

# Nuclear Expression of the Y-Box Binding Protein, YB-1, as a Novel Marker of Disease Progression in Non-Small Cell Lung Cancer<sup>1</sup>

Kotaro Shibahara, Kenji Sugio, Toshihiro Osaki, Takeshi Uchiumi, Yoshihiko Maehara, Kimitoshi Kohno, Kosei Yasumoto, Keizo Sugimachi, and Michihiko Kuwano<sup>2</sup>

Departments of Medical Biochemistry [Ko. S., T. U., M. K.] and Surgery and Science [Y. M., Kei. S.], Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, and Departments of Surgery II [Ken. S., T. O., K. Y.] and Molecular Biology [K. K.], University of Occupational and Environmental Health, Yahatanishiku, Kitakyushu 807-8555, Japan

## ABSTRACT

**Transcription factor Y-box binding protein 1 (YB-1) that binds to the inverted CCAAT box is involved not only in transcription of various genes but also in cell proliferation and DNA repair. We determined whether localization of YB-1 in either the nucleus or cytoplasm could serve as a prognostic marker for patients with non-small cell lung cancer (NSCLC). In 196 NSCLC patients, expression of YB-1 protein in the nucleus or cytoplasm was immunohistochemically evaluated. Of the 196 tumors examined, 88 (44.9%) were positive for YB-1 expression in the nucleus. Nuclear YB-1 expression significantly correlated with T factor, lymph node metastasis, and stage of the disease. Patients with a nuclear YB-1 tumor had a poorer prognosis than did those with a cytoplasmic YB-1 tumor in all of the NSCLC patients ( $P = 0.0494$ ) and in patients with squamous cell carcinoma ( $P = 0.0313$ ) but not in patients with adenocarcinomas. Nuclear localization of the YB-1 protein may prove to be an important factor of disease progression for patients with NSCLC, in particular, in cases of squamous cell carcinoma.**

## INTRODUCTION

The human YB-1<sup>3</sup> is a member of a family of DNA binding proteins that contain a highly conserved, cold shock domain,

which interacts with inverted CCAAT boxes. Several human genes including MHC class II antigens, PCNA, epidermal growth factor receptor, DNA topoisomerase II $\alpha$ , and MDR1 contain a CCAAT box in the regulatory regions (1–6). YB-1 appears to play a critical role in cell proliferation and growth, DNA replication, cell cycle, and drug resistance, as well as malignancy. YB-1 is localized mainly in the cytoplasm but is translocated into the nucleus when human cancer cells are treated with either UV irradiation or anticancer agents (7). YB-1 is overexpressed in cisplatin-resistant cancer cell lines (8). Moreover, YB-1 specifically binds to cisplatin-modified DNA and apurinic DNA and interacts with PCNA (9) and p53 (10), which suggests that YB-1 may be involved in DNA repair and/or DNA damage response, in addition to its role as a transcription factor.

In clinical studies on YB-1, nuclear or cytoplasmic localization of YB-1 were found to be closely associated with expression of P-glycoprotein encoded by the *MDR1* gene in breast cancers, osteosarcomas, ovarian cancers (11–13), and also with DNA topoisomerase II $\alpha$  and PCNA expression in colon cancers (14). Kamura *et al.* (13) reported that patients with YB-1 nuclear-positive tumors have a poor prognosis in comparison with those with YB-1 nuclear-negative tumors in ovarian cancers. Cellular localization of YB-1 in the cytoplasm or nucleus may be associated with cellular states of malignancy or drug resistance in human tumors. However, it remains unclear if YB-1 plays important role in other human malignancies.

Lung cancer is one leading cause of cancer death in North America and in Japan. Lung cancer is divided into two morphological types: SCLC and NSCLC. About 30% of NSCLC patients have localized disease, and surgical management with long-term disease control is generally restricted to this group of patients in early stages (15). Using currently available prognostic tools, it is often difficult to predict either which surgically managed patients are at risk for an early disease relapse or which advanced stage patients may have long-term survival (15). In our present study, we determined whether expression of YB-1 could be associated with disease progression or malignant properties in NSCLC.

