Table IV. Frequency of the *Pas1* haplotypes in cases and controls

Haplotype	Polymorphism					Frequency (95% CI)					
	KRAS-1	KRAS-6	CASC1-1	CASC1-4	CASC1-5	Control	ADC				
							All	AAH absent	AAH present		
									All	1 AAH	≥2 AAHs
1	A	—	A	Arg	T	0.78 (0.75–0.82)	0.75 (0.72–0.78)	0.76 (0.73–0.80)	0.71 (0.64–0.78)	0.76 (0.67–0.84)	0.65 (0.53–0.76)
2	C	T	G	Ser	C	0.10 (0.07–0.12)	0.12 (0.10–0.14)	0.11 (0.08–0.14)	0.15 (0.10–0.21)	0.12 (0.06–0.18)	0.21 (0.11–0.30)
3	A	T	G	Ser	C	0.09 (0.07–0.12)	0.10 (0.07–0.12)	0.10 (0.07–0.12)	0.09 (0.05–0.13)	0.09 (0.03–0.14)	0.09 (0.02–0.15)

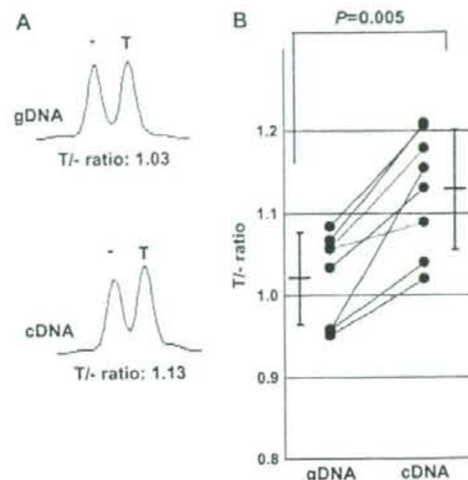

Fig. 2. Differential messenger RNA expression between two polymorphic *KRAS* alleles. (**A**) Electrophoregram for genomic DNA (gDNA) and cDNA of a representative case. Relative amounts of PCR products from the minor (T) allele to those from the major (−) allele are shown below as T/− ratios. (**B**) Relative amounts of PCR products from the minor allele to those from the major allele in genomic DNA and cDNA of non-cancerous lung tissues of eight KRAS-6 hetrozygotes. Mean ± SD for the relative amounts in genomic DNA and cDNA is also indicated with a *P* value by the paired *t*-test.

probably that *Casc1* contributes to lung ADC/adenoma susceptibility by expressing polymorphic proteins with differential activity. This result is in contrast to the case of the *Kras* gene, for which polymorphisms associated with amino acid substitution have not been found, while differential expression between polymorphic alleles was observed (18). Interestingly, the human *CASC1* gene also has a polymorphism with amino acid substitution, CASC-4. Therefore, it is possible that the polymorphism also causes a difference in the activity of CASC1 protein as in the case of mouse Casc1 protein. Thus, the functional significance of *CASC1* SNPs should be further investigated both on expression level and protein activity to clarify the involvement of the *CASC1* gene in ADC/AAH susceptibility, and such a study is in progress in our laboratory.

The present study indicated that *KRAS/Kras* polymorphisms are involved in the susceptibility to lung tumor development by causing differential expression levels of the *KRAS* oncogene, not only in mice but also in humans. Thus, a further study should be done to elucidate molecular mechanisms underlying the differential expression between susceptible and resistant *KRAS/Kras* alleles. In the present study, transcripts from the minor allele for the KRAS-6 polymorphism were shown to be more abundant than those from the major allele. However, it remains unknown whether this polymorphism is responsible for differential expression or not. The KRAS-6 polymorphism is located in a 94 kb LD region covering introns and the 3′-UTR of the *KRAS* gene (Figure 1), and 10 of other polymorphisms have been identified in this region. Notably, the ratios of the *KRAS* transcripts between the major and minor alleles for the KRAS-6 polymorphism were different among the eight cases examined; therefore, it is possible that *KRAS* expression is affected by several polymorphisms. Introns and the 3′-UTR of the *KRAS/Kras* gene contain several genomic segments with significant homologies between humans and mice (http://genome.ucsc.edu/). Thus, it is possible that such segments have common functions in the expression of *KRAS/Kras* gene, and therefore, polymorphisms in these regions are responsible for the differential levels of *KRAS/Kras* gene expression. Notably, genomic fragments of 50 bp in size encompassing the KRAS-1 and -6 polymorphisms, which showed associations with risk for ADC accompanied by multiple AAHs, did not show significant homologies with the

mouse genome. Thus, these two polymorphisms are unlikely to be responsible ones. Further functional and genetic studies on *KRAS* will give us more critical information on the involvement of *KRAS* in lung tumorigenesis in humans.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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Histological Evaluation of the Effect of Smoking on Peripheral Small Adenocarcinomas of the Lung

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Introduction: As there is little information on the histologic characteristics of adenocarcinoma in smokers, we histologically examined the effect of smoking on the carcinogenesis and progression of peripheral small lung adenocarcinomas.

