HOXB2, an Adverse Prognostic Indicator for Stage I Lung Adenocarcinomas, Promotes Invasion by Transcriptional Regulation of Metastasis-related Genes in HOP-62 Non-small Cell Lung Cancer Cells

KENTARO INAMURA[1], YUKI TOGASHI[1], HIRONORI NINOMIYA[1], TAKASHI SHIMOJI[2], TETSUO NODA[2] and YUICHI ISHIKAWA[1]

[1]Department of Pathology and [2]Genome Center, The Cancer Institute, Japanese Foundation for Cancer Research (JFCR), Koto-ku, Tokyo, Japan

Abstract. *Background: Previously, using microarray and real-time RT-PCR analysis, we established that HOXB2 is an adverse prognostic indicator for Stage I lung adenocarcinomas. HOXB2 is one of the homeobox master development-controlling genes regulating morphogenesis and cell differentiation. Materials and Methods: The molecular functions of HOXB2 were analyzed with a small interfering RNA (siRNA) approach in HOP-62 human non-small cell lung cancer (NSCLC) cells featuring high HOXB2 expression. Matrigel invasion assays and microarray gene expression analysis were compared between the HOXB2-siRNA cells and the control cells. Results: The Matrigel invasion assays showed attenuation of HOXB2 expression by siRNA to result in a significant decrease of invasiveness compared to the control cells (p=0.0013, paired t-test). On microarray gene expression analysis, up-regulation of many metastasis-related genes and others correlating with HOXB2 expression was observed in the control case. With attenuation of HOXB2 expression, down-regulation was noted for laminins alpha 4 and 5, involved in enriched signaling, and for Mac-2BP (Mac-2 binding protein) and integrin beta 4 amongst the genes having an enriched glycoprotein ontology. Conclusion: HOXB2 promotes invasion of lung cancer cells through the regulation of metastasis-related genes.*

Lung cancer is the leading cause of cancer death in men and women worldwide. The clinical outcome can be roughly predicted by pathological-stage (p-stage) and five-year survival for p-stage I cases, pathologically lacking metastases, is relatively good, ranging from 60% (1) to 90% (2). It is clearly necessary that p-stage I patients at risk of recurrence be identified and given postoperative treatment. Very recently, we found that HOXB2 may be an adverse prognostic indicator for Stage I lung adenocarcinomas (3) by screening the metastasis-related gene signature we had previously identified (4). P-stage I patients with HOXB2 up-regulation had a worse prognosis than those with HOXB2 down-regulation (p=0.0065, log-rank test), with five-year survival rates of 65.5% and 91.5% , respectively. HOXB2 is one of the 39 HOX genes in man that encode transcription factors and act as master regulators of morphogenesis and cell differentiation (5). However, the molecular functions of HOXB2 in lung cancer cells have yet to be elucidated. Hence, in this study, a small interfering RNA (siRNA) approach was therefore adopted to examine the effects in the HOP-62 human non-small cell lung cancer (NSCLC) cell line featuring high HOXB2 expression. Matrigel invasion assays and microarray gene expression analysis were also conducted for comparison between HOXB2-siRNA and control cells.

Correspondence to: Yuichi Ishikawa, Department of Pathology, JFCR Cancer Institute, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan. Tel: +81 3 35200111, Fax: +81 3 35700558, e-mail: ishikawa@jfcr.or.jp

Key Words: Lung cancer, HOXB2, invasion assay, microarray, gene expression analysis.