## MATERIALS AND METHODS

**Patients and Tumor Samples.** From April 1990 to December 1994, consecutive resected specimens from 196 Japanese patients with NSCLC included 115 adenocarcinomas and 81 squamous cell carcinomas that underwent pulmonary resection in the Department of Surgery and Science, Kyushu University, and the Department of Surgery II, University of Occupational and Environmental Health. None of the patients had received previous chemotherapy or radiotherapy. Tumor-Node-Metastasis staging designations were made according to the International System for Staging Lung Cancer (16, 17). Evalu-

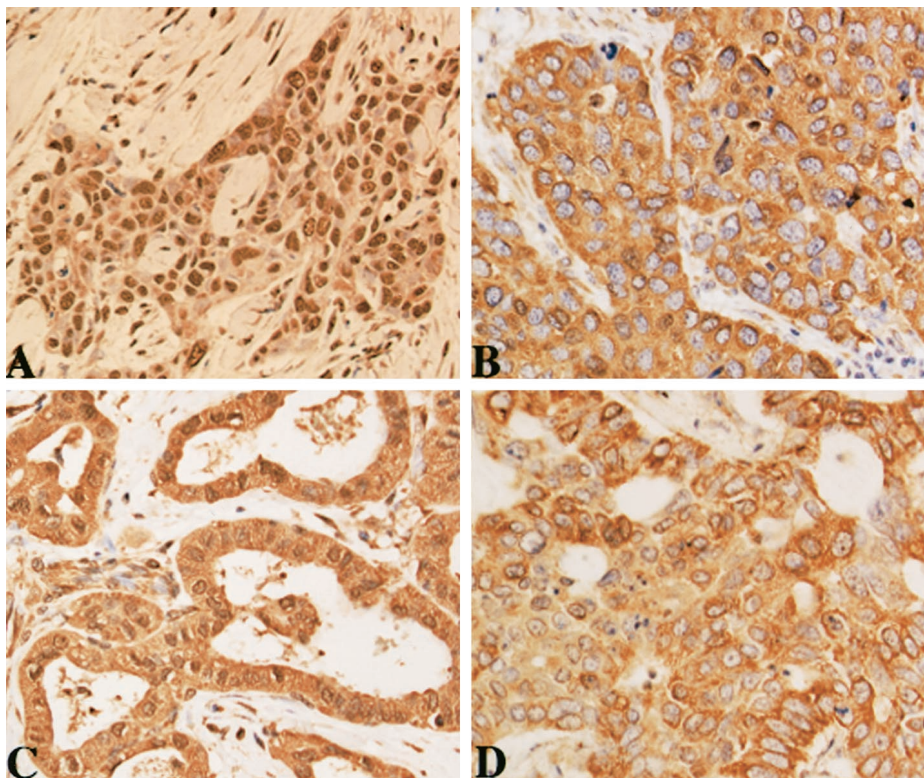
Received 3/30/01; revised 7/16/01; accepted 7/16/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by the Ministry of Education, Science, Technology, Sports and Culture of Japan Grant and also by the Ministry of Health and Welfare of Japan.

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan. Phone: 81-92-642-6100; Fax: 81-92-642-6203; E-mail: kuwano@biochem1.med.kyushu-u.ac.jp.

<sup>3</sup> The abbreviations used are: YB-1, Y-box binding protein 1; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; MDR1, multidrug-resistance 1; PCNA, proliferating cell nuclear antigen; CT, computed tomography.



