.

Clinical Materials

The study population comprised tissue samples from pretreatment endoscopic biopsies of 143 Chinese patients diagnosed with nasopharyngeal cancer, who underwent treatment at Singapore General Hospital between 2000 and 2007. Biopsy sections were cut from formalin-fixed, paraffin-embedded tissue at a thickness of 4 µm and mounted on 3-aminopropyl-tri-ethoxysilane (APES) slides. The study was approved by the Institutional Review Board, Singapore General Hospital. The age of the patients ranged from 14 to 82, with a median age of 50 years. A total of 4 patients (3%) were diagnosed with WHO Type 1 nasopharyngeal cancer, 8 (6%) with WHO Type 2 nasopharyngeal cancer and 131 (92%) had WHO Type 3 nasopharyngeal cancer. Clinicopathological data which included AJCC staging, tumor staging, nodal status and distant metastasis were only available for 135 patients (Table 1). A total of 70 patients were treated for nasopharyngeal cancer by radiotherapy whereas 64 patients were treated with radiotherapy and concurrent chemotherapy (chemoradiotherapy) using the cytotoxic drugs cisplatin and 5-fluorouracil. One patient received chemotherapy only and another received radiotherapy and adjuvant chemotherapy. Some patients received neoadjuvant chemotherapy prior to concurrent chemoradiotherapy but these cases were classified as having received chemoradiotherapy for the purpose of simplification. The median follow-up of patients was 750 days and 69 patients had recurrent tumors. The median number of days to recurrence was 566 days. A total of 12 patients died during follow-up, out of which 10 were recurrent cases.

Real-Time RT-PCR

Total RNA was extracted from cells that have reached 80–90% confluency in a 75 cm² flask using the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. Extracted RNA was converted into first-strand cDNA using SuperScript™ III Reverse Transcriptase and random hexamers from the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed with the Lightcycler (Roche), using QuantiTect SYBR Green PCR Master Mix (Qiagen) which provided a final concentration of 2.5 mM MgCl₂, and 0.5 µM of each primer. The primers for the *YB-1* gene

Table 1 Association between YB-1 immunoreactive score and clinicopathological parameters

Clinicopathological factor	Patients with intensity–percentage score ≥ 200			
	No. of patients	%	n	P
<i>Gender</i>				
Male	98	40	39	0.62
Female	37	35	13	
<i>Age (years)</i>				
<45	40	50	20	0.15
45–54	44	30	13	
≥ 55	51	37	19	
<i>Histological subtypes</i>				
WHO Type 1 and Type 2	12	42	5	
WHO Type 3	123	38	47	0.52
<i>AJCC staging^a</i>				
1	9	44	4	
2	30	43	13	
3	50	28	14	
4	42	48	20	0.24
<i>Tumor stage^b</i>				
T1	21	48	10	
T2	50	36	18	
T3	34	32	11	
T4	25	48	12	0.51
<i>Nodal status^b</i>				
N0	33	33	11	
N1	33	39	13	
N2	48	38	18	
N3	16	56	9	0.48
<i>Distant metastasis^a</i>				
M0	123	41	50	
M1	8	13	1	0.11

^aUnavailable information for 4 cases.^bUnavailable information for 5 cases.

were AAGTGATGGAGGGTGCTGAC (forward) and TTCTTCATTGCCGTCTCTC (reverse). The primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were GAAGGTGAA GGTCGGAGTCAACG (forward) and TGCCATGGG TGGAAATCATATTGG (reverse). Cycling conditions were: 15 min at 95°C, 45 cycles at 94°C for 15 s, annealing at 60°C for 25 s, extension at 72°C for 12 s. Melting curve analysis was subsequently performed to verify the specificity of the amplicon. The specificity of the YB-1 and G3PDH primers were also verified by running the amplicons on a 1% agarose gel. The $2^{-\Delta\Delta C_T}$ method for the quantification of mRNA was used to analyze the data.

Western Blot Analysis

Proteins from CNE-2 cells and MDA-MB-231 cells were extracted using cell lysis buffer M-PER[®] Mammalian Protein Extraction Reagent with Halt

Protease Inhibitor Cocktail and EDTA solution (Pierce) according to manufacturer's instructions and quantified using the Microtiter Bio-Rad Protein Assay protocol (Bio-Rad Laboratories). Bovine serum albumin diluted in nanopure water to a range of concentrations was used as a protein standard. Each of protein samples (20 μ g) from CNE-2 cells and MDA-MB-231 cells were analyzed by SDS-polyacrylamide gel electrophoresis. Following electrophoresis, the protein was transferred onto a polyvinylidene difluoride membrane by electroblotting for 45 min at 15 V. After overnight incubation in 5% nonfat milk in 0.1% Tween-20 in Tris-buffered saline (TBST; Tris, NaCl) at 4°C with shaking to block nonspecific binding and subsequent washes with TBST, the membrane was incubated with anti-YB-1 antibody (1:1000) for 1.5 h at room temperature. The anti-YB-1 antibody was raised in a rabbit by injecting a synthetic peptide K13 having the sequence MSSEAETQQPPAAPPCC which corresponds to the N terminus of human YB-1 protein (accession number: P67809). The membrane was washed with TBST, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:6000; Sigma-Aldrich) for 1 h at room temperature. Specific binding was detected by enhanced chemiluminescence (Amersham Biosciences). The membrane was incubated with monoclonal mouse β -actin primary antibody (1:6000; Amersham Biosciences) for 1.5 h and horseradish peroxidase-conjugated anti-mouse IgG (1:10 000; Amersham Biosciences) for 1 h at room temperature, which served as the loading control.