Materials and Methods

Cell line and cell culture. Human NSCLC HOP-62 cells were obtained from the National Cancer Institute (Bethesda, MD, USA) and cultured in DMEM medium (SIGMA, St. Louis, MO, USA) supplemented with 1% penicillin/streptomycin and 5% bovine calf serum (BCS) (HyClone, Logan, UT, USA) at 37˚C in a humidified incubator under 5% CO_2. High HOXB2 expression of the HOP-62 cells was checked and confirmed (data not shown).

siRNA transfection. For siRNA-mediated gene knockdown, the HOP-62 cells were transfected with DharmaFECT I (Dharmacon, Lafayette, CO, USA) according to the protocol of the manufacturer. The cells were transfected with a HOXB2 siRNA duplex, custom

synthesized by Dharmacon. The targeted sequence (sense strand) was 5'-GCCUUUAGCCGUUCGCUUAUU-3'. A non-targeting siRNA (NTA siRNA) (Dharmacon) was used as a negative control. Approximately 2×10^5 cells were cultured in 6-well plates in DMEM containing 5% BCS for 24 h. The siRNAs were dissolved in siRNA buffer to a final concentration of 100 μM. After 72 or 112 h treatment, the transfected cells were harvested and analysed.

Real-time RT-PCR analysis. Real-time RT-PCR analysis was performed using an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) and TaqMan gene expression assay (Applied Biosystems) for HOXB2 (assay ID: Hs00609873_g1) according to the manufacturer's instructions. TaqMan beta-actin pre-developed reagent (Applied Biosystems) was applied for normalization. The real-time RT-PCR experiments were performed as described previously (3).

Western blot analysis. The total proteins were obtained by homogenization of the cells in lysis buffer and protein concentrations were determined according to the Bradford method (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were subjected to SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Western blots were performed using an antibody against HOXB2 (goat polyclonal anti-HOXB2, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an antibody against beta-actin (mouse monoclonal anti-beta-actin, 1:1000, SIGMA). Electrochemiluminescence (ECL) reagents (Amersham, Little Chalfont, UK) were used for detection.

Matrigel invasion assays. Matrigel invasion assays were performed using Biocoat Matrigel invasion chambers (Becton Dickinson, Bedford, MA, USA) with 8 μm pore size polyethylene terephthalate (PET) membranes coated with Matrigel matrix in 24-well dishes. 5×10^4 cells (HOXB2 siRNA, NTA siRNA, Non-treatment, respectively) were placed in the Matrigel chambers and control inserts without Matrigel. After rehydration of the Matrigel chamber, 5×10^4 cells were placed in the Matrigel chambers and control insert and incubated for 24 h at 37˚C with 5% CO_2. The lower compartment contained 5% BCS as a chemoattractant. After incubation for 24 h at 37˚C with a 5% CO_2 atmosphere, the non-invading cells were removed from the upper surface of the membrane by scrubbing and the cells on the lower surface were stained with a Diff-Quick kit (Sysmex, Kobe, Japan). The invading cells were counted manually. Matrigel invasion assays were performed five times and the data analyzed statistically by paired *t*-test.

Microarray experiments and data analysis. For the microarray experiments, HOP-62 cells were used at 72 or 112 hours after transfection with HOXB2 siRNA or NTA siRNA. In total, five samples were examined in the microarray analysis. The same Agilent Japanese Foundation for Cancer Research custom-made human oligonucleotide microarray consisting of 22,575 probe sets as applied previously was used and the microarray experiments were conducted as described earlier (4). The data were analyzed using GeneSpring (Silicon Genetics, Redwood City, CA, USA) after log-transformation and LOWESS normalization. For the initial gene filtering, the genes for which data were obtained from all the samples, and in which all the samples had an absorption measurement of more than 300, both in the tumor signal and in the reference channel were selected. This yielded a set of 12,176 genes. For the down-regulated genes with attenuation of HOXB2, the genes for which HOXB2 siRNA cells showed less than 0.8-fold the expression levels of NTA siRNA cells were selected. This yielded a set of 298 genes. For the up-regulated genes with attenuation of HOXB2, the genes for which HOXB2 siRNA cells showed more than 1.3-fold the expression levels of the NTA siRNA cells were selected. This yielded a set of 166 genes.

