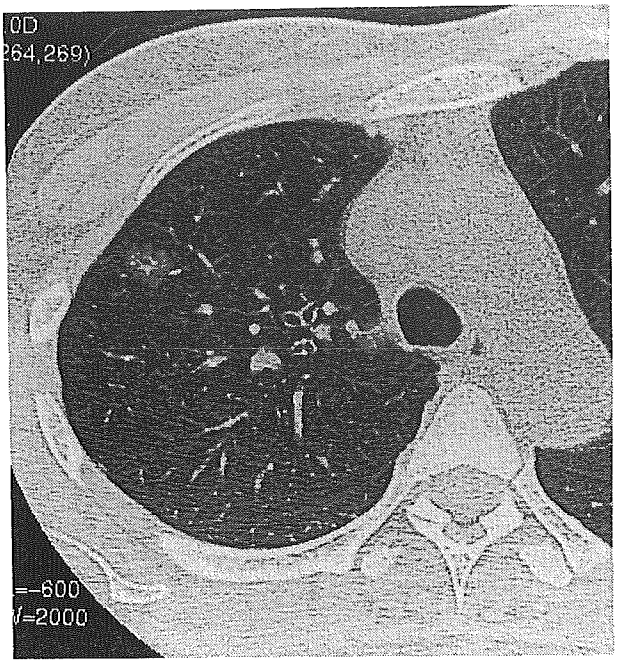

図7　すりガラス様陰影の内部にわずかな充実部を有する細気管支肺胞上皮癌(43歳，女性)
高分解能CT上，右肺上葉$S^3_a$末梢にすりガラス様陰影主体の結節を認める．内部にはごくわずかだが高吸収域を認める．病理標本上は，中心部の虚脱線維化巣に線維芽細胞増生がわずかにみられ野口C型と診断された．

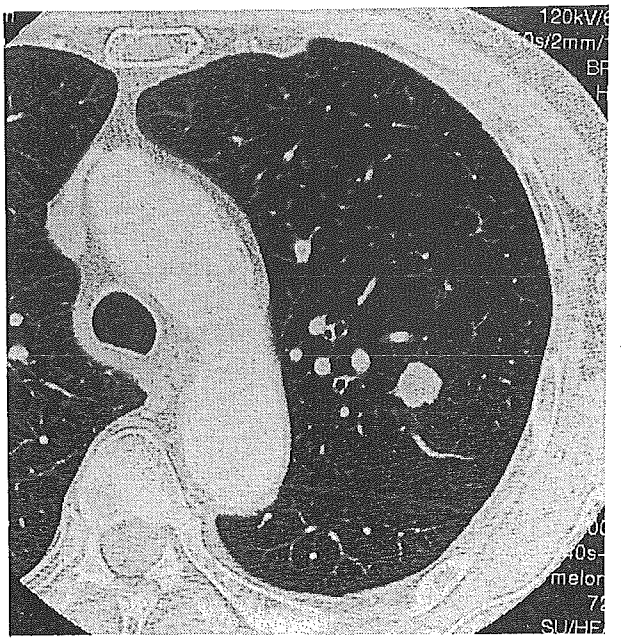

図8　すりガラス様陰影を有しない小結節(73歳，男性)
左肺上葉S1＋2cに境界明瞭な結節を認める．高分解能CTでも，辺縁部にまったくすりガラス様陰影をもたない．病理標本で低分化型腺癌(野口D型)と診断された．

いとCTではすりガラス状陰影をもたない結節としてみられることもあり，低分化型腺癌や扁平上皮癌との鑑別が困難である．またC型とB型の識別もCT上では困難なことが多く，また病理標本上ですら判定に難渋する場合がある(図4)．

D型(低分化型腺癌)(図8)，E型(腺管型腺癌)(図9)，F型(圧排性および破壊性進展を伴う乳頭状腺癌)は，いずれも辺縁部にすりガラス状陰影をもたない結節としてみられ，おおむね細気管支肺胞上皮癌からの鑑別は可能なことが多いが，一方で，通常発育の速い扁平上皮癌や小細胞癌，肉芽腫などの炎症性腫瘤との鑑別が困難なことが多い．

以上のように，高分解能CT所見からおおむね野口分類を推定することはできるが，現状のCTでは細胞レベルや細胞配列までの診断はできないので，おのずと限界がある．しかし，HRCT上のすりガラス影に着目することで，肺腺癌の診断のみならず，腺癌の浸潤程度やリンパ節転移の診断に応用可能である．広範囲にすりガラス状陰影をもつ腺癌は，浸潤傾向がない細気管支肺胞上皮癌か浸潤があっても微細なものにとどまり，通常リンパ節転移がないために，これらの切除例の予後は良好である[11)~13)]．したがって，高分化腺癌のうち，術前のHRCT上のすりガラス影の大きさや割合によって，縮小手術の可能性を検討する段階にきている．

## 異型腺腫様過形成とそのCT所見

末梢型肺腺癌との境界病変，あるいは前癌病変としてとらえられている疾患概念としては，肺の異型腺腫様過形成(atypical adenomatous hyperplasia；AAH)がある[14)]．AAHは，末梢気道上皮の異形成で，細気管支肺胞腺腫とも表現できる病変である．AAHは，クララ細胞あるいはII型肺胞上皮に類似する末梢気道上皮の上皮内異型増殖病変で，多くは長径10mm以下の微小な病変である．AAHの組織学的特徴は，領域性をもつ病変で腺癌のような単調な細胞増殖を示さず，核異型を示すが腺癌ほどでなく，細胞配列も腺癌よりもややまばらで，異型細胞間に異型性に乏しい細胞が介在することがある．肺胞壁の肥厚は軽度で，ときに炎症細胞浸潤もみられるとされるが[15)16)]，腺癌との境界については現時

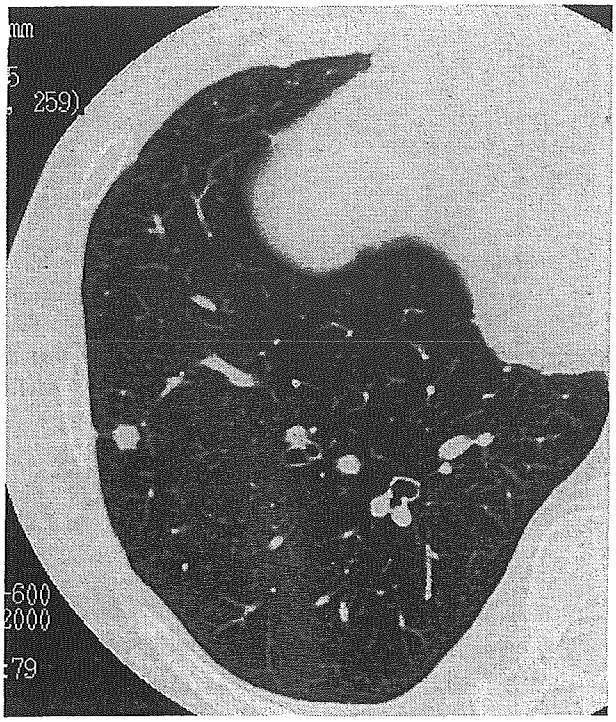


図9　すりガラス様陰影を有しない小結節(68歳，男性)
右肺下葉に境界明瞭な結節を認める．高分解能CTでも辺縁部にまったくすりガラス様陰影をもたない．病理標本で腺管型腺癌(野口E型)と診断された．

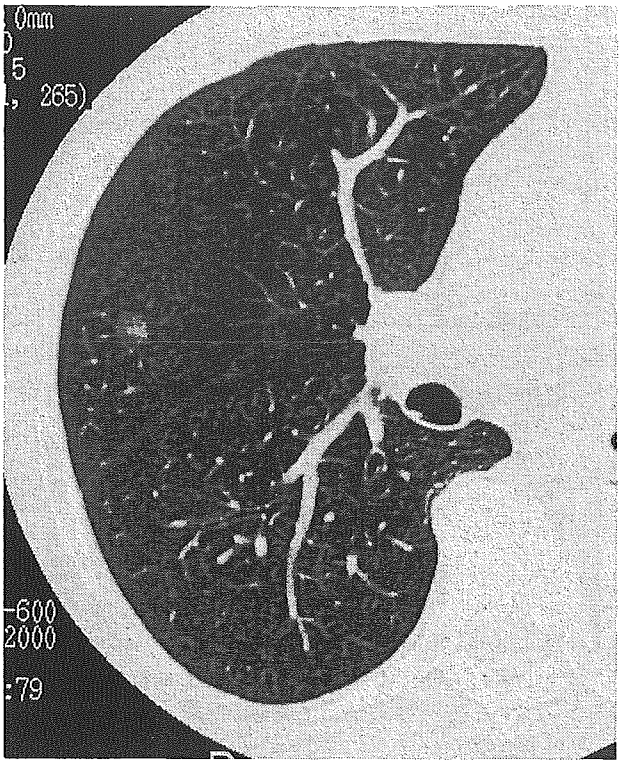


図10　異型腺腫様過形成(68歳，男性)
高分解能CT上，右中葉$S^4_a$に大きさが8×8 mm大の境界明瞭な円形の孤立性限局性のすりガラス影を認める．すりガラス影の内側を肺静脈が貫通しているのが描出されている．摘出標本にて異型腺腫様過形成と診断された．

点では必ずしも明確な診断基準が確立されているとはいい難い．

AAHは，これまで肺腺癌切除例などの切除肺で偶然発見されることが多く，病変が微小なため画像でとらえられることが稀であった．しかし，前述の通りCTによるスクリーニングで発見される症例の報告や，マルチスライスCTの導入で，肺癌切除前のCTで肺癌以外の部位に小さなすりガラス影としてAAHが発見される機会が増えてきた．AAHは単純X線写真では描出されず，CTで微小な病変として認識可能である．通常AAHは，CT上10mm以下のほぼ円形の限局性のすりガラス陰影(濃度)(ground glass attenuation；GGAあるいはground glass opacity；GGO)としてみられ，周囲肺との境界は比較的明瞭であることが多いが，病変が小さくなるにつれ境界自体評価困難になることがある(図10)．Bronchioloalveolar adenoma(BAA)としてのCTに関する報告では，小さな限局性のすりガラス陰影としてみられる，とされており[17]，これらは5 mm以下の小さなAAHでも同様である[18]．

AAHと腺癌との境界については，現時点では病理学上も必ずしも明確な診断基準が確立されているとはいい難い状況の中で，CTなどの画像のみでAAHと腺癌を鑑別することは困難といわざるを得ない．AAHはA型の腺癌よりもやや小さい傾向にあるという報告もあるが[19]，この報告ではAAHは腺癌に合併したものを対象としており，病理上で孤立性のAAHと診断される症例は，前述の野口のA型の腺癌と同様，CT上では10mm内外の円形の限局性のすりガラス陰影として描出され，画像上での明確な鑑別は困難である．図2にAAHとの鑑別が問題になる肺腺癌の症例を示している．

## 肺小結節に対する術前の生検の位置づけ

肺末梢の結節に対する開胸によらない生検としては，気管支鏡を用いた経気管支的診断とCTなどをガイドにした経皮的な針生検が主な方法である．このうちわが国では経気管支的な診断