*Fig. 1* Immunohistochemical analysis of YB-1. *A*, squamous cell carcinoma and *C*) adenocarcinoma showed nuclear YB-1 expression. The nuclei of the tumor cells was strongly stained. *B*, squamous cell carcinoma and *D*) adenocarcinoma showed cytoplasmic YB-1 expression. The cytoplasm but not the nuclei was stained. ( $\times 200$ ).

ation of the staging of the disease was done by chest X-ray film, chest CT, upper abdominal CT, brain CT, radionuclide bone scanning, and so forth. We performed chemotherapeutic treatments against unresectable or inoperable advanced stage patients. The stage IIIA disease is operable except for the advanced, bulky N2 disease, and the stage IIIB disease with N3 lesion is usually considered as unresectable disease. However, when solitary synchronous lesions are observed in the same lobe as the primary tumor (T4, stage IIIB) without N3 lesion, both lesions were resected. Complete clinical and follow-up information was available for all of the patients. At the time these data were analyzed, the median follow-up time for the 83 survivors was 75.6 months (range, 25–110). Included were 131 men and 65 women with ages ranging from 37 to 82 years (median 67.5 years).

**Antibodies and Immunohistochemical Analysis of YB-1 Expression.** Antibody to YB-1 was prepared against a 15-amino acid synthetic peptide in the COOH-terminal domain (residues 299–313) as described (8). Immunohistochemistry was done using a streptavidin-biotin-peroxidase complex method (12). Sections (6- $\mu\text{m}$ -thick) cut from formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene and rehydrated in ethanol. The sections were pretreated for 15 min at 100°C in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 15 min. The sections were then treated at 4°C overnight with primary antibody (1:100 dilutions) followed by staining using streptavidin-biotin-peroxidase kits (Nichirei, Tokyo, Japan). The sections were stained with diaminobenzidine solution and then counterstained with hematoxylin.

The results were evaluated independently by two observers (Ko. S. and Ke. S.), who had no knowledge of clinical data and other immunohistochemical-related data. Negative controls were processed using a control IgG as the primary antibody. Paraffin sections of breast cancer were served as positive controls for YB-1 (11, 12).

**Statistical Analysis.** Clinicopathological data were stored in an IBM 3090 mainframe computer (IBM, Armonk, NY). The Biomedical Computer Program was used for all of the statistical analysis (18). The Biomedical Computer Program P4F and P3S programs were used for  $\chi^2$  and Mann-Whitney tests to compare characteristics between groups. The Biomedical Computer Program P1L program was used for the Kaplan-Meier analysis of survival rates and the Mantel-Cox test was used to test for equality of survival curves. The Biomedical Computer Program P2L program was used for simultaneous multivariate adjustments of all of the covariates by the Cox regression analysis (19). The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

We first determined if YB-1 was localized in the nucleus or the cytoplasm in all of the 196 tumors. Two representative examples used for immunostaining showed localization of YB-1 in the nucleus (Fig. 1, *A* and *C*) and cytoplasm (Fig. 1, *B* and *D*), respectively. In all of the 196 tumors, tumor cells but not surrounding normal stromal cells reacted with anti-YB-1 antibody in the cytoplasm. Of the 196 tumors, 88 (44.9%) showed intense YB-1 expression in the nuclei of tumor cells, and these

Table 1 Clinicopathological factors and nuclear YB-1 expression in all NSCLC and each histological subtype