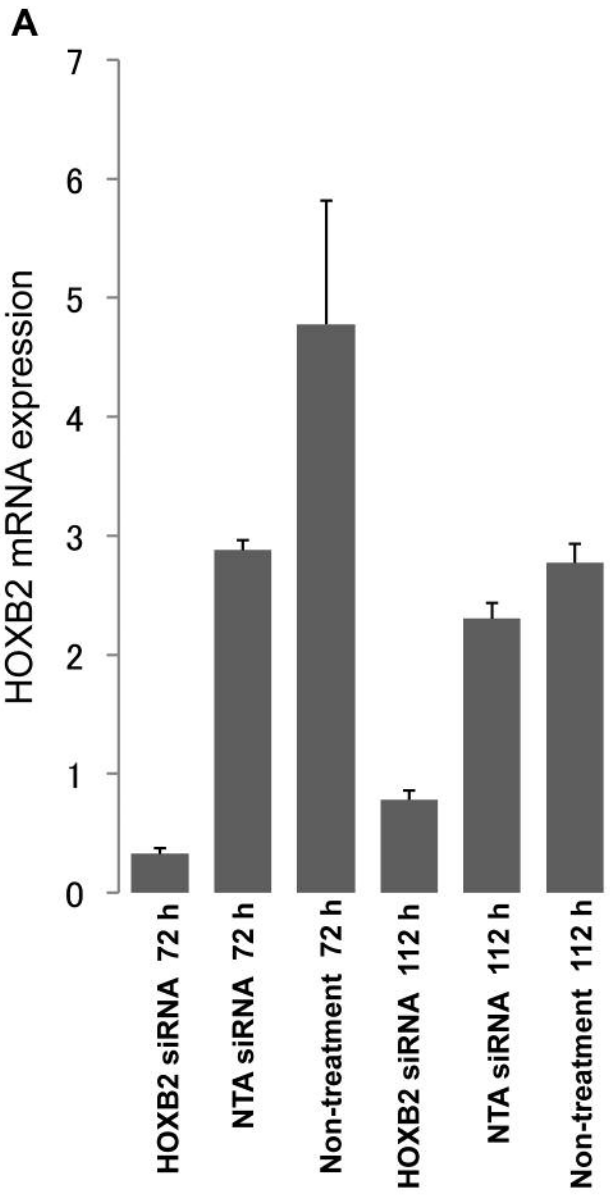

Figure 1. *continued*

DAVID ontological analysis. Functional annotations were performed using the DAVID (database for annotation, visualization and integrated discovery) program (6) (http://david.abcc.ncifcrf.gov/) and statistically tested (EASE Score, modified Fisher's exact test) for significant coregulation (overrepresentation) of the identified genes. The Functional Annotation Chart of the DAVID program at the SP_PIR_Keywords was used.