がもっとも普及しているが，大きさが1cm以下の小型肺癌に対する確定診断率は必ずしも満足すべきものではなく，高分解能CTを詳細に読影して気管支鏡を行っても44%との報告がある[19]．CTガイド下針生検でも，1cm以下の結節に対しては70%程度と診断能だけからみれば気管支鏡よりも比較的良好な結果の報告もあるが[20]，実際1cm内外の小病変に対して針生検や気管支鏡による生検では，すべての症例にわたって診断に足る検体を病変部から採取することは必ずしも容易でなく，限界があるといわざるを得ない．気管支鏡下生検や経皮的針生検で，末梢の小結節から良性疾患に特異的な検体が得られた場合は良性病変と診断可能で，結果として開胸術を回避できるという大きな利点を有する．しかし，生検に関する最大の問題は，悪性所見を示す検体が採取されなかった場合，「悪性でない」と断定できない場合があることである[21]．

「生検をして悪性所見が得られたから手術，生検をして悪性所見が病理学上あるいは細胞診上証明されないが画像上やはり癌が疑われるから手術」という立場をとるのであれば，この生検は治療方針に影響を与えない検査法ということになる．したがって，画像で臨床病期Ⅰ期の肺癌が強く疑われ，生検の結果にかかわらず開胸術になると考えられる手術可能な肺癌の場合は，術前の生検は全例に必要ではないという考え方ができる[22]．

2003年の国立がんセンター中央病院呼吸器外科で，肺癌または肺癌疑いで手術になった症例は445例で，そのうち269例(60.5%)が術前に確定診断を得ず画像所見で開胸術となった．その術前未確定診断症例269例のうち250例(93%)は術後最終病理診断が悪性であり，結果的に良性腫瘤と診断されたものは19例(7%)であった．開胸術全例における悪性腫瘍の割合は95.7%で，良性腫瘤の割合は4.3%であった．術前に確定診断をつけずに開胸術を行う場合は，できるかぎり良性腫瘤の手術例を少なくする努力が必要であり，この際に画像診断が重要な役割をはたす．

## 高分解能CTでの肺小結節のマネージメント

まず，CTで小さい結節や淡い結節をみつけた場合，ヘリカルスキャンを用いて小病変部を連続的なHRCTを撮影する．①境界明瞭な結節で，すりガラス様陰影を有しない場合と，②結節全体がすりガラス様陰影を示す場合，③辺縁部がすりガラス様陰影を示す場合に大きく分けて考える．

単純X線写真でとらえられず，高分解能CTで境界明瞭な結節としてみられる場合は，多くは1cm未満の小腫瘤である．この場合は，通常多くは炎症性肉芽腫か肺内リンパ節であることが多い．メイヨークリニックのデータでも，CT検診参加者の69%に石灰化のない結節がみられ(しかも95%が径8mm以下の小結節)，このうち98%以上が結果的に良性結節と診断されている[4]．もちろん肺転移や良性腫瘍もありえるが，肺転移の場合は原発巣の情報があれば診断は比較的容易であり，現実に増大する小結節で最終的に悪性であると診断されるものは，既知の悪性腫瘍がある場合が多い[23]．肺内リンパ節は，中下葉の胸膜直下か1cm以内に存在する，長径12mm以下の境界明瞭な小結節という特徴を有するので，診断が比較的容易である[24]．

1cm内外の小結節のうちで，鑑別上もっとも問題になるのは，肺原発の低分化型腺癌や扁平上皮癌であり，これらは小さいとき境界明瞭な結節を示すことが多い．しかし，通常これらの肺癌は，増大が急速であるため短期間の画像の経過観察で診断できることが多い．充実性結節の場合は，われわれは約1か月後の経過観察を行っている．ただし，これらの病変を経過観察してよいかどうかは問題の残る部分であるが，1cm未満の小結節の場合は，画像上で経過を観察するのが現実的実際的である[24][25]．

結節全体がすりガラス影を示す場合，径2cm程度でも単純X線写真でとらえることは通常困難な場合がある．15mm以下ですりガラス影の割合が70%以上であると，X線での検出が困難になる[26]．境界明瞭な小円形のすりガラス様陰影の場合でかつ内部に充実部をまったくもたず，経過

観察で消退傾向がみられない場合は，限局性の細気管支肺胞上皮癌(野口A型)かあるいはAAHであることがほとんどである[13)27)28)]．これらは新生物であることにはほぼ間違いないが，これらは急速な増大が確認されないのも特徴である[24)]．したがって，このような病変に対しては手術によるすみやかな摘出よりも，患者の年齢などを十分に考慮しCTで経過観察をして，増大がないかぎり観察を続けるというのも一法である．

辺縁部がすりガラス影を示し内部に小結節(充実部分)をもつタイプも，周辺部に肺胞上皮進展部を伴う腺癌である特異性が高い[13)]．ただし，これも稀に限局性の炎症の治癒過程で同様の画像所見を示す場合もあり注意が必要である．この形状を示す肺癌は，放置すると緩徐とはいえ発育進展するため，切除を念頭にいれた診断治療方針が必要である．また，辺縁部のすりガラス影の部分が全周性でなく部分的な場合もあり，読影には注意を要する．ただし，器質化肺炎などとの鑑別が難しい場合，経過観察を行うことになるがその際は，肺癌であっても高分化腺癌の可能性が高いため，経過観察は3か月後程度でよいと思われる．

画像上肺結節がみられ，肺癌が否定できないからただちに開胸生検を勧めるということになると，画像診断のレベルが低い場合多くの良性腫瘤に対する開胸が行われることになりそれだけ多くの患者に不利益となる．画像診断が病変の存在をとらえるのみならず，患者に対する治療方針のマネージメントに大きく参画する場面で，画像診断の重要性と責任が問われることになる．

## おわりに

肺癌の早期発見のため，あるいは胸部疾患のスクリーニングとしてのCTが普及しつつあり，そのために肺の小結節が単純X線写真より数多く発見される時代に入りつつある．これらの数多くの小結節から，末梢性肺癌をみつけ診断するためには，スクリーニングCTを丁寧に読影し，その上で疑わしい病変部分に対して高分解能CTを追加して撮影あるいは再構成し読影することが重要である．高分解能CTの読影にあたっては，小さく淡い末梢性肺癌の画像上特徴に熟知し，生検に頼らない責任ある画像診断が要求される．

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**Original Paper**

# CK20 expression, CDX2 expression, *K-ras* mutation, and goblet cell morphology in a subset of lung adenocarcinomas

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## Abstract

**There are data in the literature that suggest a close relationship between the expression of CK20 and CDX2, *K-ras* mutations, and goblet cell morphology. The present study has examined these factors in a cohort of 264 non-small cell lung cancers. Thirteen of 212 adenocarcinomas expressed CK20; 29 expressed CDX2; *K-ras* mutation was identified in 28; and goblet cell features were present in 19. These four factors correlated with each other in a complex way and therefore a logistic regression model was constructed. Significant correlations were found between CK20 and CDX2 expression, and between *K-ras* mutation and goblet cell morphology, and there was a marginal correlation between CDX2 immunoreactivity and goblet cell morphology. These four features have also been commonly detected in colorectal, pancreato-biliary, and ovarian mucinous carcinomas, suggesting that these adenocarcinomas may be prototypical, independent of the organ of origin. Furthermore, as high and uniform expression of CDX2 was characteristic of metastatic colorectal cancer, weak and/or focal CDX2 expression should alert surgical pathologists to the possibility of primary lung adenocarcinoma, especially in the presence of goblet cell morphology. However, some lung adenocarcinomas may express CDX2 strongly: in this case, CK20 also tends to be positive.**


**Keywords: CK20; CDX2; *K-ras*; goblet cells; mucinous carcinoma; non-small cell carcinoma**

## Introduction

Lung cancers, especially adenocarcinomas, are characterized by a high degree of morphological heterogeneity, which implies both intra- and inter-tumoural diversity. Morphologically, lung adenocarcinomas show a wide range of cellular features and are subdivided into a number of categories including type II pneumocyte type, Clara cell type, bronchial surface epithelium type, and bronchial gland type, according to the scheme of cellular classification of lung adenocarcinomas proposed by Shimosato [1]. Goblet cell adenocarcinoma is one such subtype, about which interesting data have been reported. Several types of adenocarcinoma in the WHO classification, including mucinous type of bronchioloalveolar carcinoma, mucinous (colloid) carcinoma, mucinous cystadenocarcinoma, and adenocarcinoma, mixed mucinous type, belong to this category.

Close correlations have been suggested between the occurrence of mucinous bronchioloalveolar carcinomas (BACs) and mutations in the *K-ras* proto-oncogene [2,3]. Tsuchiya *et al* reported that goblet cell adenocarcinomas exhibited a very high rate of *K-ras* mutation in comparison with other types of adenocarcinoma [3]. Marchetti *et al* found *K-ras* mutations in ten out of ten mucinous BACs, but only in 34 of 98 non-mucinous adenocarcinomas [4].

Another interesting finding in mucinous carcinomas is differential expression of cytokeratins. Clinically, the expression of cytokeratin 20 (CK20), as well as CK7 and thyroid transcription factor-1 (TTF-1), has been used to discern primary from metastatic adenocarcinomas in the lung [5–10]. CK20 expression is restricted to a limited group of adenocarcinomas: it is detectable in almost all colon cancers, about half of gastric and pancreato-biliary carcinomas, and about 30% of transitional cell carcinomas. CK20 expression is rare in lung cancer, with less than 8% of lung adenocarcinomas positive [5,9,10]. However, some have reported that the expression in lung tumours is linked to a particular histological subtype. Shah *et al* [11] reported that 17 of 19 mucinous BACs showed immunoreactivity for CK20, whereas only ten of 80 adenocarcinomas NOS and four of 14 non-mucinous BACs were positive. Goldstein and Thomas

[12] also reported differential immunoreactivity for CK20 between mucinous and non-mucinous BACs; seven of 14 mucinous BACs were positive for CK20, in contrast to only one of 26 non-mucinous BACs.

*CDX2* is a homeobox gene related to the *Drosophila Caudal* gene and encodes a transcription factor that plays an important role in pattern formation in the developing embryo and induction of intestine-specific genes [13]. Transfection of *CDX2* into an undifferentiated intestinal cell line resulted in arrest of proliferation, followed by morphological and phenotypic differentiation to mature intestinal epithelial-like cells, suggesting that the expression of this gene affects the proliferation and differentiation of intestinal cells [14]. Indeed, *CDX2* (+/−) heterozygous mice develop colonic hamartomas and polyps, whereas *CDX2* (−/−) homozygous mice are embryonic lethal. In adult human tissue, CDX2 is specifically expressed in the intestinal epithelium and most colorectal cancers are positive for CDX2. In addition, 50% of gastric cancers express CDX2 in association with their intestinal phenotype and positivity for CDX2 was observed in some pancreato-biliary carcinomas [15,16]. Whereas expression in organs derived from the mesodermal component of the digestive tract during development is understandable, ovarian mucinous carcinomas, which are positive for both *K-ras* mutations and CK20 expression, are also positive for CDX2.