Factors	NSCLC			Adenocarcinoma			Squamous cell carcinoma		
	No. <sup>a</sup>	YB-1 <sup>b</sup>	P <sup>c</sup>	No. <sup>a</sup>	YB-1 <sup>b</sup>	P <sup>c</sup>	No. <sup>a</sup>	YB-1 <sup>b</sup>	P <sup>c</sup>
Total	196	88		115	63		81	25	
Sex									
Male	131	53	0.0760	62	33	0.7168	69	20	0.3801
Female	65	35		53	30		12	5	
T factor									
pT1	63	28	0.0005 <sup>d</sup>	49	24	0.1348	14	4	0.0099 <sup>d</sup>
pT2	86	30		38	19		48	11	
pT3	18	7		6	3		12	4	
pT4	29	23		22	17		7	6	
N factor									
pN0	111	41	0.0025 <sup>d</sup>	68	34	0.3376	43	7	0.0005 <sup>d</sup>
pN1	42	18		18	10		24	8	
pN2	41	28		27	18		14	10	
Unknown	2	1		2	1		0	0	
Tumor stage									
I	98	35	0.0004 <sup>d</sup>	61	30	0.2131	37	5	0.0008 <sup>d</sup>
II	33	10		16	7		17	3	
III	58	39		34	23		24	16	
IV	7	4		4	3		3	1	
Histological grade									
Well	53	22	0.4757	40	19	0.4422	13	3	0.3583
Moderately	97	43		44	27		53	16	
Poor	37	20		27	15		10	5	
Unknown	9	3		4	2		5	1	
Histological subtype									
Adenocarcinoma	115	63	0.0009 <sup>d</sup>						
Squamous cell carcinoma	81	25							

<sup>a</sup> Total no. of patients.

<sup>b</sup> YB-1, no. of patients with nuclear localization of YB-1.

<sup>c</sup>  $\chi^2$  test was used to analyze the data.

<sup>d</sup>  $P < 0.01$ .

were interpreted to be nuclear YB-1 tumors. The remaining 108 tumors (55.1%) showed YB-1 expression only in the cytoplasm of the tumor cells, and these were considered to be cytoplasmic YB-1 tumors. However, we could not observe any apparent expression of YB-1 in the normal lung tissues when normal lung tissues were determined (data not shown).

Table 1 shows the relationship between nuclear YB-1 expression and clinicopathological characteristics. There was a significant correlation between nuclear YB-1 expression and T factor, lymph node metastasis, pathological stages, and histological subtype ( $P < 0.01$ ; Table 1). In cases of squamous cell carcinoma, there were significant correlations between nuclear YB-1 expression and T factor, lymph node metastasis, and pathological stages ( $P < 0.01$ ; Table 1). In cases of adenocarcinoma, there were no significant correlations between nuclear YB-1 expression and any clinicopathological factors.

We next asked if nuclear localization of YB-1 is associated with the postoperative survival time. The lung cancer-related 5-year survival rates were 42.8% in the nuclear YB-1 expression and 58.7% in the cytoplasmic YB-1 expression, and patients with nuclear YB-1 expression had a significantly poorer prognosis than did those with cytoplasmic YB-1 expression in all of the NSCLC patients ( $P = 0.0494$ ; Fig. 2A). The survival rate was also based on the histology. In the cases of squamous cell carcinoma, the 5-year survival rate was 24.4% in the nuclear YB-1 expression and 53.3% in the cytoplasmic YB-1 expres-

sion. The nuclear YB-1 expression in squamous cell carcinoma significantly correlated with a poorer prognosis more so than did the cytoplasmic YB-1 expression ( $P = 0.0313$ ; Fig. 2B). By contrast, there was no significant correlation regarding survival time between nuclear and cytoplasmic YB-1 expression in adenocarcinoma (Fig. 2C). To additionally evaluate prognostic factors on overall survival, a multivariate Cox regression analysis was carried out; only T factor and lymph node metastasis showed an independent prognostic marker (Table 2). However, nuclear YB-1 expression could not independently predict the prognosis.

## DISCUSSION

We evaluated the intracellular localization of YB-1 in the nucleus or in the cytoplasm in NSCLCs and analyzed the correlation between the nuclear YB-1 expression and clinicopathological characteristics and the prognosis. We found that nuclear YB-1 expression significantly correlated with T factor, lymph node metastasis, and malignant stages in all of the NSCLCs and in squamous cell carcinomas but not in adenocarcinomas. We also found that patients with nuclear YB-1 expression had a poorer prognosis in all of the 196 patients with NSCLC, histologically, and that nuclear YB-1 expression in squamous cell carcinoma meant a significantly unfavorable prognosis compared with cytoplasmic YB-1 expression. How-