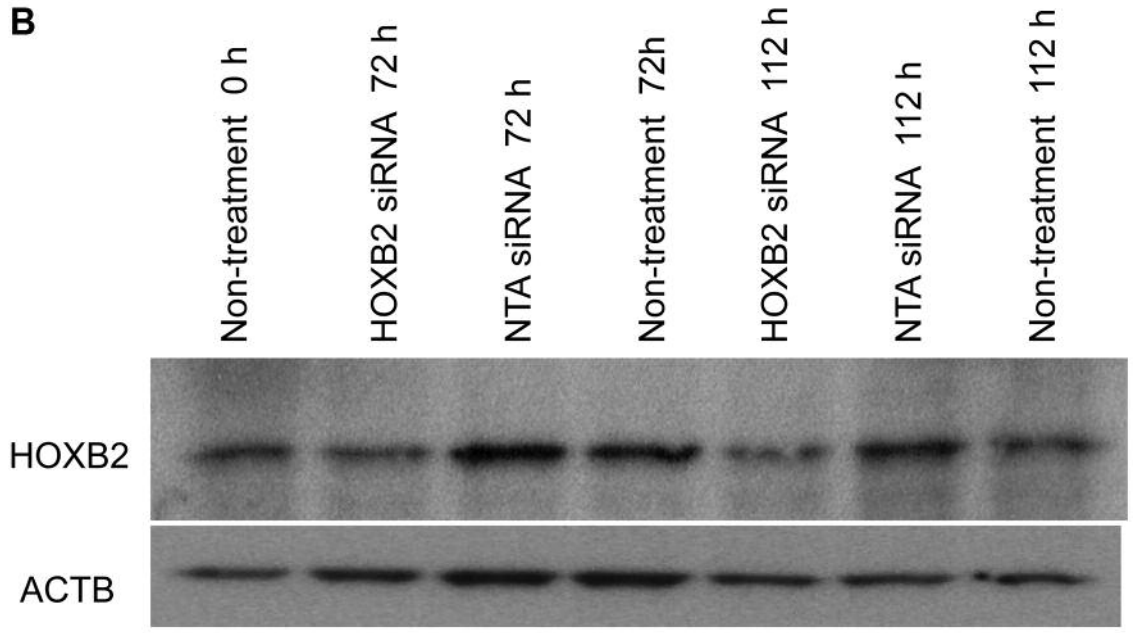

Figure 1. *HOXB2 gene attenuation by siRNA transfection in HOP-62 cells. (A) Real time RT-PCR analysis. mRNA levels are expressed as means+SD relative to the reference samples, normalized to beta-actin. (B) Western blot analysis. HOXB2 siRNA, NTA (non-targeting) siRNA transfected cells; non-treatment: untransfected control cells.*

Results

Effect of HOXB2 siRNA transfection on lung cancer cells. As shown in Figure 1A, lung cancer HOP-62 cells transfected with HOXB2 siRNA displayed a significant reduction (10-30%) in HOXB2 mRNA expression compared to those with the NTA siRNA. On Western blot analysis (Figure 1B), the HOP-62 cells with HOXB2 siRNA displayed some degree of reduction in the HOXB2 protein expression compared with the NTA siRNA and the non-treatment cells.

Effect of HOXB2 expression on invasion of lung cancer cells. Compared to the control cells with NTA siRNA, the HOP-62 cells with HOXB2 siRNA showed a significant decrease of invasiveness in the Matrigel invasion assay (Figure 2; p=0.0013, paired t-test).

Transcriptional effects of reduced HOXB2 expression on microarray analysis. Mac-2 binding protein (*Mac-2BP*), melanoma antigen family D, 1 (*MAGED1*), and family with sequence similarity 83, member D (*FAM83D*), were identified within the 75 metastasis-related genes we had previously identified (4). The expression of all three genes was closely related with HOXB2 expression in the lung carcinomas. The most down-regulated and up-regulated genes are shown in Table I. As expected, *HOXB2* was the most down-regulated gene. The DAVID ontological analysis using the down- or up-regulated genes ascertained the transcriptional effects of HOXB2 expression (Table II).

Discussion

The present study with the siRNA approach and a human NSCLC cell line showed attenuation of HOXB2 to cause a significant decrease in invasiveness. Furthermore, on microarray gene expression analysis, many metastasis-related genes, some with ontologies linked to HOXB2 expression were found to be altered. Thus HOXB2 may act to promote metastasis in human lung cancer.

Among the genes down-regulated with the attenuation of HOXB2 expression, particular ontologies, such as signal (laminins alpha 4 and 5), glycoprotein (Mac-2BP), transmembrane (integrin beta 4), and lysosome (cathepsin L and cathepsin K) links, were characteristically included. Laminins, a family of extracellular matrix glycoproteins, have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis (7). Gene microarray analysis has indicated that up-regulation of laminin alpha 4 could be important for the glial tumor progression (8). Most recently, Vainionpaa *et al.* have reported that this form has a de-adhesive function and thus may play a role in detachment, migration and invasion of