Immunohistochemistry

For immunohistochemical staining of nasopharyngeal cancer cells *in vitro*, CNE-2 nasopharyngeal cancer cells were seeded into a Lab-Tek[®] 4-Chambered Coverglass (Nalge Nunc) at a density of 5×10^4 cells per ml. When cells have reached 70–80% confluency, they were fixed 1 ml of cold 4% paraformaldehyde (pH 7.4) for 8 min. For the human nasopharyngeal cancer tissues, paraffin sections were dewaxed in xylene, histoclear, equal parts histoclear and absolute ethanol, respectively, and rehydrated with ascending series of ethanol. The sections were then heated in 10 mM citrate buffer (0.1 mM citric acid, 0.1 M sodium citrate; pH 6.0) for 15 min in a 700 W microwave oven for the purpose of antigen retrieval. The slides were washed in PBS-TX (0.05 M PBS, 0.1% Triton-X 100) thrice and then incubated with 3% hydrogen peroxide in methanol at room temperature for 15 min to quench endogenous peroxidase. The slides were then washed thrice with PBS-TX before incubation with 5% normal goat serum for 1 h at room temperature to block nonspecific antibody binding, followed by incubation with primary anti-K13 antibody (1:300) overnight at room temperature. Detection of bound primary

antibody was by adding biotinylated anti-rabbit immunoglobulin (1:200) for 45 min and avidin-biotin peroxidase complex (Vectastain[®] ABC Kit; Vector Laboratories) for 45 min at room temperature. The sections were visualized by incubating the slides in freshly prepared diaminobenzidine solution (10× DAB, TBS, 30% H₂O₂) for 10 min and counterstained with Shandon's haematoxylin before mounting.

Immunohistochemical Evaluation

The stained slides were viewed using the ×10 and ×40 objective lens of a light microscope to determine the overall percentage and intensity of staining. YB-1 immunopositivity was defined as the presence of any specific staining in the cytoplasm and/or nucleus. The intensity of staining was scored as 0 (no detectable immunoreactivity), 1+ (weak staining), 2+ (moderate staining), and 3+ (strong staining). The intensity–percentage score was derived from the product of staining intensity and percentage scores.

Statistical Analysis

The SPSS 12.0 statistical package was used for statistical analysis. The intensity–percentage score was dichotomized to categorize patients into those with an intensity–percentage score of ≥200 (approximate topmost tertile) or <200 (lowest and middle tertiles). Correlations were examined using Fisher's exact test or Pearson's χ^2 -test and Mann-Whitney test or unpaired *t*-test. Recurrence-free survival and overall survival were determined using the Kaplan–Meier method and the survival curves were compared using log-rank statistics. Hazard ratios (and 95% confidence intervals) of the association of high YB-1 immunoreactivity (intensity–percentage score ≥200) with probabilities of recurrence and survival were estimated using Cox proportionate hazard regression models including age, sex, histopathological subtype, AJCC staging, and treatment as covariates. A *P*-value of <0.05 was considered significant.

Results

Expression of YB-1 *In Vitro*

CNE-2 nasopharyngeal cancer cells were found to have a higher level of YB-1 mRNA expression (Figure 1a) although not statistically significant as compared to MDA-MB-231 breast cancer cells (which was used as a positive control).^{14,15} The specificity of the YB-1 and G3PDH primers were verified by melting curve analyses (not shown) and running the amplicons on a 1% gel (Figure 1b). The specificity of the anti-K13 antibody for human YB-1 protein in CNE-2 cells and MDA-MB-231 cells are