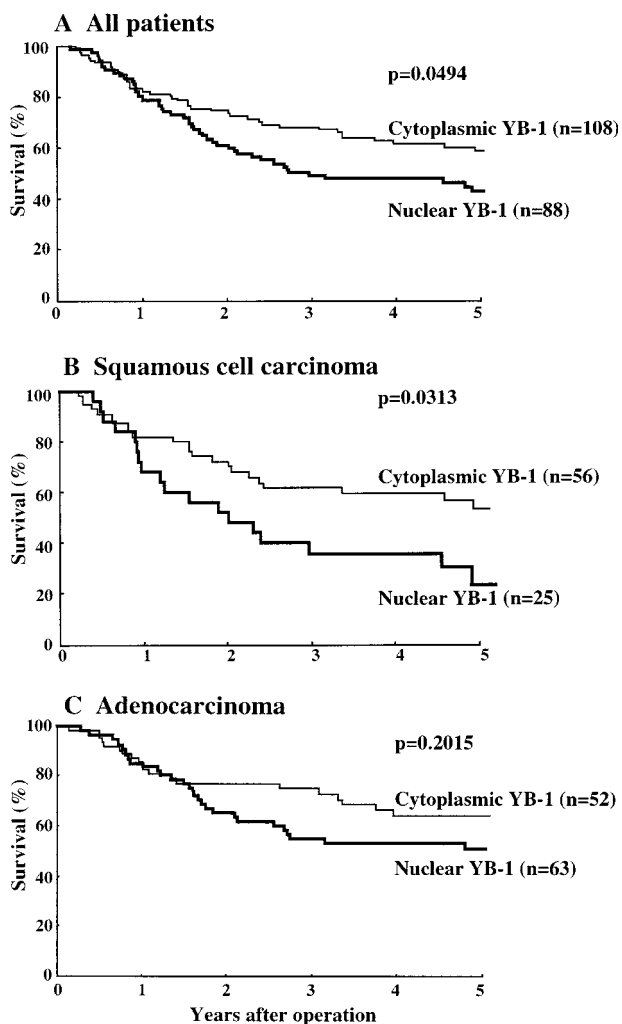


Fig. 2 Survival curves with respect to nuclear or cytoplasmic YB-1 expression. A, in all NSCLC patients. B, patients with squamous cell carcinoma. C, patients with adenocarcinoma.

Table 2 Multivariate analysis on disease-related survival (Cox proportional hazard model)<sup>a</sup>

Factor	Relative risk (95% confidence interval)	P
Lymph node metastasis (+ vs. -)	3.8815 (2.4297–6.2006)	<0.0001
Tumor stage (T1 vs. T2, T3, T4)	1.4229 (1.1557–1.7518)	0.0011
YB-1 (positive vs. negative)	0.9849 (0.6188–1.5676)	0.9490

<sup>a</sup> A multivariate analysis was performed using a Cox proportional hazard model with the following covariates: tumor stage, lymph node metastasis, distant metastasis, histological type, differentiation and expression of YB-1.

ever, there was no significant correlation between nuclear YB-1 expression and prognosis in patients with adenocarcinoma. Thus, the nuclear localization of YB-1 might occur during the process of the tumor progression especially in squamous cell carcinomas, the result being an unfavorable prognosis. How-

ever, the multivariate analysis showed that the YB-1 expression was not an independent prognostic factor for all of the NSCLC and squamous cell carcinoma (data not shown). Kamura *et al.* (13) reported that there was a significant correlation between nuclear YB-1 localization and disease-free survival time in patients with ovarian serous carcinoma. Our data suggest that the nuclear localization of YB-1 can be of prognostic significance in some types of malignant tumors.