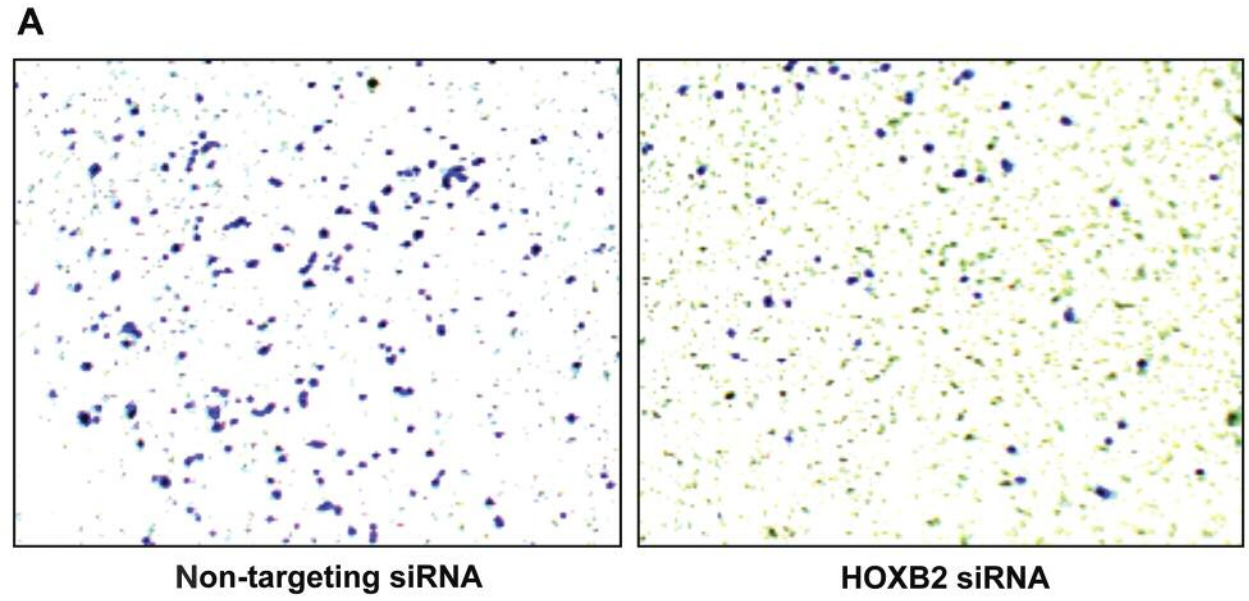

Figure 2. *Matrigel invasion assay (A) Representative photomicrographs showing invasive capability of HOP-62 cells. (B) Percentage of cells migrating through the membrane. HOXB2 siRNA and NTA (non-targeting) siRNA transfected cells after 72 h treatment.*

renal cell carcinomas (9). Pouliot *et al*. reported that laminin alpha 5 had an important role in promoting epidermal proliferation and migration (10). Mac-2BP is a secreted glycoprotein playing a role in cell–cell and cell–extracellular matrix adhesion. Marchetti *et al*. reported that expression of Mac-2 BP correlated with distant metastasis and predicted survival in stage I NSCLC patients (11). Integrins mediate cell–matrix or cell–cell adhesion and transduce signals that regulate gene expression and cell growth. In fact, integrin beta 4 stimulates proliferation and survival of epithelial cells and is overexpressed in human carcinomas (12). The lysosomal cysteine protease cathepsin L is synthesized in various carcinomas including NSCLCs and may facilitate tumor invasion and metastasis (13). Most recently, Rapa *et al*. have found cathepsin K to be selectively expressed in the stroma of lung adenocarcinomas but not in bronchioloalveolar carcinomas (14). Overall, these down-regulated genes and ontologies are all associated with metastasis or oncogenesis, providing convincing evidence that HOXB2 has a meaningful transcriptional role in expression of metastasis-related genes.