These previous studies reported various pieces of information regarding the relationships between *K-ras* mutation, CK20 expression, goblet cell morphology, and CDX2 expression in lung cancer and in carcinomas in other sites. In this study, using a consecutive series of 264 non-small cell lung cancers, we attempted to organize and integrate the data on lung cancers and compared these with carcinomas at other sites. The results suggest the existence of an adenocarcinoma prototype, in which a subset of lung cancers may be included.

## Materials and methods

### Patients

A series of 264 consecutive, non-small cell carcinomas presenting between September 2000 and December 2002 at the Department of Pathology and Molecular Diagnostics, Aichi Cancer Center, Nagoya, Japan were used for the present study, after obtaining the approval of the institutional review board and patients' written informed consent. To exclude the possibility that the lung tumours were metastatic, all of the patients who underwent surgical resection were routinely examined by abdominal computed tomography and further analysis was performed when abnormal findings were obtained. Twenty-one metastatic colorectal carcinomas were used as controls. In addition, a spectrum of 32 neuroendocrine tumours of the lung was examined, comprising six typical carcinoid tumours, two atypical carcinoid tumours, eight large cell neuroendocrine carcinomas, and 16 small cell carcinomas. Clinical information was collected from the database and pathological staging was determined according to the *AJCC Cancer Staging Manual* [17].

### Tissue microarrays

Four spots were selected per tumour and tissue microarrays were constructed using an MTA-1 manual tissue arrayer (Beecher Instruments, Inc, Silver Spring, MD, USA). Briefly, selected spots of the donor paraffin wax block were punched with a 0.6-mm-diameter coring needle, and transferred and arrayed in the recipient block using the arrayer. Serial 4-μm-thick sections on coated slide glasses were prepared for immunohistochemical analysis, as described previously [18,19].

### Immunohistochemistry

Immunohistochemical examination proceeded according to the standard avidin–biotin–peroxidase complex method using monoclonal antibodies against CDX2 (CDX2-88; Biogenex, San Ramon, CA, USA), CK20 (Ks 20.8; DAKO, Copenhagen, Denmark), and TTF-1 (8G7G3, DAKO). Antigens were retrieved by autoclave for CDX2 and TTF-1, or by trypsin treatment for CK20. The tissue array was used for screening and all of the positive cases or cases with suspicious positive reactions were examined using regular large sections. Absence of false-negative cases in the tissue microarray was confirmed similarly with regular large sections, using 20 each of the cases evaluated as negative. Selected large sections were evaluated semi-quantitatively with the following criteria. More than a moderate intensity of signal was considered as positive and the proportion of positive cells was scored as 0, negative; 1, less than 25% of positive tumour cells; 2, 26–50%; 3, 51–75%; and 4, 76% or more.

### Relative quantification by real-time RT-PCR

First-strand cDNAs were synthesized from DNase I-treated total RNA extracts, using Superscript II (Invitrogen, Carlsbad, CA, USA) and random hexamer primers (Roche Applied Science, Alameda, CA, USA). Real-time quantitative PCR amplifications were performed using a Smart Cycler system (SC-100, Cepheid, Sunnyvale, CA, USA). The reactions were performed using QuantiTect SYBR Green PCR kits (Qiagen, Valencia, CA, USA). The primer sequences for CDX2 were forward, 5′-CCGAACAGGGACTTGTTTAGAG- 3′ and reverse, 5′-CTCTGGCTTGGATGTTACACAG-3′. In each reaction, standard samples diluted up to 1/1000 of cDNA from a colon cancer cell line, SW480, were run with unknown tumour samples. Quantitative values of each sample relative to HT-29, which is known to have minimal expression, were compared with those of $\beta$-actin.

### Mutation status of K-ras

Frozen tissues from the tumour specimens were dissected macroscopically to enrich tumour cells in the extracted tissues, followed by extraction of total RNA with the RNAeasy kit (Qiagen). Using a standard RT-PCR procedure, *K-ras* was amplified using the following primers: forward, 5′-GGCCTGCTGAAAATGACTGA- 3′ and 5′-TCTTGCTAAGTCCTGAGCCTGTT-3′. The products were directly sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

### Statistical analysis

The $\chi^2$ test for independence and the unpaired $t$-test were used to compare the gene expression data. Logistic regression models were constructed to analyse complex relationships using SYSTAT software (SYSTAT Software Inc, Richmond, CA, USA). $p < 0.05$ was considered statistically significant.

## Results

### *K-ras* mutation and expression of CDX2 and CK20 in non-small cell lung cancer

Careful screening using tissue microarray and validation with regular whole sections revealed that 13 and 32 of 264 non-small cell lung cancers were positive for CK20 and CDX2 expression, respectively. The distribution of expression among the histological subtypes is listed in Table 1. All of the tumours expressing CK20 and CDX2 were adenocarcinomas except for three CDX2-positive squamous carcinomas. The intensity of the positive reactions in the primary lung adenocarcinomas was variable, whereas 21 metastatic cancers of colorectal origin were uniformly and intensely positive. The distribution of reactions and representative pictures are shown in Figures 1 and 2. The mean positive score for lung adenocarcinomas was significantly lower than that of metastatic colorectal cancers, and most of the reactions were regional. However, in a limited number of the lung adenocarcinomas, uniform positive reactions appeared to mimic those of metastatic colon cancers. Examination of clinical charts revealed no history of advanced gastrointestinal carcinoma prior to undergoing surgery for lung cancer in the patients with tumours positive for these markers.

*K-ras* mutations were observed in 30 of 251 tumours. The majority of the mutations were at codon 12 (26 of 30) and the others included one at codon 13 and three at codon 61. The significant association of *K-ras* mutations with adenocarcinomas (28 of 30 mutations found) is consistent with previous reports [20,21].

**Table 1.** Expression status in non-small cell lung cancers and metastatic colon cancers

| | CK20 expression | CDX2 expression | *K-ras* mutations |
|---|---|---|---|
| Adenocarcinomas | 13/212 | 29/212 | 28/203 |
| Squamous cell carcinomas | 0/41 | 3/41 | 1/39 |
| Adenosquamous carcinomas | 0/5 | 0/5 | 1/5 |
| Large cell carcinomas | 0/6 | 0/6 | 0/4 |
| Metastatic colon cancers | 20/21 | 21/21 | 8/19 |

### Correlation of the expression of CK20, CDX2, and mutated *K-ras* with goblet cell morphology

Because *K-ras* mutations and CK20 expression have been reported in association with goblet cell morphology, we examined the relationship between these features in lung adenocarcinomas. Goblet cell adenocarcinomas were observed in 19 of the 212 tumours. This was associated with a higher frequency of *K-ras* mutations ($p < 0.05$) and CDX2 expression ($p < 0.05$). Similarly, a statistically significant relationship was found between CDX2 and CK20 expression ($p < 0.01$), and *K-ras* tended to be mutated more frequently in CDX2-expressing adenocarcinomas ($p = 0.06$). The clinicopathological features of the four groups are summarized in Table 2. The relationships were associated with each other and we therefore constructed logistic regression models to analyse these complex relationships, together with various clinicopathological features (Table 3). CK20 expression was associated only with CDX2 expression and *K-ras* mutations only with goblet cell morphology, respectively. CDX2 expression was significantly associated with CK20 expression, male sex, a tumour diameter of more than 30 mm, and lack of TTF-1 expression; there was a marginal association with goblet cell morphology. Goblet cell morphology was related significantly to lack of TTF-1 expression and marginally with *K-ras* mutation. These results suggested three kinds of relationship: first, a close association between CK20 and CDX2; second, correlations between *K-ras* mutations and goblet cell morphology; and third, a weak association between CDX2 expression and goblet cell morphology.

These findings raised the question of whether a small proportion of adenocarcinomas were individually positive for all of these markers, or whether a subset of adenocarcinomas specifically expressed some of the markers. The former possibility suggests the presence of a distinct form of adenocarcinoma, whereas in the latter case, expression represents a vague tendency for a certain differentiation feature. The results revealed that tumours expressing just one or two markers were most frequent and that only two expressed all markers.

### CDX2 mRNA expression in lung cancers

In contrast to previous studies, we observed CDX2 expression in lung tumours. To confirm this,

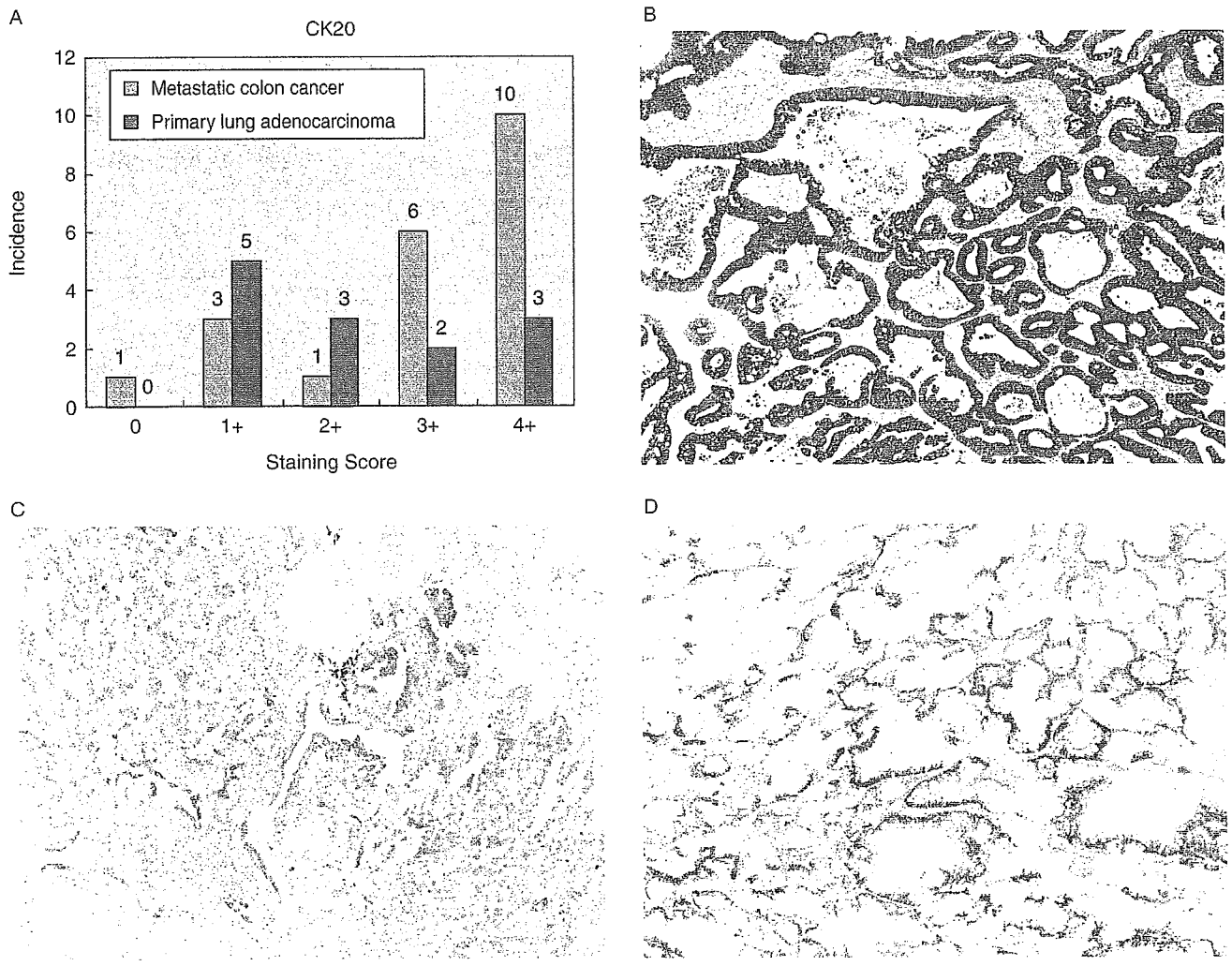



**Figure 1.** CK20 expression in metastatic colon cancers and lung adenocarcinomas. The histogram (A) indicates the distribution of CK20-postive scores between the metastatic colon cancers and lung adenocarcinomas examined: mean scores are 3.0 and 2.2, respectively. The numbers on the tops of the bars indicate the frequency of each staining category. Representative sections are shown for a score of 4+ in a metastatic colon cancer (B), a score of 2+ in a lung adenocarcinoma (C), and a score of 4+ in a lung adenocarcinoma (D)