One could argue why nuclear localization of YB-1 is associated with an unfavorable prognosis for patients with NSCLC. Translocation of YB-1 into the nucleus from the cytoplasm is often observed in human cancer cells in response to genotoxic stress such as UV irradiation or anticancer agents (7). The nuclear localization of YB-1 may not only act as a transcription factor of various genes that are closely associated with DNA replication, cell proliferation, and drug resistance but also exert SOS signaling to protect cells or DNA integrity from genotoxic factors such as cisplatin, mitomycin C, and ionizing irradiation (7, 8, 20). The nuclear translocation of YB-1 is closely associated with *MDR1* gene expression in breast cancers, osteosarcomas, and ovarian cancers (11–13). Moreover, nuclear localization of YB-1 might up-regulate expression of PCNA, epidermal growth factor receptor, DNA polymerase  $\alpha$ , and thymidine kinase genes, resulting in promotion of DNA synthesis and cell growth of cancer cells (1, 2, 21). YB-1 expression correlates with PCNA and DNA topoisomerase II $\alpha$  expression in colon cancers (14). Our research demonstrated that introduction of antisense YB-1 cDNA resulted in enhanced drug sensitivity to anticancer agents (8) and also inhibition of cell growth in the culture and tumor enlargement of xenografts in nude mice<sup>4</sup>. The close association of nuclear localization of YB-1 with clinicopathological characteristics and prognosis in NSCLC might partly relate to transcriptional activation of genes involving DNA replication and cell proliferation. However, it remains unclear how YB-1 itself is involved in malignant behavior of lung cancer cells as well as other tumor types.

In conclusion, nuclear YB-1 expression correlated with T factor, lymph node metastasis, and malignant stages and indicated an unfavorable prognosis. This result indicates that YB-1 protein is an important marker of disease progression in NSCLC, especially in squamous cell carcinoma. Determination of the intracellular localization of YB-1 may prove useful to predict malignancy or disease progression in NSCLC.

**ACKNOWLEDGMENTS**

We thank Mariko Ohara for editorial help and Junko Tsuchihashi for statistical analysis.

**REFERENCES**

- Ladomery, M., and Sommerville, J. A role for Y-box proteins in cell proliferation. *Bioessays*, 17: 9–11, 1995.
- Wolffe, A. P., Tafuri, S., Ranjan, M., and Familari, M. The Y-box factors: a family of nucleic acid binding proteins conserved from *Escherichia coli* to man. *New Biol.*, 4: 290–298, 1992.

<sup>4</sup> K. Kohno, T. Uchiumi, and M. Kuwano, unpublished data.