Among the genes up-regulated with attenuation of HOXB2 expression, gene ontologies such as nuclear protein (HEX, hematopoietically expression homeobox) and phosphorylation (MTAP, methylthioadenosine phosphorylase) were characteristically included. Puppin *et al*. have reported a similar difference in expression and localization between normal

Table I. *The genes most down-regulated and up-regulated by the attenuation of HOXB2 expression.*

Fold change	Accession	Symbol	Description
10 genes most down-regulated by the attenuation of HOXB2 expression			
0.400	NM_002145	HOXB2	Homeobox B2
0.545	NM_000088	COL1A1	Collagen, type I, alpha 1
0.579	NM_000929	PLA2G5	Phospholipase A2, group V
0.604	NM_003012	SFRP1	Secreted frizzled-related protein 1
0.604	NM_006936	SUMO3	SMT3 suppressor of MIF two 3 homolog 3 (*S. cerevisiae*)
0.628	XM_001129279	SLC45A1	Solute carrier family 45, member 1
0.632	NM_001974	EMR1	Egf-like module containing, mucin-like, hormone receptor-like 1
0.660	NM_016422	RNF141	Ring finger protein 141
0.663	NM_001511	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
0.682	NM_004413	CDK10	Cyclin-dependent kinase (CDC2-like) 10
10 genes most up-regulated by the attenuation of HOXB2 expression			
1.701	NM_003546	HIST1H4L	Histone cluster 1, H4l
1.662	AI005198	PDE4DIP	Phosphodiesterase 4D interacting protein (myomegalin)
1.586	NM_016639	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A
1.516	AK024522	GPRC5A	G protein-coupled receptor, family C, group 5, member A
1.507	NM_031844	HNRPU	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)
1.493	NM_031954	KCTD10	Potassium channel tetramerisation domain containing 10
1.490	NM_002451	MTAP	Methylthioadenosine phosphorylase
1.482	NM_002729	HHEX	Homeobox, hematopoietically expressed
1.481	NM_016090	RBM7	RNA-binding motif protein 7
1.478	NM_001018067	SERBP1	SERPINE1 mRNA-binding protein 1

Fold changes were calculated from the data after 72 hours of transfection with HOXB2 siRNA.

mammary gland and breast carcinomas (15). Recently, Hellerbrand *et al*. have reported a functional role of MTAP inactivation in the development and invasiveness of hepatocellular carcinomas (16).

There is some degree of similarity between morphogenesis and oncogenesis. For example, carcinoembryonic proteins, such as alpha-fetoprotein and carcinoembryonic antigen, are characteristically expressed in both instances. Furthermore, cellular motility with invasion or metastasis of cancer cells is reminiscent of that found during embryonic development. The difference lies in biomedical homeostasis. From this standpoint, oncogenesis can be ascribed to an incomplete borrowing of the morphogenetic program. Since HOX genes are master regulators of morphogenesis and cell differentiation during embryogenesis (5), the finding that they may contribute to malignancy is therefore entirely feasible.

HOX genes are expressed in a spatiotemporal fashion during embryonic development and act as master transcription–regulating factors (5). Hence disordered spatial information may lead to the destruction of coordinated cell society. Indeed, there is increasing evidence of differences in expression of HOX genes between tumors and their normal counterpart tissues. In the normal human adult lung, HOX genes are predominantly expressed from the 3' end of clusters A and B, particularly HOXA5, HOXB2 and HOXB6 (17). With commercially

Table II. *Ontological categories over-represented by genes down-regulated and up-regulated by the attenuation of HOXB2 expression.*

Term	Count	%	*P*-value
Down-regulated genes (DAVID 256IDs)			
Signal	96	37.5%	8.1E-31
Glycoprotein	96	37.5%	3.3E-21
Transmembrane	87	34.0%	1.4E-13
Membrane	85	33.2%	3.9E-12
Lysosome	11	4.3%	1.0E-08
Up-regulated genes (DAVID 157IDs)			
Nuclear protein	41	26.1%	1.3E-06
Phosphorylation	29	18.5%	6.2E-06
Nucleotide-binding	24	15.3%	9.0E-06
ATP-binding	20	12.7%	2.7E-05
Inflammatory response	5	3.2%	4.1E-04

P-value, modified Fisher's exact test.

available polyclonal antibodies against HOXB2, cross reaction against carbonic anhydrase is unfortunately observed so that a more specific anti-HOXB2 antibody is required. The aberrant expression of HOX genes has, however, been implicated in leukemias (18) and various solid tumors including renal (19), colonic (20), ovarian (21), mammary (22), and pulmonary carcinomas (23). The overexpression of HOXD3 is known to induce the coordinated expression of metastasis-related genes in lung cancer cells (23). A recent study revealed ectopic HOXB2 expression in pancreatic malignancies and a proportion of their precursor lesions, pancreatic intraepithelial neoplasias, were possibly associated with a poor prognosis (24). In a previous study, we observed HOXB2 overexpression not only in the central but also in the peripheral zones of node-positive primary tumors (4). Malignant potential associated with HOXB2 expression might thus be acquired early in tumorigenesis.