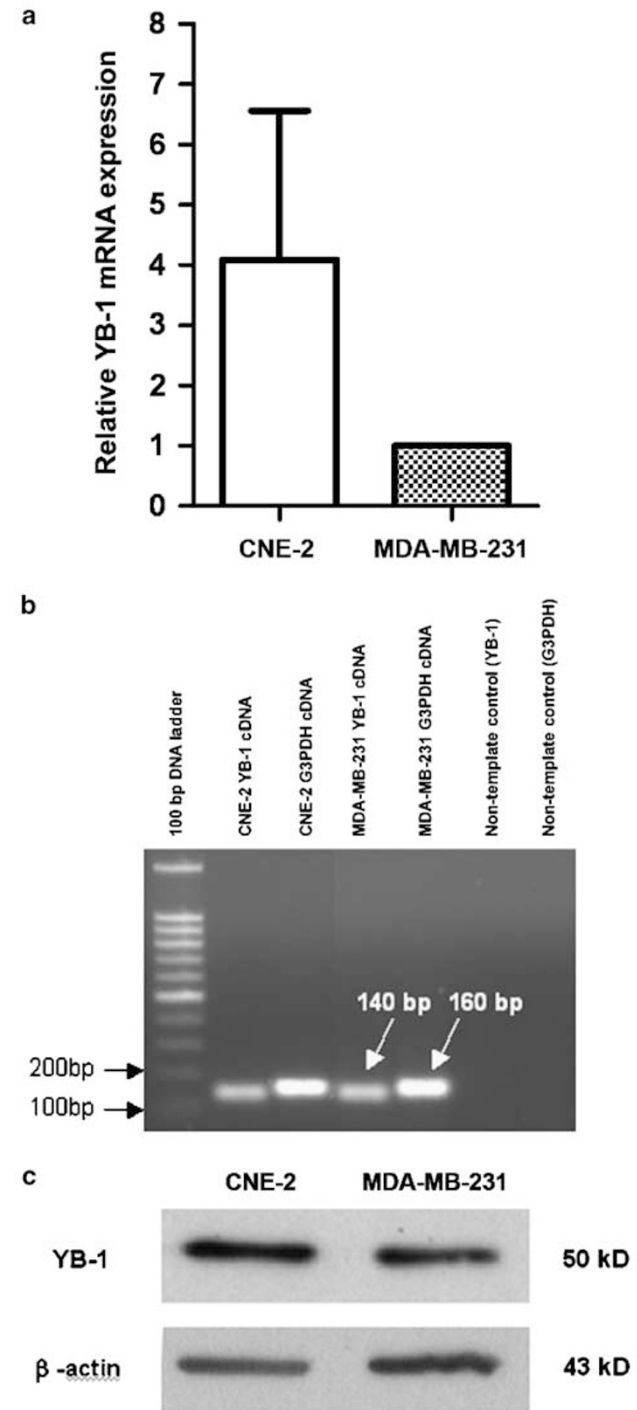


Figure 1 YB-1 expression in CNE-2 nasopharyngeal cancer cells. (a) YB-1 mRNA expression as determined by quantitative real-time RT-PCR. MDA-MB-231 breast cancer cells were designated as reference cell line. Relative mRNA expression is expressed as fold difference using the $2^{-\Delta\Delta C_T}$ method. Columns, means of quadruplicate experiments; bars, s.e. (b) Gel electrophoresis photograph of real-time RT-PCR products depicting the 140 bp amplicon of YB-1 and the 160 bp amplicon of G3PDH. No bands were detected for the negative controls. (c) Western blot analysis of YB-1 proteins in CNE-2 cells and MDA-MB-231 cells. Antibody against β -actin was incubated with the same blot for normalization of proteins loaded per well. A single band on the western blot corresponding to human YB-1 protein (50 kDa) was observed in each lane.

shown in Figure 1c. Positive YB-1 immunostaining in CNE-2 nasopharyngeal cancer cells was observed to be predominantly cytoplasmic (Figure 2a) with no staining seen in the negative control (Figure 2b).

Immunohistochemical Analysis in Nasopharyngeal Cancer Patient Tumor Samples

Of 143 patient tissue sections, 137 (96%) were immunopositive for YB-1. Seven (4%) nasopharyngeal cancer tissues showed negative YB-1 staining (Figure 2c). Among the YB-1-positive tumors, immunostaining was observed to be predominantly cytoplasmic and the intensity of staining was variable. A total of 36 (25%) tissues had low YB-1 expression (1+) (Figure 2d), 44 (31%) with moderate expression (2+) (Figure 2e) and 57 (40%) with high expression (3+) (Figure 2f).

Association of YB-1 Immunohistochemical Staining With Clinicopathological Parameters

There was no significant statistical correlation between high YB-1 expression (intensity–percentage score ≥ 200) and gender, age, histopathological subtype, AJCC staging, tumor staging, nodal status and distant metastasis as seen in Table 1 (clinicopathological data was only available for 135 patients).

Correlation of YB-1 Immunohistochemical Staining With Tumor Recurrence

Tumors with higher YB-1 expression (median intensity–percentage score = 240) as observed in pretreatment biopsies had a higher recurrence risk of nasopharyngeal cancer compared to those with lower expression (median intensity–percentage score = 97.5) ($P < 0.001$; Figure 3a). Within the group which received radiotherapy, 43 (61%) of them had tumor recurrence. Thirty-two (74%) of these patients with recurrence had high YB-1 expression (intensity–percentage score ≥ 200). In addition, the median intensity–percentage score of patients who received radiotherapy and had tumor recurrence (intensity–percentage score = 270) was found to be significantly higher than nonrecurrent cases (intensity–percentage score = 85) ($P < 0.001$; Figure 3b). Of 64, 24 (38%) patients who were administered with chemoradiotherapy had tumor recurrence and within the tumor recurrence group, 10 (42%) of them had high YB-1 expression (intensity–percentage score ≥ 200). Moreover, the median intensity–percentage score in recurrent cases (intensity–percentage score = 185) was significantly higher than that of nonrecurrent cases (intensity–percentage score = 82.5) ($P = 0.0035$; Figure 3c).