expression relative to that of a colon cancer cell line, HT29, was examined using real-time RT-PCR in three normal lungs, three metastatic colon cancers, five CDX2-positive lung adenocarcinomas, and five CDX2-negative lung adenocarcinomas (Figure 3). No or negligible transcript levels were detected in normal lung and CDX2-negative lung adenocarcinomas, whereas CDX2-positive lung adenocarcinomas and colorectal cancers showed, respectively, 10.7 and 36.6 times as much transcript relative to HT29 cells. In the lung adenocarcinomas with positive CDX2 expression, relative transcript values varied from 0.1 to 49.3, which correlated well with the staining scores.

### CDX2 expression in neuroendocrine carcinomas

We noted that, in addition to lung adenocarcinomas, some of the neuroendocrine tumours expressed CDX2. This was observed in six of 24 high-grade neuroendocrine tumours (25%), including large cell neuroendocrine carcinomas and small cell lung cancers (Figure 4). Although the expression in about half of the positive cases was restricted to a portion of the tumour (less than 25% of the tumour area), the other half demonstrated relatively uniform expression. The distribution of the staining scores and representative scoring 3+ is shown in Figure 4.

## Discussion

To date, pieces of information in the literature have indicated a close relationship between the expression of CK20 and CDX2, *K-ras* mutations, and goblet cell morphology. CK20 is expressed commonly in colorectal adenocarcinomas, ovarian mucinous carcinomas, transitional cell carcinomas, gastric adenocarcinomas, and pancreatic carcinomas [5,9,10]. CDX2 is expressed in colorectal adenocarcinomas, ovarian mucinous carcinomas, gastric adenocarcinomas, pancreatic ductal carcinomas, and biliary duct carcinomas [15,16,22]. *K-ras* is frequently mutated in pancreatic carcinomas, colorectal carcinomas, ovarian mucinous

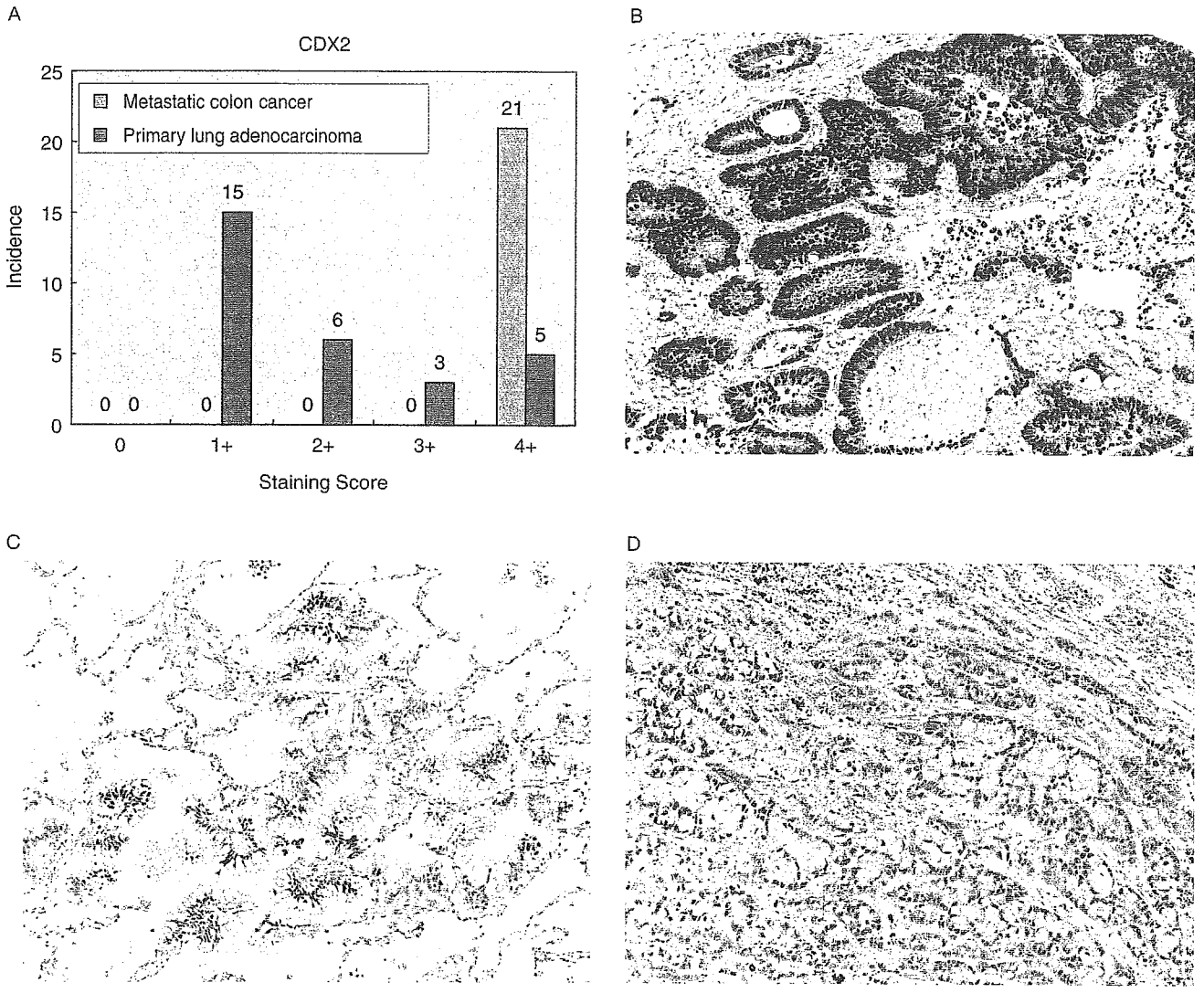



**Figure 2.** CDX2 expression in metastatic colon cancers and lung adenocarcinomas. The histogram (A) indicates the distribution of CDX2-postive scores between metastatic colon cancers and lung adenocarcinomas: mean scores are 4.0 and 1.9, respectively. The numbers on the tops of the bars indicate the frequency of each staining category. Representative figures are shown for a score of 4+ in a metastatic colon cancer (B), a score of 2+ in a lung adenocarcinoma (C), and a score of 4+ in a lung adenocarcinoma (D)

carcinomas, and endometrial carcinomas. Therefore, colorectal carcinomas, ovarian mucinous carcinomas, pancreatic carcinomas, and some endometrial carcinomas share the four properties examined in the present study and these tumours make up a significant proportion of human adenocarcinomas. These findings suggested that overlap of these four features is rather common and a cancer with these features may represent one of the adenocarcinoma prototypes, which is independent of the organ of origin. Characteristics of this prototype may be cell type of 'intestinal' goblet cells. Ichikawa *et al* reported that intestinal-type mucinous ovarian, but not endocervical, tumours exhibit *K-ras* mutation, suggesting a specific association of this mutation with 'intestinal' goblet cell-type adenocarcinomas [23]. Indeed, although endocervical adenocarcinomas show goblet cell morphology, *K-ras* mutation and expression of CK20 and CDX2 are rarely detected. This kind of alteration that is specific for cellular context and/or cell type has been documented. Some skin tumours show a preference for certain genes to be mutated, ie PTCH in basal cell carcinomas [24] and CYLD in cylindroma [25]. It is of note that despite induction throughout the epidermis and skin appendages using a keratin-5 promoter, mice transgenic for *H-ras, GLI2*, and *β-catenin* developed different types of tumour: squamous cell carcinoma [26], basal cell carcinoma [27], and hair-follicle tumour [28], respectively. Another example is mice transgenic for *K-ras*. Even though the mice expressed $K\text{-}ras^{V12}$ throughout the body, hyperproliferative properties and consequent progression to cancer were induced only in the bronchioloalveolar cells in the lung. These findings suggest that the molecular mechanism of carcinogenesis is different among cell types and/or cellular contexts, and similar cell types and/or contexts may share mechanisms that result in a similar genotype and phenotype.

To our knowledge, there are only three reports concerning CDX2 expression in the differential diagnosis

**Table 2.** Patient characteristics

| | CDX2 expression | CK20 expression | *K-ras* mutation | Goblet cell morphology |
|---|---|---|---|---|
| Number of cases | 29 | 13 | 28 | 19 |
| Histological subtypes in the WHO classification | | | | |
| Mixed | 16 | 6 | 15 | 16 |
| Acinar | 8 | 4 | 5 | 3 |
| Papillary | 4 | 3 | 6 | 0 |
| Solid with mucin | 1 | 0 | 2 | 0 |
| TNM stage | | | | |
| pT(1/2/3/4) | 9/12/6/2 | 9/4/0/0 | 11/13/2/2 | 7/7/0/5 |
| pN(0/1/2/3) | 20/2/7/0 | 10/0/2/1 | 18/0/9/1 | 16/1/2/0 |
| pM(0/1) | 28/1 | 13/0 | 28/0 | 19/0 |
| Male gender | 22 | 7 | 21 | 5 |
| More than median age | 14 | 8 | 10 | 11 |
| Smoker | 21 | 8 | 21 | 7 |
| More than 30 mm of tumour size | 19 | 3 | 12 | 7 |
| Invasion beyond visceral pleura | 13 | 2 | 7 | 4 |
| Nodal metastasis | 9 | 2 | 9 | 3 |
| TTF-1 expression | 11 | 8 | 17* | 7 |

Each number indicates the frequency in each category.
* Two cases were not examined.