3. Sakura, H., Maekawa, T., Imamoto, F., Yasuda, K., and Ishii, S. Two human genes isolated by a novel method encode DNA-binding proteins containing a common region of homology. *Gene*, *73*: 499–507, 1998.
4. Furukawa, M., Uchiumi, T., Nomoto, M., Takano, H., Morimoto, R. I., Naito, S., Kuwano, M., and Kohno, K. The role of an inverted CCAAT element in transcriptional activation of the human DNA topoisomerase II $\alpha$  gene by heat shock. *J. Biol. Chem.*, *273*: 10550–10555, 1998.
5. Kohno, K., Tanimura, H., Sato, S., Nakayama, Y., Makino, Y., Wada, M., Fojo, A. T., and Kuwano, M. Cellular control of human multidrug resistance 1 (*mdr-1*) gene expression in absence and presence of gene amplification in human cancer cells. *J. Biol. Chem.*, *269*: 20503–20508, 1994.
6. Uchiumi, T., Kohno, K., Tanimura, H., Matsuo, K., Sato, S., Uchida, Y., and Kuwano, M. Enhanced expression of the human multidrug resistance 1 gene in response to UV light irradiation. *Cell Growth Differ.*, *4*: 147–157, 1993.
7. Koike, K., Uchiumi, T., Ohga, T., Toh, S., Wada, M., Kohno, K., and Kuwano, M. Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Lett.*, *417*: 390–394, 1997.
8. Ohga, T., Koike, K., No, M., Makino, Y., Itagaki, Y., Tanimoto, M., Kuwano, M., and Kohno, K. Role of the human Y box-binding protein YB-1 in cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and ultraviolet light. *Cancer Res.*, *56*: 4224–4228, 1996.
9. Ise, T., Nagatani, G., Imamura, T., Kato, K., Takano, H., Nomoto, M., Izumi, H., Ohmori, H., Okamoto, T., Ohga, T., Uchiumi, T., Kuwano, M., and Kohno, K. Transcription factor Y-box binding protein 1 binds preferentially to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen. *Cancer Res.*, *59*: 342–346, 1999.
10. Okamoto, T., Izumi, H., Imamura, T., Takano, H., Ise, T., Uchiumi, T., Kuwano, M., and Kohno, K. Direct interaction of p53 with the Y-box binding protein, YB-1: a mechanism for regulation of human gene expression. *Oncogene*, *19*: 6194–6202, 2000.
11. Bargou, R. C., Jurchott, K., Wagener, C., Bergmann, S., Metzner, S., Bommert, K., Mapara, M. Y., Winzer, K. J., Dietel, M., Dorken, B., and Royer, H. D. Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic *MDR1* gene expression. *Nature Med.*, *3*: 447–450, 1997.
12. Oda, Y., Sakamoto, A., Shinohara, N., Ohga, T., Uchiumi, T., Kohno, K., Tsuneyoshi, M., Kuwano, M., and Iwamoto, Y. Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human osteosarcoma. *Clin. Cancer Res.*, *4*: 2273–2277, 1998.
13. Kamura, T., Yahata, H., Amada, S., Ogawa, S., Sonoda, T., Kobayashi, H., Mitsumoto, M., Kohno, K., Kuwano, M., and Nakano, H. Is nuclear expression of Y box-binding protein-1 a new prognostic factor in ovarian serous adenocarcinoma? *Cancer (Phila.)*, *85*: 2450–2454, 1999.
14. Shibao, K., Takano, H., Nakayama, Y., Okazaki, K., Nagata, N., Izumi, H., Uchiumi, T., Kuwano, M., Kohno, K., and Itoh, H. Enhanced coexpression of YB-1 and DNA topoisomerase II  $\alpha$  genes in human colorectal carcinomas. *Int. J. Cancer*, *83*: 732–737, 1999.
15. Minn, J. D., Sekido, Y., and Fong, K. M. Cancer of the lung. *In*: V. T. De Vita, S. Rosenberg, and S. Hellman (eds.), *Cancer: Principles & Practice of Oncology*, 5th ed., pp. 849–949. Philadelphia: Lippincott-Raven, 1997.
16. Mountain, C. F. Revisions in the international system for staging lung cancer. *Chest*, *111*: 1710–1717, 1997.
17. Sobin, L. H., and Fleming, I. D. TNM classification of malignant tumors, 5th ed. Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer (Phila.)*, *80*: 91–100, 1997.
18. Dixon, W. J. (ed.). *BMDP Statistical Software Manual*, pp. 133–744. Berkeley: University of California Press, 1988.
19. Cox, D. R. Regression models and life tables. *JR Stat. Soc. Series B*, *34*: 187–220, 1972.
20. Ohga, T., Uchiumi, T., Makino, Y., Koike, K., Wada, M., Kuwano, M., and Kohno, K. 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.*, *273*: 5997–6000, 1998.
21. Wolffe, A. P. Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *Bioessays*, *16*: 245–251, 1994.

# Clinical Cancer Research

## Nuclear Expression of the Y-Box Binding Protein, YB-1, as a Novel Marker of Disease Progression in Non-Small Cell Lung Cancer

Kotaro Shibahara, Kenji Sugio, Toshihiro Osaki, et al.

*Clin Cancer Res* 2001;7:3151-3155.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/7/10/3151>

**Cited articles** This article cites 17 articles, 7 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/7/10/3151.full#ref-list-1>

**Citing articles** This article has been cited by 24 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/7/10/3151.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://clincancerres.aacrjournals.org/content/7/10/3151>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.