The Cox proportionate hazard regression model also established that high YB-1 immunoreactivity

(intensity–percentage score ≥ 200) was significantly correlated with increased risk (2.13 times) of recurrence compared to low YB-1 immunoreactivity (intensity–percentage score < 200) ($P = 0.01$; Table 2). Gender, age, histopathological subtype, AJCC staging and treatment modality were treated as covariates. Hence, patients with a higher YB-1 expression in their pretreatment biopsies before radiotherapy or chemoradiotherapy had a higher risk of nasopharyngeal cancer recurrence after treatment.

Survival curves were plotted by the Kaplan–Meier method, and the statistical differences between the curves were evaluated by the log-rank test. A higher YB-1 expression (intensity–percentage score ≥ 200) was associated with an increased probability of nasopharyngeal cancer recurrence (Figure 4a). On the other hand, there was no significant statistical correlation between high YB-1 expression and overall survival ($P = 0.56$; Figure 4b). This could, however, be due to the small number of deaths ($n = 12$) in this study.

Discussion

This is the first study to analyze YB-1 expression in human nasopharyngeal cancer tissues using molecular and immunohistochemical methods. Expression of YB-1 mRNA and protein were demonstrated in CNE-2 nasopharyngeal cancer cells which were derived from a patient with poorly differentiated nasopharyngeal cancer. This histological subtype accounts for more than 90% of all nasopharyngeal cancers diagnosed worldwide.¹⁶ In this study, more than 95% of the nasopharyngeal cancer tissues which were primarily WHO Type 3 nasopharyngeal cancer were observed to be positively stained for the YB-1 protein. The YB-1 staining was seen to be predominantly cytoplasmic in CNE-2 cells and nasopharyngeal cancer patient tissues. Studies conducted in breast cancer, non-small cell lung cancer, ovarian cancer and osteosarcoma have shown the presence of concomitant nuclear YB-1 expression together with cytoplasmic localization of the protein.^{12,17–19}

We have not found distinct nuclear YB-1 staining in CNE-2 cells and nasopharyngeal cancer tissues. Although it is possible that the anti-K13 antibody which was used in this study, recognizes only the cytoplasmic epitope of YB-1, we consider the possibility unlikely because translocation of YB-1 to the nuclei in human HeLa cells has been detected by immunofluorescence using the same anti-YB-1 antibody (Matsumoto K, unpublished results). The YB-1 protein has been previously reported to be predominantly located in the cytoplasm in normal and tumor cells throughout the cell cycle (in association with an anchoring protein in the cytoplasm at a binding site on the C-terminal tail domain), but the protein is known to translocate into