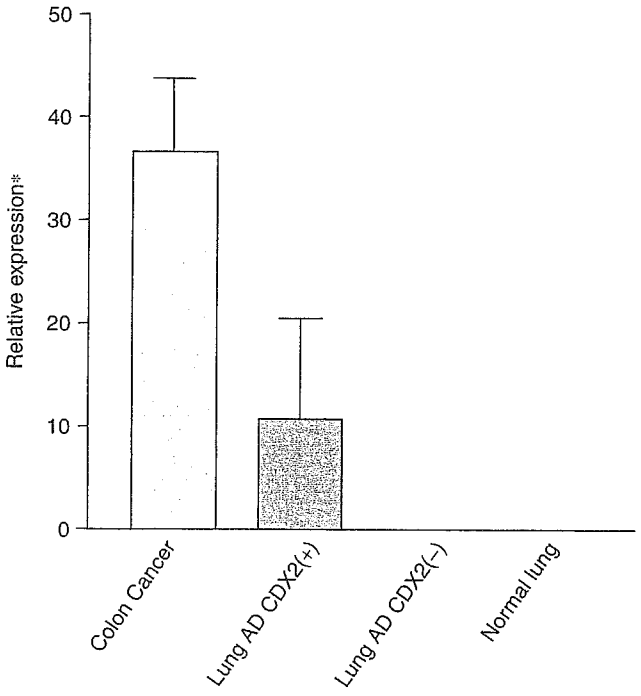
**Table 3.** Four logistic regression models for the analysis of relationships

| | Dependent variables | | | |
|---|---|---|---|---|
| **Independent variables** | **CK20 expression** | **CDX2 expression** | ***K-ras* mutations** | **Goblet cell morphology** |
| CK20 expression | — | < 0.01 | 0.11 | 0.16 |
| CDX2 expression | < 0.01 | — | 0.43 | 0.25 |
| *K-ras* mutations | 0.30 | 0.49 | — | *0.06* |
| Goblet cell morphology | 0.43 | *0.05* | 0.02 | — |
| More than median age | 0.80 | 0.73 | 0.10 | 0.23 |
| Male gender | 0.20 | < 0.01 | 0.20 | 0.12 |
| Smoker | 0.49 | 0.52 | 0.16 | 0.38 |
| Tumour diameter > 30 mm | 0.40 | 0.03 | 0.74 | 0.80 |
| Invasion to visceral pleura | 0.22 | 0.27 | 0.52 | 0.19 |
| Pathological stage I (early stage) | 0.73 | 0.86 | 0.67 | 0.33 |
| Nodal metastasis | 0.78 | 0.40 | 0.39 | 0.10 |
| TTF-1 expression | 0.53 | 0.02 | 0.85 | < 0.01 |

Each value indicates a $p$ value for the comparison of independent variables (rows) with individual models (columns). For example, the CK20 column indicates that although CD20 expression is potentially influenced by various factors listed in rows (independent variables), the logistic regression model for CK20 expression (second column) reveals a statistically significant association with CDX2 expression ($p = 0.01$) but not the other factors.
Underlines and italics represent statistically significant and marginal correlations, respectively.

between primary lung adenocarcinoma and metastatic cancers of colorectal origin [15,16,22]. The present study is in agreement with those in terms of the marked difference in CDX2 expression between primary and metastatic adenocarcinomas; high and uniform expression was characteristic of colorectal cancers. However, as shown with real-time PCR, expression is not completely absent from primary lung adenocarcinomas. Indeed, one of the previous three reports documented one case each of 1+ and 2+ CDX2 staining among 33 primary lung adenocarcinomas examined [15]. Furthermore, other non-gastrointestinal tumours were also positive for CDX2. Franchi *et al* demonstrated uniform expression in most ethmoid sinus adenocarcinomas, which were also positive for CK20 and goblet cell morphology [29]. It is therefore reasonable to suggest that CDX2 can be expressed in this specific subset of lung adenocarcinomas, as a reflection of the characteristics of the cell- or cancer-type. This also alerts surgical pathologists to a pitfall in differential diagnosis using CDX2 immunohistochemistry.

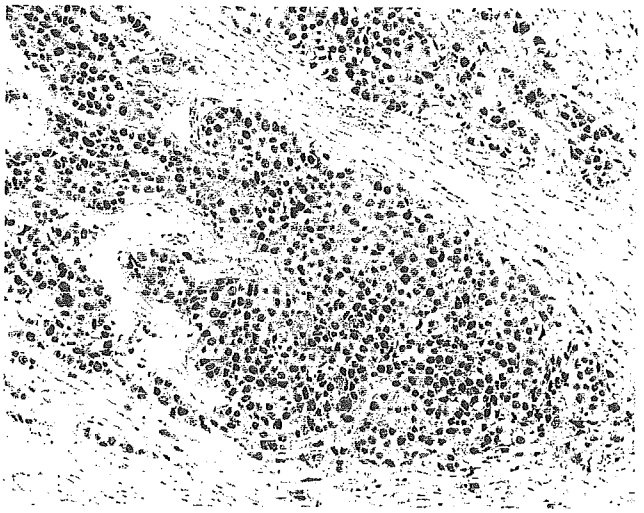
It is of note that high-grade neuroendocrine carcinomas also expressed CDX2. It is well known that *K-ras* is rarely mutated in small cell lung cancers [21] and that high-grade neuroendocrine tumours are negative for CK20 [9]. Thus, CDX2 is not expressed in association with a certain adenocarcinoma prototype. Rather, this finding may represent aberrant expression of lineage-specific molecules in pulmonary high-grade neuroendocrine carcinomas. We have reported




| Samples | n= | Average | SEM |
|---|---|---|---|
| Colon cancer | 3 | 36.6 | 7.1 |
| Normal lung | 3 | 0.0 | 0.0 |
| Lung AD, CDX2(+) | 5 | 10.7 | 9.7 |
| Lung AD, CDX2(−) | 5 | 0.0 | 0.0 |

* Relative to expression of HT29; SEM, standard error of the mean

**Figure 3.** Expression of CDX2 transcripts in colon, normal lung, and lung cancers. CDX2 transcript levels correlated well with protein expression, evaluated by immunohistochemistry



| Score | n= | 0 | 1+ | 2+ | 3+ | 4+ |
|---|---|---|---|---|---|---|
| TC | 6 | 6 | 0 | 0 | 0 | 0 |
| AC | 2 | 1 | 0 | 1 | 0 | 0 |
| LCNEC | 8 | 5 | 1 | 0 | 2 | 0 |
| SCLC | 16 | 13 | 2 | 0 | 1 | 0 |

TC, typical carcinoid; AC, atypical carcinoid;
LCNEC, large cell neuroendocrine carcinoma;
SCLC, small cell lung cancer

**Figure 4.** CDX2 expression in a spectrum of pulmonary neuroendocrine tumours. Most of the CDX2-positive tumours were high-grade neuroendocrine tumours (large cell neuroendocrine tumors and small cell lung cancers). Six of the 24 tumours (25%) were positive for CDX2. A representative picture scoring 3+ is shown in the upper panel

tissue-specific expression of TTF-1 [30] and MGB1 (unpublished observations) in peripheral lung and breast, respectively. Unexpectedly, both molecules were expressed in pulmonary high-grade neuroendocrine carcinomas. Similar findings were obtained by others [31,32]. Furthermore, ectopic expression of c-kit and the stem cell factor is common in small cell lung carcinomas [33,34]. We suspect that aberrant expression of these molecules is related to the nature of this tumour, which is characterized by its undifferentiated or primitive morphology. Shamblott *et al* reported that human pluripotent stem cells derived from primordial germ cells simultaneously express markers of neural, vascular, haematopoietic, muscle, and endodermal lineages [35]. This is not an artefact of culture conditions, because Akashi *et al* have reported similar multi-lineage expression in haematopoietic stem cells, which were freshly prepared using Dynabeads and a fluorescence-activated cell sorter [36]. Expression of lineage-specific molecules in pulmonary high-grade neuroendocrine tumours may have some association with the multi-lineage gene expression of stem cells.

The frequency of *K-ras* mutation in the present study (14%) appeared to be low compared with that of pervious studies, in which the mutation was detected in about 30–40% of lung adenocarcinomas. This low frequency is not due to our RNA-based analysis, because *p53* mutation was detected with a prevalence similar to previous studies using the same methods and the same RNA samples (data not shown). There may be ethnic or geographical differences in the prevalence of *K-ras* mutation, because Hunt *et al* reported an increased prevalence of the mutation in African-Americans around the Mississippi river [37], and a low frequency of *K-ras* mutation in Japanese subjects has been reported previously by us [38] and in other Japanese studies [3,39].

In summary, we have demonstrated that there is a subset of lung adenocarcinomas that frequently show expression of CK20 and CDX2, *K-ras* mutations, and/or goblet cell morphology. These phenotypes are commonly observed in other organs, suggesting that adenocarcinomas with these features are one of the prototypes that are independent of the organ of origin. The study also calls the attention of surgical pathologists to CDX2 expression in lung adenocarcinomas. Whereas CDX2 expression is very high and uniform in colorectal carcinomas, as reported previously, some lung adenocarcinomas may express CDX2. In this case, CK20 also tends to be positive, so that the differential diagnosis should be integrated with other findings. In addition to lung adenocarcinomas, pulmonary high-grade neuroendocrine tumours may also be positive for CDX2.

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ORIGINAL PAPERS

# Maspin expression in normal lung and non-small-cell lung cancers: cellular property-associated expression under the control of promoter DNA methylation

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**Maspin has been demonstrated to be a suppressor of invasion and cell motility *in vitro*, whereas *in vivo* analyses have reported that increased expression of maspin is associated with malignant behavior. The present study examined maspin expression in normal lung and non-small-cell lung cancers. Only proximal airway cells in the normal lung expressed maspin, and the expression was associated with decreased methylation. This association was also observed in non-small-cell lung cancers, but the expression was quite different among histologic subtypes; 20 of 21 squamous cell carcinomas showed intense, uniform expression, whereas the expression status varied among adenocarcinomas. Of the 119 adenocarcinomas, 60 were negative, 23 positive and 36 showed a heterogeneous expression pattern. The expression was inversely correlated with markers of peripheral airway cells. Taken together, the results suggest that maspin may be expressed in association with the proximal airway cell type. It is of note that the heterogeneous expression pattern of maspin is quite distinctive, showing geographic positivity in the individual tumors. Separate analysis of methylation status in positive and negative portions of individual tumors provided an instance of intratumor diversity associated with promoter DNA methylation.**
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## Introduction

The mammary serine protease inhibitor maspin was first isolated by Zou *et al.* (1994) as a defective molecule in breast carcinoma cells by differential display analysis. Maspin exhibits significant homology to the serpin superfamily of serine protease inhibitors, which includes the plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2), and α1-antitrypsin. Recent analyses *in vitro* have suggested an inhibitory effect on tumor invasion and metastasis. Cell motility and invasion are inhibited with transfection of the maspin gene into cancer cell lines, and the transplantation of the transfectants in nude mice led to reduced tumorgenicity and decreased metastatic potential (Sheng *et al.*, 1994; Zou *et al.*, 1994; Sheng *et al.*, 1996; Zou *et al.*, 2000). The mechanism underlying maspin's inhibitory activity remains controversial, but recent reports suggested that it does not directly inhibit matrix-degrading proteases, but rather functions as a regulator of plasminogen-tissue-type plasminogen activator complex (Bass *et al.*, 2002). On the other hand, it has been shown that maspin has two consensus p53-binding sequences in its promoter region, and p53 regulated maspin expression, indicating that maspin is one of the target genes of p53 pathway (Zou *et al.*, 2000). Furthermore, maspin was shown to have an inhibitory effect on tumor angiogenesis (Zhang *et al.*, 2000) and a sensitizing effect on apoptosis (Jiang *et al.*, 2002). Despite a tumor suppressing role *in vitro*, clinicopathological analysis using *in vivo* tumors failed to demonstrate the role, and maspin-expressing tumors tend to show more malignant behavior, including shorter survivals, in breast cancers (Umekita *et al.*, 2002; Bieche *et al.*, 2003), pancreatic cancers (Maass *et al.*, 2001) and ovarian cancers (Sood *et al.*, 2002).