The establishment of prognostic factors for lung adenocarcinomas is a high priority (1, 25). Recently, Potti *et al*. proposed a "lung metagene model" that could identify individuals at increased risk of disease recurrence with stage-IA NSCLCs (26) and a prospective randomized clinical trial is planned to test this possibility. Although many prognostic factors have been found retrospectively, most have yet to be prospectively validated and the necessity of clinical evaluation now should be stressed. However, challenges remain. For example, it has been argued (27) that the histological classification of NSCLCs by Potti *et al*. into squamous cell carcinoma and adenocarcinoma cannot be predictive for prognosis in general. Sun *et al*. have demonstrated that the histological grade is a significant predictor of both recurrence and survival (28). Some integration of microarray gene expression analysis and classical histopathology is therefore required.

In conclusion, the present cellular functional analysis indicated metastasis-related activity for HOXB2 so that p-stage I patients with up-regulation of this particular form should be regarded as being at high risk of recurrence and a poor prognosis.

Acknowledgements

The authors thank Ms. Kyoko Nakazawa, Ms. Kazuko Yokokawa, Ms. Kimie Nomura, Mr. Motoyoshi Iwakoshi, Ms. Miyuki Kogure, and Ms. Tomoyo Kakita for their technical assistance, and Ms. Yuki Takano for her secretarial expertise.

Parts of this study were supported financially by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, and the Japan Society for the Promotion of Science, and grants from the Ministry of Health, Labour and Welfare, the Smoking Research Foundation, the National Institute of Biomedical Innovation, and the Vehicle Racing Commemorative Foundation.

References

1 Brundage MD, Davies D and Mackillop WJ: Prognostic factors in non-small cell lung cancer: a decade of progress. Chest *122*: 1037-1057, 2002.

2 Miyoshi T, Satoh Y, Okumura S *et al*: Early-stage lung adenocarcinomas with a micropapillary pattern, a distinct pathologic marker for a significantly poor prognosis. Am J Surg Pathol *27*: 101-109, 2003.

3 Inamura K, Togashi Y, Nomura K *et al*: Up-regulation of PTEN at the transcriptional level is an adverse prognostic factor in female lung adenocarcinomas. Lung Cancer *57*: 201-206, 2007.

4 Inamura K, Shimoji T, Ninomiya H *et al*: A metastatic signature in entire lung adenocarcinomas irrespective of morphological heterogeneity. Hum Pathol *38*: 702-709, 2007.

5 Gehring WJ and Hiromi Y: Homeotic genes and the homeobox. Annu Rev Genet *20*: 147-173, 1986.

6 Dennis G Jr, Sherman BT, Hosack DA *et al*: DAVID: Database for annotation, visualization, and integrated discovery. Genome Biol *4*: P3, 2003.

7 Timpl R and Brown JC: The laminins. Matrix Biol *14*: 275-281, 1994.

8 Ljubimova JY, Lakhter AJ, Loksh A *et al*: Overexpression of alpha4 chain-containing laminins in human glial tumors identified by gene microarray analysis. Cancer Res *61*: 5601-5610, 2001.

9 Vainionpaa N, Lehto VP, Tryggvason K *et al*: Alpha4 chain laminins are widely expressed in renal cell carcinomas and have a de-adhesive function. Lab Invest *87*: 780-791, 2007.