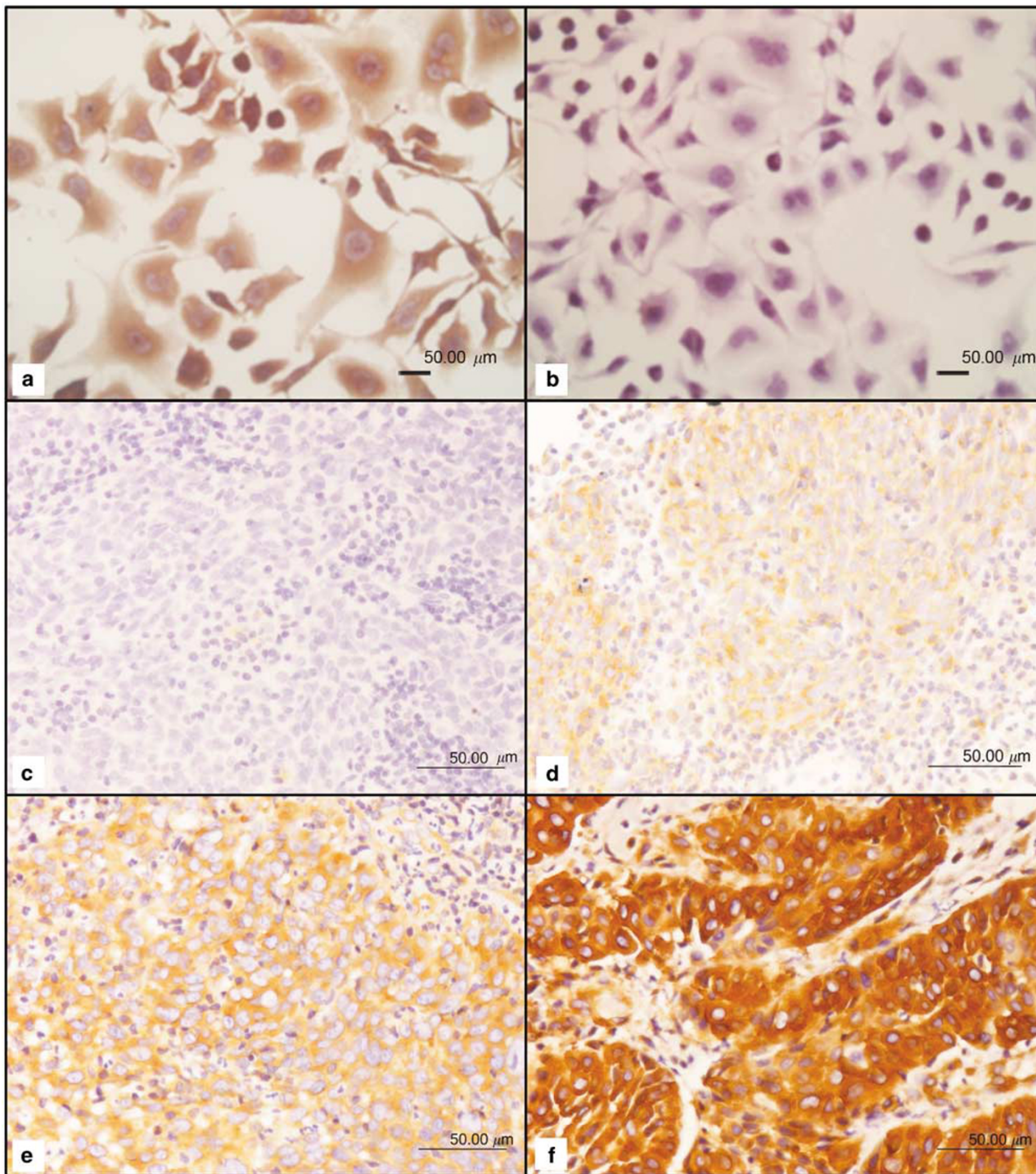


Figure 2 Immunohistochemical expression of YB-1 in CNE-2 cells and nasopharyngeal cancer tissue samples. (a) Predominantly cytoplasmic staining of YB-1 in CNE-2 cells. Nuclei counterstaining was performed using hematoxylin. (b) Negative control in CNE-2 cells. (c) Negative staining (intensity = 0) in nasopharyngeal cancer tissues. (d) Weakly positive staining (1+) in nasopharyngeal cancer tissues. (e) Moderate staining (2+) in nasopharyngeal cancer tissues. (f) Strong staining (3+) in nasopharyngeal cancer tissues. Scale bars, 50 μ m.

the nucleus during the G₁ to S phase transition.^{20,21} Zhang *et al*²² have further demonstrated that nuclear localization of YB-1 requires wild-type p53.

YB-1 has been established as a prognostic marker in several human malignancies. Nuclear YB-1

expression has been linked to poorer prognosis compared to tumors with cytoplasmic YB-1 expression in breast cancer, non-small cell lung cancer, ovarian cancer, synovial sarcoma and hepatocellular carcinoma.^{15,17,18,23–25} Huang *et al*²⁶ found a signifi-