Promoter DNA hypermethylation is one of the epigenetic mechanisms to silence certain genes. So far, a number of genes have been shown to be inactivated by this gene silencing (Jones and Baylin, 2002; Laird, 2003), whereas the silencing was predominantly reported in the cancer tissues, but not in normal tissues. Although the involvement of DNA methylation in X-chromosome inactivation (Mohandas *et al.*, 1981) and genomic imprinting (Li *et al.*, 1993) has been widely accepted, tissue-specific regulation of gene expression in normal tissues mediated by DNA methylation has long been speculated. Recently, Futscher *et al.* (2002) first demonstrated that the tissue-specific expression of maspin was

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controlled by DNA methylation. They described a close correlation between maspin expression and the absence of DNA methylation using various normal tissues, and this expression in immortalized cells was restored by treatment with 5-aza-2′-deoxycytidine. In addition to normal tissues, the gene silencing of maspin was observed in breast cancers (Domann *et al*., 2000; Maass *et al*., 2002), suggesting a contribution to breast carcinogenesis.

Lung cancers, especially adenocarcinomas, are characterized by a high degree of morphological heterogeneity, which in turn implies both intra- and intertumor diversities. We have been interested in and have analysed the diversities. Recently, we revealed that thyroid transcription factor-1, TTF-1, serves as a lineage marker for peripheral airway cells, including type I and II pneumocytes (Yatabe *et al*., 2002). Furthermore, analysis of various cancer-associated genes, including p53 (Nishio *et al*., 1997), cyclin D1 (Nishio *et al*., 1997), RB (Nishio *et al*., 1997), $p27^{Kip1}$ (Yatabe *et al*., 1998b) and COX1 (Yatabe *et al*., 1998a; Achiwa *et al*., 1999), suggests a different molecular pathway for carcinogenesis in lung adenocarcinomas between cells with and without TTF-1 expression. This result implies that one of the intertumor heterogeneities of lung adenocarcinoma is represented by putative original cells, that is, peripheral airway cell-derived carcinomas and the others. This distinction is supported by the expression profiling analysis (Bhattacharjee *et al*., 2001; Garber *et al*., 2001). Unsupervised hierarchical clustering, based on the molecular signature, classified lung adenocarcinomas largely into two subtypes, which were delineated by TTF-1. The present study also focused on the diversities. First, we confirmed cell-type-specific expression of maspin, and then we examined lung tumors, revealing that maspin is expressed in a highly heterogeneous fashion in lung adenocarcinomas, similar to the morphology. We found that the expression of maspin is associated with cell type in lung tissue, while intratumor diversity of maspin is associated with regional promoter hypermethylation. There are few references in the literature concerning DNA methylation associated with the intratumor diversity (Graff *et al*., 2000; Nass *et al*., 2000; Markl *et al*., 2001), and this study therefore provides new information regarding maspin expression, which might shed light on the complex mechanism of the metastatic process.

## Results

### *Cell-type-specific expression of maspin in the normal lung*

Tissue-specific expression, including airway epithelium, has been previously reported (Futscher *et al*., 2002), but there are various types of the epithelium covering airway tracts. Therefore, we further examined which cell types in the airway epithelium expressed maspin. Immunohistochemical analysis demonstrated a characteristic expression pattern, and this expression was restricted to the basal cells of the bronchial epithelium (Figure 1 (a1)) and myoepithelium of the bronchial glandular acini (Figure 1 (a2)). This was in sharp contrast to the peripheral portion of the lung parenchyma; none of the cells in the peripheral lung, such as type I and II pneumocytes, were positive for maspin (Figure 1 (a4)). In the bronchiolar cells that connect the bronchus to the peripheral lung, a number of maspin-expressing cells were decreased (Figure 1 (a3)).

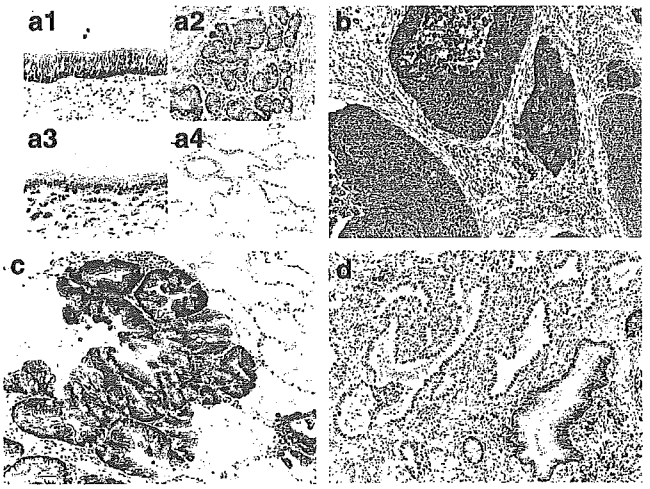


**Figure 1** Maspin expression in normal lung tissue (**a**) from a proximal airway (a1) to peripheral lung parenchyma (a4). Basal cells of the bronchial surface epithelium (a1), and myoepithelium of the bronchial glands (a2), expressed maspin. Positive signal frequency was reduced as being close to the alveolar spaces (bronchioles, A3), and no expression was detected in the alveolar spaces (a4). Squamous cell carcinoma (**b**) demonstrates intense, uniform expression, whereas the expression was varied among adenocarcinomas. A similar positive pattern was seen in mucinous bronchioloalveolar carcinoma (**c**). However, an ordinary peripheral type of adenocarcinoma is completely negative for maspin despite the positive internal control for bronchial epithelium (**d**)

### *Maspin expression in lung cancers*

Similar to the normal lung, a cell-type-specific pattern of maspin expression was observed in non-small-cell lung cancers (Table 1). All of the squamous cell carcinomas, except a single case, expressed maspin intensively and very uniformly (Figure 1b), whereas adenocarcinomas demonstrated a variety of expression patterns (Figure 1c and d). Of the 119 adenocarcinomas, 60 (50.4%) were negative for maspin, while 23 (19.3%) demonstrated a uniform, and 36 (30.3%) a heterogeneous expression pattern for maspin. The heterogeneous expression pattern of maspin was quite distinctive, and details are discussed later.

Columnar cell-specific expression in normal lung tissue prompted us to explore the biological significance of maspin expression in pulmonary adenocarcinomas. First, we examined the clinicopathological characteristics of the maspin-positive adenocarcinoma, which is summarized in Table 2. Although maspin expression was not associated with sex, smoking status or

pathological stage, maspin expression was more frequently observed in advanced local tumor status (pT). In the scheme of lung cancer staging classification, pT is determined by tumor size and an extent of local invasion. Individual analysis of tumor size and local invasiveness revealed that the correlation of maspin expression with pT was primarily dependent on the prevalence of maspin expression in larger tumors, but not that of locally invasive tumors. In addition, the presence of lymph node metastasis was independent of maspin expression.

We have recently proposed the subclassification of lung adenocarcinoma into two major subtypes of peripheral airway adenocarcinoma and the remainder, which are delimited by TTF-1. Interestingly, maspin expression is inversely correlated with TTF-1 expression, being consistent with proximal airway-specific expression of maspin in the normal lung. This was confirmed by the expression status of surfactant precursor protein B, which is known as a differentiation marker for pneumocytes. p53 alteration is frequent in maspin-expressing adenocarcinomas, and is also consistent with less-frequent p53 alteration in TTF-1-positive adenocarcinomas. Therefore, this suggests that maspin is expressed in association with cellular type of the nonperipheral airway epithelium even after malignant transformation. Selective expression of maspin in normal proximal airway cells suggests that maspin may be expressed in association with proximal airway cell type. The K-*ras* mutation is also prevalent in maspin-expressing adenocarcinomas.

**Table 1** Maspin expression of non-small-cell lung cancers

| | *Maspin expression* | | |
|---|---|---|---|
| | *Mostly negative* | *Heterogeneous* | *Mostly positive* |
| Adenocarcinoma | 60 | 36 | 23 |
| Squamous cell carcinoma | 0 | 1 | 20 |
| Large cell carcinoma | 0 | 1 | 2 |
| Adenosquamous ca. | 0 | 0 | 1 |

*Heterogeneous expression of maspin in lung cancers*

During the examination, we found that the heterogeneous pattern of maspin expression was distinct from the common expression pattern; the expression pattern was very geographic, that is, a part of the tumor intensely expressed maspin, but the other was completely negative (Figure 2). In 36 of the 119 adenocarcinoma cases examined, maspin expression varied among the tissue-microarrayed cores in individual tumors. Therefore, we further analysed with regular whole sections, and confirmed the geographic expression of maspin in 36 adenocarcinoma cases. This heterogeneous expression appears to be inconsistent with the idea of proximal airway-cell-type-associated expression of maspin, because characteristic uniform expression of TTF-1 was observed in 25 of 36 adenocarcinomas with heterogeneous maspin expression (Table 2). On this matter, following two points were addressed. First, we examined the expression status of maspin in preneoplastic lesions and *in situ* carcinoma of a peripheral type of adenocarcinoma. All five atypical adenomatous hyperplasia and five nonmucinous bronchioloalveolar carcinomas (carcinoma *in situ*) were positive for TTF-1, but negative for maspin. Second, clinicopathological characteristics of the heterogeneous expression among the TTF-1-positive, peripheral airway cell-associated adenocarcinomas were examined. Heterogeneous expression was associated with higher histologic grade ($\chi^2$ test, $P=0.02$) and lower frequency of surfactant expression (Fisher's exact test, $P=0.03$), suggesting that maspin heterogeneity was prevalent in less-differentiated tumors in the category of peripheral airway cell-associated adenocarcinomas. These findings imply that maspin should be considered as a differentiation marker rather than a lineage marker.