10 Pouliot N, Saunders NA and Kaur P: Laminin 10/11: an alternative adhesive ligand for epidermal keratinocytes with a functional role in promoting proliferation and migration. Exp Dermatol *11*: 387-397, 2002.

11 Marchetti A, Tinari N, Buttitta F *et al*: Expression of 90K (Mac-2 BP) correlates with distant metastasis and predicts survival in stage I non-small cell lung cancer patients. Cancer Res *62*: 2535-2539, 2002.

12 Mercurio AM and Rabinovitz I: Towards a mechanistic understanding of tumor invasion – lessons from the alpha 6 beta 4 integrin. Semin Cancer Biol *11*: 129-141, 2001

13 Chauhan SS, Goldstein LJ and Gottesman MM: Expression of cathepsin L in human tumors. Cancer Res *51*: 1478-1481, 1991.

14 Rapa I, Volante M, Cappia S *et al*: Cathepsin K is selectively expressed in the stroma of lung adenocarcinoma but not in bronchioloalveolar carcinoma. A useful marker of invasive growth. Am J Clin Pathol *125*: 847-854, 2006.

15 Puppin C, Puglisi F, Pellizzari L *et al*: HEX expression and localization in normal mammary gland and breast carcinoma. BMC Cancer *6*: 192, 2006.

16 Hellerbrand C, Muhlbauer M, Wallner S *et al*: Promoter-hypermethylation is causing functional relevant down-regulation of methylthioadenosine phosphorylase (MTAP) expression in hepatocellular carcinoma. Carcinogenesis *27*: 64-72, 2006.

17 Golpon HA, Geraci MW, Moore MD *et al*: HOX genes in human lung: altered expression in primary pulmonary hypertension and emphysema. Am J Pathol *158*: 955-966, 2001.

18 Hatano M, Roberts CW, Minden M *et al*: Deregulation of a homeobox gene, HOX11, by the t(10;14) in T-cell leukemia. Science *253*: 79-82, 1991.

19 Cillo C, Barba P, Freschi G *et al*: HOX gene expression in normal and neoplastic human kidney. Int J Cancer *51*: 892-897, 1992.
20 De Vita G, Barba P, Odartchenko N *et al*: Expression of homeobox-containing genes in primary and metastatic colorectal cancer. Eur J Cancer *29A*: 887-893, 1993.
21 Naora H, Yang YQ, Montz FJ *et al*: A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells. Proc Natl Acad Sci USA *98*: 4060-4065, 2001.
22 Cantile M, Pettinato G, Procino A *et al*: *In vivo* expression of the whole HOX gene network in human breast cancer. Eur J Cancer *39*: 257-264, 2003.
23 Miyazaki YJ, Hamada J, Tada M *et al*: HOXD3 enhances motility and invasiveness through the TGF-beta-dependent and -independent pathways in A549 cells. Oncogene *21*: 798-808, 2002.
24 Segara D, Biankin AV, Kench JG *et al*: Expression of HOXB2, a retinoic acid signaling target in pancreatic cancer and pancreatic intraepithelial neoplasia. Clin Cancer Res *11*: 3587-3596, 2005.
25 Singhal S, Vachani A, Antin-Ozerkis D *et al*: Prognostic implications of cell cycle, apoptosis, and angiogenesis biomarkers in non-small cell lung cancer: a review. Clin Cancer Res *11*: 3974-3986, 2005.
26 Potti A, Mukherjee S, Petersen R *et al*: A genomic strategy to refine prognosis in early-stage non-small-cell lung cancer. N Engl J Med *355*: 570-580, 2006.
27 Sun Z and Yang P: Refining prognosis in non-small-cell lung cancer. N Engl J Med *356*: 190; author reply 190-191, 2007.
28 Sun Z, Aubry MC, Deschamps C *et al*: Histologic grade is an independent prognostic factor for survival in non-small cell lung cancer: an analysis of 5018 hospital- and 712 population-based cases. J Thorac Cardiovasc Surg *131*: 1014-1020, 2006.

Received March 3, 2008
Revised May 19, 2008
Accepted May 21, 2008