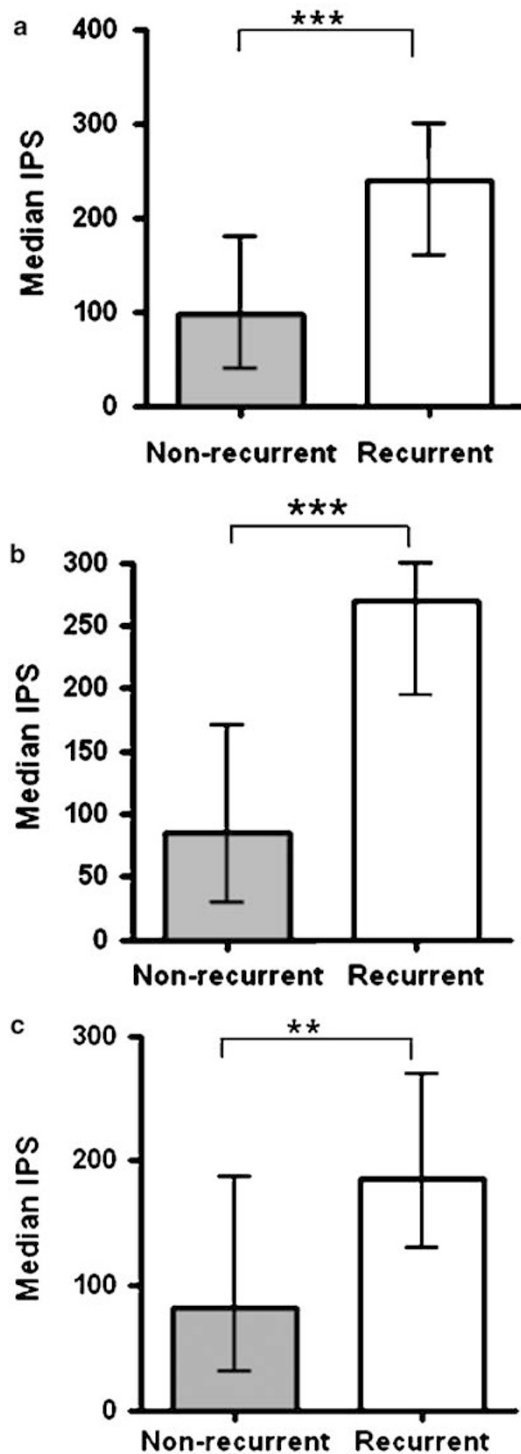


Figure 3 Median intensity percentage score of YB-1 immunostaining in non-recurrent and recurrent nasopharyngeal cancer cases. (a) All nasopharyngeal cancer patients. ****P*-value <0.001. (b) Nasopharyngeal cancer patients treated with radiotherapy. ****P*-value <0.001. (c) Nasopharyngeal cancer patients treated with chemoradiotherapy. ***P*-value <0.01.

cant correlation between coexpression of YB-1 and Pgp and poorer survival in ovarian cancer. In this study, YB-1 immunostaining had no significant

correlation with age, sex, histopathological subtype, AJCC staging, tumor grade, nodal status and distant metastasis. However, we observed that high YB-1 expression was significantly associated with tumor aggressiveness and earlier tumor recurrence after therapy in nasopharyngeal cancer.

In this study, we showed that high YB-1 expression was significantly correlated with a reduction in radiosensitivity and chemoradiosensitivity, accounting for the high recurrence rate in nasopharyngeal cancer. YB-1 plays a significant role in the mediation of chemoresistance in many cancers, primarily by mechanisms involving *mdr1* expression^{13,27,28} and increased potential of DNA repair by molecules such as DNA topoisomerase.²⁸ There is also evidence that YB-1 binds specifically to RNA containing 8-oxoguanine, cisplatin-modified and apurinic DNA and interacts with proliferating cell nuclear antigen and p53, enhancing DNA repair.^{29–31}

Recent biomarkers evaluated for predicting radiosensitivity and chemosensitivity in nasopharyngeal cancer include heat-shock protein Gp96 (HSP Gp96), growth differentiation factor 15 (GDF15), basic helix-loop-helix transcription factor TWIST, cytokeratin 8 (CK 8), latent membrane protein-1 (LMP-1) and inhibitor of differentiation/DNA-binding 1 (Id-1). Gp96 which belongs to the HSP 9 family and GDF-15, a member of the transforming growth factor- β superfamily were found to be upregulated in radioresistant nasopharyngeal cancer cell lines.³² Enhanced expression of TWIST and ectopic expression of Id-1 have been separately found to protect nasopharyngeal cancer cells from taxol-induced cell death.^{33–35} Wang *et al*³⁶ have reported that silencing of the *CK8* gene increased cisplatin toxicity in HONE1 nasopharyngeal cancer cells. Mei *et al*³⁷ have observed that there was enhanced chemosensitivity to bleomycin and cisplatin, following siRNA-mediated knockdown of LMP1 in EBV-positive C666-1 cells. To our knowledge, this is the first time that YB-1 has been reported to mediate radioresistance and chemoradioresistance in nasopharyngeal cancer.

A pervasive question on the minds of medical and radiation oncologists is how to identify patients with locally advanced nasopharyngeal cancer who could benefit from intensified systemic therapies.⁹ In this regard, YB-1 has the potential to be used as a biomarker to assess therapeutic efficacy and to predict treatment response, thus aiding selection of the most appropriate therapy to improve clinical outcome and minimize nasopharyngeal cancer recurrence. YB-1 could also be a promising target molecule for the development of novel combinatorial therapeutic strategies aimed at overcoming resistance to cancer therapy. Cisplatin-based combination therapy which is the current standard first-line therapy could potentially be more efficacious against advanced stage or recurrent nasopharyngeal cancer when combined with a strategy to inhibit YB-1 expression or abrogate its function.

Table 2 Association of YB-1 intensity (intensity–percentage score ≥ 200) with recurrence and survival

	Wald statistic ^a	Degree of freedom	P-value	Hazard ratio	95% confidence interval	
Recurrence	6.619	1	0.01	2.13	1.20	3.79
Survival	0.344	1	0.56	1.68	0.30	9.54

^aTest of significance of the regression coefficient.