Characteristics of maspin-positive portions in the individual tumors were then analysed. Although there was some unexpected expression, two morphologically characteristic portions tended to be positive for maspin: a portion showing solid growth pattern (Figure 2b), and a portion with infiltrating cancer cells in small clusters (Figure 2d). Some adenocarcinomas contained either solid or infiltrating patterns, while the other

**Table 2** Clinicopathologic features of adenocarcinoma in relation to maspin expression

| | *Maspin expression* | | | |
|---|---|---|---|---|
| | *Negative* | *Heterogeneous* | *Positive* | P-*value* |
| *n* | 60 | 36 | 23 | |
| Female/male | 35/25 | 16/20 | 11/12 | 0.38 |
| Smoker/nonsmoker | 33/27 | 16/20 | 7/16 | 0.12 |
| pStage (Stage I/>Stage I) | 40/20 | 18/18 | 15/8 | 1.00 |
| pT1/>pT1 | 32/28 | 10/26 | 7/16 | 0.02 |
| Size $\leqslant$30 mm/>30 mm | 44/16 | 18/18 | 9/14 | <0.01 |
| Local pleural invasion | 41/19 | 19/17 | 16/17 | 0.25 |
| pN0/>pN0 | 41/19 | 23/13 | 19/4 | 0.30 |
| TTF-1 +/− | 52/8 | 25/11 | 4/19 | <0.01 |
| SPPB +/− | 46/14 | 16/20 | 4/19 | <0.01 |
| p53 wild type/mutated | 38/18 | 25/10 | 8/13 | 0.03 |
| K-*ras* wild type/mutated | 53/2 | 32/3 | 15/6 | <0.01 |

simultaneously had both patterns. It is of note, however, that maspin expression was not always associated with these features of tumor growth, in that not all of the invasive portions and/or solid portions were positive for maspin (Figure 3). The portions with and without the expression were morphologically indistinguishable. The findings raised a question about which portion was associated with metastasis. We therefore compared the expression pattern between primary and metastatic sites in order to ascertain the role of maspin in metastasis. Of the 36 adenocarcinomas with heterogeneous expression, 13 tumors metastasized to the lymph nodes, with only 12 able to be examined. The results are summarized in Figure 3, and the expression status in half of the 12 tumors was decreased compared to that of the corresponding lymph nodes.

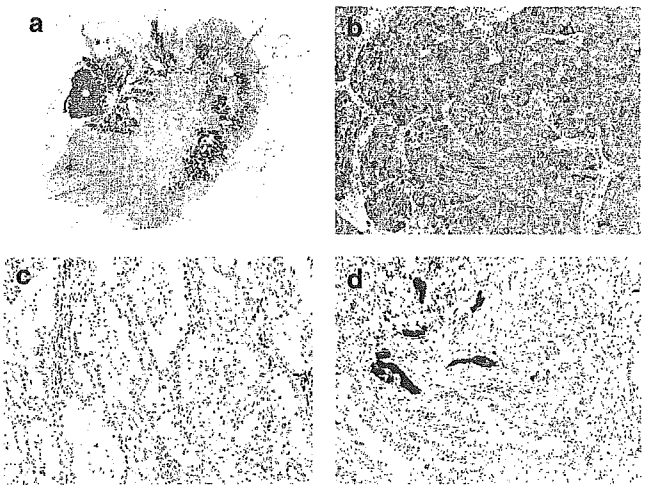


**Figure 2** (**a**) Representative case with heterogeneous expression of maspin. Gross appearance demonstrates a characteristic geographic pattern of expression (**a**). The solid growth area shows intense, uniform positivity for maspin (**b**) and the tubular growth area is completely negative (**c**). In part, infiltrating cancer cells in small clusters are intensely positive for maspin (**d**)

*Maspin expression associated with promoter DNA methylation*

The relationship between the expression and methylation status was examined in lung cancer cell lines. The promoter region of the maspin gene was densely methylated in five of 13 cell lines (Table 3 and Figure 4). All of the five cell lines showing dense promoter methylation were shown to be negative for maspin expression using real-time PCR. Conversely, all of the cell lines without detectable transcripts demonstrated promoter methylation, except for two cell lines, ACC-LC-172 (SCLC) and VMRC-LCD (adenocarcinoma).

In the normal tissues, no expression was observed in the peripheral lung and, consistently, the promoter region of the maspin gene in the tissues was densely methylated. Methylation of the six peripheral lung tissues reached 89.6% on average. Similarly, 91.5% of CpG sites examined were methylated in lymph nodes, where no maspin expression was detected. In contrast, percentage methylation of microdissected bronchial

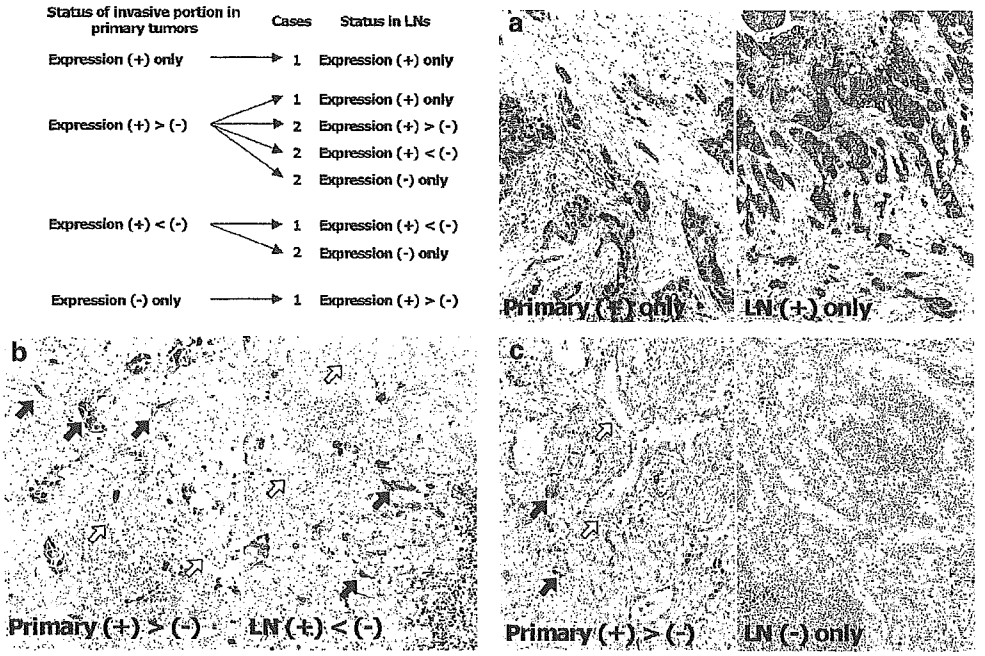


**Figure 3** Summarized results of the comparison of expression status between primary and metastatic sites in cases with heterogeneous expression and lymph node metastasis. Half of the tumors showed an identical or increased expression pattern between primary and metastatic sites, whereas decreased expression in metastatic sites was observed in the other half. Three representative pictures (**a–c**) of maspin expression in primary tumors (left, labeled as primary) and the corresponding lymph nodes (right, labeled as LN) are displayed. Arrows indicate maspin-positive (closed) and -negative (open) tumor cells

**Table 3** Summary of methylation status with bisulfite genomic sequencing

| | N | *Expression* | *% Methylation* |
|---|---|---|---|
| *Cell lines*[a] | | *Maspin mRNA/18s rRNA* | |
| Small cell carcinoma | 3 | 0/2/0[b] | 0/0/100[c] |
| Adenocarcinoma | 4 | 232/29/17/1 | 0/0/0/0 |
| Adenocarcinoma | 2 | 0/0 | 95/0 |
| Squamous cell carcinoma | 3 | 0/0/11 | 90/98/0 |
| Large cell carcinoma | 1 | 0 | 58 |
| *Normal tissues* | | *IHC results* | |
| Peripheral lung | 6 | Almost negative | 85/88/88/90/92/95 |
| Bronchial epithelium | 2 | Positive in basal cells | 32/43 |
| Lymph node | 2 | Completely negative | 91/92 |
| *Tumors* | | *IHC results* | |
| Squamous cell carcinoma | 6 | Mostly positive | 1/1/1/2/3/4 |
| Adenocarcinoma | 6 | Mostly positive | 0/0/1/7/23/18 |
| Adenocarcinoma | 7 | Mostly negative | 0/34/35/46/65/91/98 |
| Adenocarcinoma | 3 | Positive portion | 23/1/37[d] |
| | | Negative portion | 46/39/71 |

[a]Cell lines used include ACC-LC172, ACC-LC80, SK-LC2 for SCLC, SK-Lu-1, ACC-LC319, RERF-LC-MT, SK-LC3 for AD-mRNA positive, ACC-LC94, VMRC-LCD for AD-mRNA negative, RERF-LC-AI, SK-MES1, QG56 for squamous cell carcinoma, and Calu6 for large cell carcinoma. [b]In order corresponding to that listed in footnote a. [c]In order corresponding to that in column expression. [d]Positive and negative portions are separately displayed from case 1–3

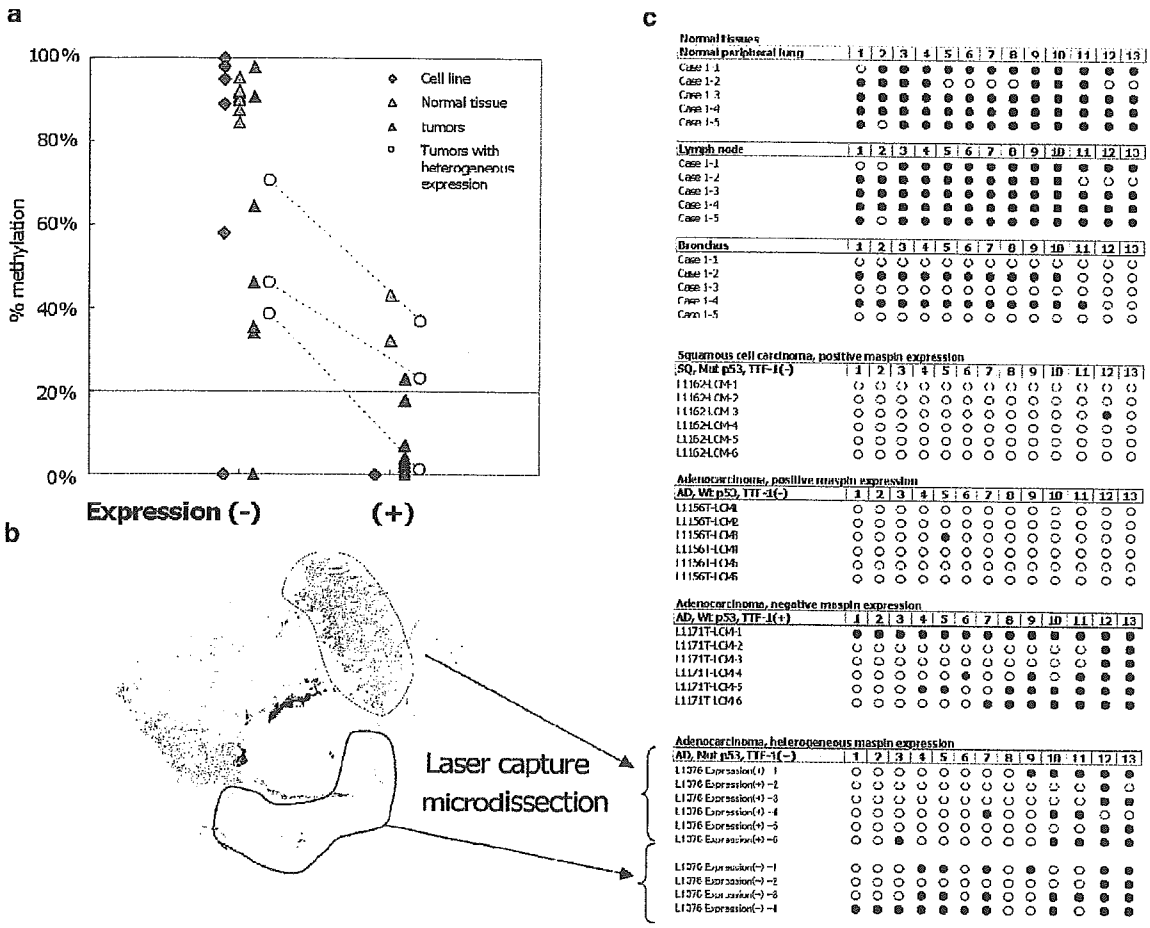


**Figure 4** Relationship between promoter DNA methylation and expression status in normal lung tissue, lung cancer cell lines and non-small-cell carcinoma *in vivo* (**a**). Lines in the chart between positive and negative expression indicate the shift in methylation patterns between positive and negative portions in individual cases with heterogeneous expression, which were examined separately. A representative frozen section examined is shown (**b**). Positive and negative portions were separately microdissected and examined. Representative results are listed in (**c**)

epithelium from two individuals was 32.3 and 43.1%. Bronchial epithelium was only a part of the lung tissue that expressed maspin, but the expression was limited to the basal layer. The whole layer of bronchial epithelium was able to be microdissected, and thus, the results represented the methylation status of mixed bronchial epithelium with and without maspin expression. These results indicated that methylation and expression are well correlated, suggesting cell-type-associated promoter DNA methylation.