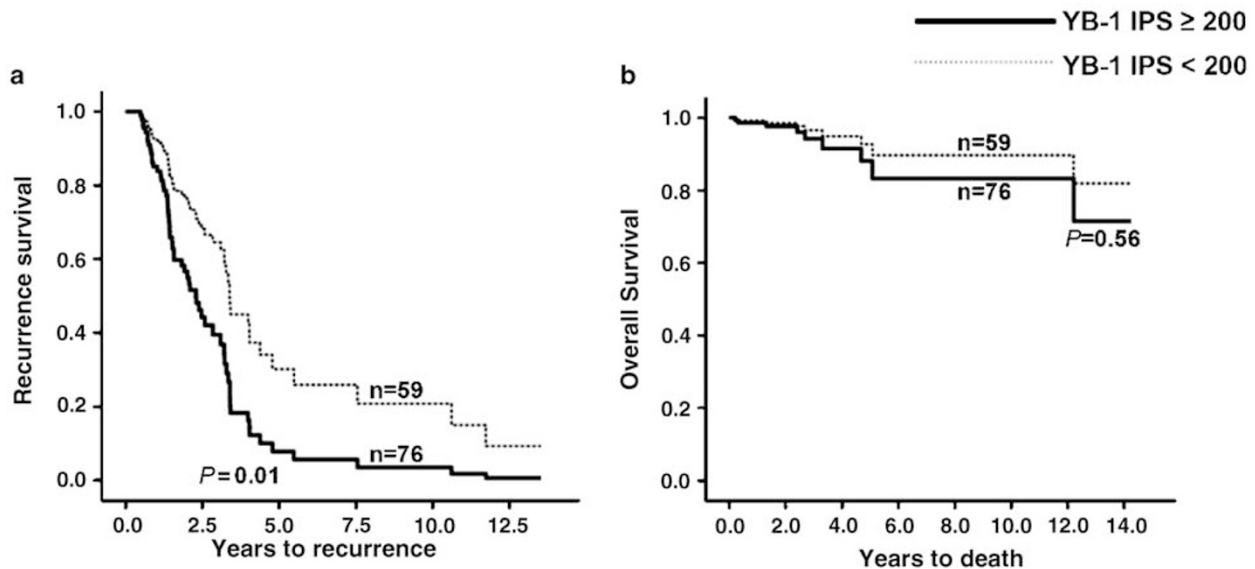


Figure 4 Kaplan–Meier plots of (a) recurrence-free survival and (b) overall survival in nasopharyngeal cancer patients with follow-up data ($n = 134$).

Acknowledgements

We are grateful to Dr Joseph Wee, National Cancer Centre of Singapore (NCC) for his advice, Dr M Olivo (NCC) for the gift of the CNE-2 cell line and Ms SL Bay for technical assistance. This project was supported by the Singapore National Medical Research Council Grant NMRC/1019/2005 (B-HB) and the Singapore Cancer Syndicate MS0004 and MS 04R (P-HT).

Disclosure/conflict of interest

None.

References

- 1 Ferlay J, Bray F, Pisani P, *et al*. Globocan 2000: Cancer Incidence, Mortality and Prevalence Worldwide (Version 1.0). IARC Cancer Base No. 5. IARC Press: Lyon, 2001.
- 2 Chan JKC, Pilch BZ, Kuo TT, *et al*. Tumours of the nasopharynx: introduction. In: Barnes L, Eveson JW, Reichart P, Sidransky D (eds). World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours. IARC Press: Lyon, 2005, pp 83–84.

- 3 Marks JE, Phillips JL, Menck HR. The national cancer database report on the relationship of race and national origin to the histology of nasopharyngeal carcinoma. *Cancer* 1998;83:582–588.
- 4 Lo KW, To KF, Huang DP. Focus on nasopharyngeal carcinoma. *Cancer Cell* 2004;5:423–428.
- 5 Lee AW. Contribution of radiotherapy to function preservation and cancer outcome in primary treatment of nasopharyngeal carcinoma. *World J Surg* 2003;27:838–843.
- 6 Reddy SP, Raslan WF, Gooneratne S, *et al*. Prognostic significance of keratinization in nasopharyngeal carcinoma. *Am J Otolaryngol* 1995;16:103–108.
- 7 Ma BB, Hui EP, Chan AT. Systemic approach to improving treatment outcome in nasopharyngeal carcinoma: current and future directions. *Cancer Sci* 2008;99:1311–1318.
- 8 Teo PM, Chan AT. Treatment strategy and clinical experience. *Semin Cancer Biol* 2002;12:497–504.
- 9 Guigay J. Advances in nasopharyngeal carcinoma. *Curr Opin Oncol* 2008;20:264–269.
- 10 Kohno K, Izumi H, Uchiumi T, *et al*. The pleiotropic functions of the Y-box binding protein YB-1. *Bioessays* 2003;25:691–698.
- 11 Matsumoto K, Wolffe AP. Gene regulation by Y-box proteins: coupling control of transcription and translation. *Trends Cell Biol* 1998;8:318–323.
- 12 Bargou RC, Jürchott K, Wagener C, *et al*. Nuclear localization and increased levels of transcription factor

- YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. *Nat Med* 1997;3:447–450.
- 13 Ohga T, Uchiyama T, Makino Y, *et al*. Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. *J Biol Chem* 1998;273:5997–6000.
 - 14 Huang JX, Tan PH, Li KB, *et al*. Y-box binding protein, YB-1, as a marker of tumor aggressiveness and response to adjuvant chemotherapy in breast cancer. *Int J Oncol* 2005;26:607–613.
 - 15 Janz M, Harbeck N, Dettmar P, *et al*. Y-box factor YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumour biologic factors HER2, uPA and PAI-1. *Int J Cancer* 2002;97:278–282.
 - 16 Zhang S, Gao X, Zeng Y. Cytogenetic studies on an epithelial cell line derived from poorly differentiated nasopharyngeal carcinoma. *Int J Cancer* 1983;31:587–590.
 - 17 Shibahara K, Sugio K, Osaki T, *et al*. Nuclear expression of the Y-box binding protein, YB-1, as a novel marker of disease progression in non-small cell lung cancer. *Clin Cancer Res* 2001;7:3151–3155.
 - 18 Kamura T, Yahata H, Amada S, *et al*. Is nuclear expression of Y box-binding protein protein-1 a new prognostic factor in ovarian serous adenocarcinoma? *Cancer* 1999;85:2450–2454.
 - 19 Oda Y, Sakamoto A, Shinohara N, *et al*. Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human osteosarcoma. *Clin Cancer Res* 1998;4:2273–2277.
 - 20 Jürchott K, Bergmann S, Stein U, *et al*. YB-1 as cell cycle regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. *J Biol Chem* 2003;278:27988–27996.
 - 21 Koike K, Uchiyama T, Ohga T, *et al*. Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Lett* 1997;417:390–394.
 - 22 Zhang YF, Homer C, Edwards SJ, *et al*. Nuclear localization of Y-box factor YB1 requires wild-type p53. *Oncogene* 2003;22:2782–2794.
 - 23 Gessner C, Woischwill C, Schumacher A, *et al*. Nuclear YB-1 expression as a negative prognostic marker in non-small cell lung cancer. *Eur Respir J* 2004;23:14–19.
 - 24 Oda Y, Ohishi Y, Saito T, *et al*. Nuclear expression of Y-box-binding protein-1 correlates with P-glycoprotein and topoisomerase II α expression, and with poor prognosis in synovial sarcoma. *J Pathol* 2003;199:251–258.
 - 25 Yasen M, Kajino K, Kano S, *et al*. The up-regulation of Y-box binding proteins (DNA binding protein A and Y-box binding protein-1) as prognostic markers of hepatocellular carcinoma. *Clin Cancer Res* 2005;11:7354–7361.
 - 26 Huang X, Ushijima K, Komai K, *et al*. Co-expression of Y box-binding protein 1 and P-glycoprotein as a prognostic marker for survival in epithelial ovarian cancer. *Gynecol Oncol* 2004;93:287–291.
 - 27 Kuwano M, Oda Y, Izumi H, *et al*. The role of nuclear Y-box binding protein 1 as a global marker in drug resistance. *Mol Cancer Ther* 2004;3:1485–1492.
 - 28 Chaudhary PM, Roninson LB. Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J Natl Cancer Inst* 1993;85:632–639.
 - 29 Hasegawa SL, Doetsch PW, Hamilton KK, *et al*. DNA binding properties of YB-1 and dbpA: binding to double-stranded, single-stranded, and abasic site containing DNAs. *Nucleic Acids Res* 1991;19:4915–4920.
 - 30 Ise T, Nagatani G, Imamura T, *et al*. Transcription factor Y-box binding protein 1 binds preferentially to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen. *Cancer Res* 1999;59:342–346.
 - 31 Okamoto T, Izumi H, Imamura T, *et al*. Direct interaction of p53 with the Y-box binding protein, YB-1: a mechanism for regulation of human gene expression. *Oncogene* 2000;19:2955–2966.
 - 32 Chang JT, Chan SH, Lin CY, *et al*. Differentially expressed genes in radioresistant nasopharyngeal cancer cells: gp96 and GDF15. *Mol Cancer Ther* 2007;6:2271–2279.
 - 33 Wang X, Ling MT, Guan XY, *et al*. Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells. *Oncogene* 2004;23:474–482.
 - 34 Cheung HW, Ling MT, Tsao SW, *et al*. Id-1-induced Raf/MEK pathway activation is essential for its protective role against taxol-induced apoptosis in nasopharyngeal carcinoma cells. *Carcinogenesis* 2004;25:881–887.
 - 35 Zhang X, Wang Q, Ling MT, *et al*. Anti-apoptotic role of TWIST and its association with Akt pathway in mediating taxol resistance in nasopharyngeal carcinoma cells. *Int J Cancer* 2007;120:1891–1898.
 - 36 Wang Y, He QY, Tsao SW, *et al*. Cytokeratin 8 silencing in human nasopharyngeal cells leads to cisplatin sensitization. *Cancer Lett* 2008;265:188–196.
 - 37 Mei YP, Zhou JM, Wang Y, *et al*. Silencing of LMP1 induces cell cycle arrest and enhances chemosensitivity through inhibition of AKT signaling pathway in EBV-positive nasopharyngeal carcinoma cells. *Cell Cycle* 2007;6:1379–1385.