We then examined the correlation of expression and methylation status in tumors *in vivo* (Table 3). As in normal tissue, six of the squamous cell carcinoma and six adenocarcinomas with uniform maspin expression had almost no methylated CpG sites in the promoter region of maspin gene (2.0 and 8.2% on average, respectively). Methylation patterns in seven adenocarcinomas without maspin expression were contrasted, and the promoter region was methylated in six of the seven tumors. In three adenocarcinomas with heterogeneous expression, portions with and without maspin expression were separately microdissected, and the methylation status was independently accessed. The portions without detectable maspin expression contained a more intensely methylated promoter than that with maspin expression (Figure 4). The results suggested that the expression of maspin was correlated with methylation status both between and within tumors.

## Discussion

The current study demonstrated that maspin is not expressed in the lung parenchyma, but specifically in bronchial epithelium, of which basal cells were the predominant source of expression. As promoter DNA methylation in the bronchial epithelium was contrasted to that in lung parenchyma, this suggests that the expression is associated with promoter DNA methylation. This observation extends the finding of Futscher *et al.* (2002) that promoter hypermethylation plays a role in the maintenance of cell-specific expression. Differences in whole genomic methylation patterns among various tissues with restriction landmark genomic scanning (Kawai *et al.*, 1993) also supports the hypothesis.

It has been widely accepted that promoter DNA hypermethylation is one of the mechanisms that inactivate tumor suppressor gene. Although maspin is frequently documented to function as a tumor suppressor *in vitro*, current work suggests that the expression status of maspin in non-small-cell lung cancers is reflected in its expression in corresponding normal lung tissues. Selective expression of maspin in normal proximal airway cells suggests that maspin may be expressed in association with proximal airway cell type. Indeed, uniform expression of maspin in squamous cell carcinoma is consistent with the idea that squamous cell carcinoma is likely to be derived from proximal airway cells through the metaplasia–dysplasia–carcinoma sequence. We have recently reported that TTF-1 is useful for the distinction of peripheral airway cell-derived adenocarcinomas (Yatabe *et al.*, 2002). The expression status of TTF-1 is inversely correlated with that of maspin in adenocarcinomas, suggesting that maspin expression is not associated with the role of tumor suppressor, but that simply is a reflection of the cell type of the proximal airway cells. This may explain the discrepancies between several reports examining tumors *in vivo* and suggesting a role *in vitro*. For example, in breast cancers, two articles have reported that maspin expression is associated with a higher histological grade, lack of estrogen receptor expression and poor prognosis (Umekita *et al.*, 2002; Bieche *et al.*, 2003). Nevertheless, breast cancer cell lines, which were transfected with the maspin gene, suppressed motility and invasion *in vitro* (Zou *et al.*, 1994; Sheng *et al.*, 1996). Linkage of the discrepant findings may be present in the fact that maspin expression is restricted to the basal/myoepithelium of the ducts in the normal breast. According to the molecular profiles, cDNA microarray analysis subdivides breast cancers into three subtypes, which include luminal, basal and ERBB2$^+$ types (Perou *et al.*, 2000; Sorlie *et al.*, 2003). One of the subtypes, the basal cell type, is characterized by (1) high expression of the basal cell markers, cytokeratin 5 and 17; (2) negative expression of estrogen receptor and progesterone; and (3) clinical aggressiveness. Although the expression status of maspin is not available in the classification, the characteristics of this basal cell type resembles those of maspin-expressing tumors, suggesting that maspin expression in breast cancers may also be simply reflected in certain cellular properties rather than tumor-suppressive function of maspin. This hypothesis does not deny the finding observed *in vitro* and, indeed, maspin expression was decreased in metastatic cancer cells in the lymph nodes, as shown in this study.

Another point of interest is that intratumor heterogeneity is partly mediated by promoter DNA methylation. We have previously reported that the allelic loss of 2q, 9p and 22q, which are associated with the advanced stage of tumors, varied in individual tumors, and that the diversity is related to morphological tumor grade (Yatabe *et al.*, 2000). The present study revealed that gene silencing by DNA methylation is another contributor to intratumor heterogeneity. In general, DNA methylation is stable and inheritable over cell divisions. However, selective pressure during the tumor progression may alter the pattern. Indeed, a reversible shift in methylation pattern between monolayer and spheroid culture has been reported (Graff *et al.*, 2000). A recent article by Kang *et al.* (2003) proposed the hypothesis that metastasis is enhanced by intratumor clonal diversity superimposed on a background of poor-prognosis signature property.

Infiltrating cancer cells to the stroma in small clusters are conceivable as a source of metastasis. In the current study, when the expression status is compared between the clusters and metastatic cancer cells, decreased expression is observed in half of the cases with heterogeneous expression and lymph node metastasis.

This is compatible with the results *in vitro* that maspin functions as an invasion suppressor. However, in another comparison with a low-grade lesion and the infiltrating portion of individual tumors, the infiltrating portion expressed maspin. These findings appear to be in conflict. A similar complex issue was addressed by Graff *et al.* (2000). E-cadherin is known to be heterogeneously expressed throughout all stages of malignant progression, including primary and metastatic tumors. They revealed a drift in methylation pattern of E-cadherin between monolayer and spheroid culture *in vitro*, suggesting a dynamic, reversible process of methylation in tumors. Based on the results, they speculated that a portion of tumor cells with decreased E-cadherin expression preferentially infiltrate to the stroma and metastasize to the lymph nodes. This is followed by an expansion of the E-cadherin-expressing portion in lymph nodes to survive in the microenvironment, because restoration of E-cadherin facilitates proliferation and cell survival through cell-to-cell interaction (Day *et al.*, 1999). Thus, the status in lymph nodes appears heterogeneous. Maspin may follow a similarly complex scenario. Alternatively, different processes may be involved between heterogeneous maspin expression in tumors and decreased expression in lymph nodes. Preferential expression of maspin in high histological grade tumors and in solid/infiltrating portions in individual tumors suggests the following hypothesis. Generally, maspin is expressed in accordance with the cell type. As tumors progress, the methylation maintenance system is impaired, and it alters some expression, including maspin, that results in increased heterogeneity in the individual tumors. Then, metastatic clone(s) is selected among the heterogeneous cell population according to its metastatic potential, and metastasizes to lymph nodes. This explains the findings obtained, and lack of expression in preneoplastic and *in situ* lesions of the peripheral type of adenocarcinoma supports the hypothesis.

Maspin is one of the target genes of the p53 pathway. It has been shown that mutated p53 lacks an ability to induce maspin (Zou *et al.*, 2000), and that restoration of wild-type p53 and inhibition of DNA methylation by 5-aza-doxycytidine treatment reactivate maspin expression (Oshiro *et al.*, 2003). In the current study, correlation between maspin expression and p53 status in lung cancers was not clear, and the expression status of maspin was not affected by p53 status in cases without promoter methylation. This implies that cell-type-specific expression of maspin might function differently from induced maspin that is mediated by p53. However, two cell lines and a case of adenocarcinoma, showing a negative expression of maspin (despite no promoter hypermethylation), exhibited mutated p53. p53 may modulate the expression in some occasions. In contrast, K-*ras* status was associated with maspin expression in lung adenocarcinoma. It has been reported that mucinous bronchioloalveolar carcinoma (BAC) preferentially harbors a K-*ras* mutation (Tsuchiya *et al.*, 1995; Marchetti *et al.*, 1996). Mucinous BAC is composed of neoplastic cells resembling goblet cells, which are present in the proximal airway tracts in the normal tissue. Although the mucinous BAC spreads over the peripheral lungs in a manner similar to lepidic growth, a marker of peripheral airway cells, TTF-1, is mostly negative (Goldstein and Thomas, 2001; Lau *et al.*, 2002), suggesting proximal airway-associated tumors of mucinous BAC. Therefore, prevalence of K-*ras* mutations in maspin-positive adenocarcinoma might represent maspin expression in association with cell type of proximal airway cells.

It is of no doubt that the accumulation of genetic and epigenetic alterations generates a tumor. The alterations are not always the same among a certain group of tumors. Indeed, a metastatic tumor is categorized as being adjacent to the primary tumor among any other tumors with unsupervised hierarchical clustering of expression profiles. The current study used a cross-sectional analysis to illustrate the difference in methylation patterns in individual tumors. The analysis gives the ability to reveal real differences independent of the background of the alterations. The analysis can be applied to various methods, including chromatin immunoprecipitation and expression profile analysis, and the results may shed light on complex phenomena in tumors *in vivo*.

## Materials and methods

### *Patients*

A series of 145 consecutive, non-small-cell carcinoma cases presenting between September 2000 and December 2002 at the Department of Pathology and Molecular Diagnostics, Aichi Cancer Center, Nagoya, Japan were used for the present study. In addition, five cases of atypical adenomatous hyperplasia and five cases of nonmucinous BAC were examined to determine the expression status in premalignant and *in situ* neoplasia. Pathological staging was determined according to the AJCC Cancer Staging Manual (Greene *et al.*, 2002).

### *Tissue microarray*

In order to represent a whole tissue, four regions were selected per tumor, and tissue microarrays were constructed with an MTA-1 manual tissue arrayer (Beecher Instruments, Inc., Silver Spring, MD, USA). Briefly, selected regions of the donor paraffin block were punched with a 0.6 mm core needle, transferred and arrayed in the recipient block using the arrayer. Serial 4 $\mu$m thick sections were then placed on coated glass slides for immunohistochemical analysis.

### *Immunohistochemistry*

Immunohistochemical examination was performed with the standard avidin–biotin–peroxidase complex method using the monoclonal antibodies against maspin (G167-70, BD Bioscience Pharmingen, San Diego, CA, USA), TTF-1 (8G7G3, DAKO, Copenhagen Denmark) and surfactant precursor protein B (19H7, Novocastra, Newcastle upon Tyne, UK). Antigens were retrieved by autoclave. Some of the tissue cores, which were missing during the procedure, or unable to be evaluated, were excluded from the analysis. Each core stained was evaluated semiquantitatively for the following criteria. A greater than moderate intensity of signal was