Methods: Two hundred thirty-six consecutive patients with peripheral adenocarcinoma of the lung 30 mm or less in diameter were studied. Prognosis, histology, and location of the adenocarcinoma were compared among patients with a Brinkman index (B.I.) of 0, 1 to 500, and more than 500.

Results: The ratio of smokers to nonsmokers was 1.4:1. The rate of carcinogenesis in the upper region of the lung (S1-3) was 1.4 times as high that in the lower region (S4-10) in smokers, but almost equal in the two regions in nonsmokers. Outcome tended to be worse in patients with a B.I. of more than 500 than in those with a B.I. of less than or equal to 500 for adenocarcinomas 30 mm or less in diameter (p 0.0855), and was significantly worse for adenocarcinomas 20 mm or less in diameter (p 0.0359). Patients with a high B.I. tended to have invasive adenocarcinoma (IAC) without a bronchioloalveolar carcinoma (BAC) component (IAC BAC) or IAC with a BAC component (IAC BAC) rather than noninvasive adenocarcinoma. For adenocarcinomas as a whole, B.I. was correlated with several pathologic prognostic factors, including pathologic stage, lymphatic permeation, vascular invasion, presence of a solid component, necrosis, and modified scar grade, particularly in the upper region. Specifically, in IAC BAC, B.I. was correlated with modified scar grade and the presence of a solid component. In IAC BAC, B.I. was correlated with the presence of a solid component and necrosis.

Conclusions: Small adenocarcinoma in smokers seems to occur frequently in the upper region of the lung, shows invasive features more frequently, and shows greater progression and dedifferentiation than that in nonsmokers. Tobacco-smoking may have an effect on the carcinogenesis and multistep progression of peripheral lung adenocarcinoma 30 mm or less in diameter.

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Key Words: Smoking, Lung, Adenocarcinoma, Histology.

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Among several histologic types of lung carcinoma, a gradual increase in the incidence of peripheral adenocarcinoma has been reported,[1,2] although the effect of smoking on the carcinogenesis and progression of peripheral adenocarcinoma is unclear. The relative risk for lung cancer among smokers over nonsmokers is reported to be 2 to 10 for adenocarcinoma, and more than 10 for squamous cell carcinoma and small cell carcinoma.[3] Squamous cell carcinoma and small cell carcinoma tend to occur in the upper lobe,[4–8] but there have been few reported studies on the location of adenocarcinoma.[8,9]

Several studies have indicated that the prognosis of adenocarcinoma[10–12] or nonsmall nonsquamous cell carcinoma[13] in smokers is worse than that in nonsmokers. Suzuki et al.[14] and Morita and Urano[15] reported that the incidence of moderately to poorly differentiated adenocarcinoma was higher in smokers than in nonsmokers, but no detailed histologic study has yet examined the relationship between smoking status and histologic changes in these cancers.

With regard to the progression of lung adenocarcinoma, Shimosato et al.[16] and Clayton[17] indicated that bronchioloalveolar carcinoma might progress to mixed subtypes with a BAC component (sclerosing bronchioloalveolar carcinoma). On the other hand, papillary, acinar, solid adenocarcinoma, and mixed subtypes without a BAC component were considered to be de novo carcinoma,[18] or a progressed form of mixed subtypes with a BAC component.

In the present study, we pathologically examined the effect of smoking on the carcinogenesis and progression of peripheral small lung adenocarcinoma, with reference to tumor location.

PATIENTS AND METHODS

We reviewed 236 consecutive patients with peripheral lung adenocarcinomas 30 mm or less in diameter, who underwent lobectomy at the National Cancer Center Hospital between 1984 and 1990. Peripheral lung adenocarcinoma was defined as a tumor located in a fourth branching bronchus or more peripheral region. Recently, now that the prevalence of smoking is decreasing, the incidence of adenocarcinoma with a predominantly bronchioloalveolar carcinoma (BAC) com-

ponent has been increasing and that of invasive adenocarcinoma (IAC) without a BAC component (IAC BAC) has been decreasing. Therefore, to examine the relationship of smoking to invasiveness of adenocarcinoma, we selected the period when the prevalence of smoking and the incidence of overt IAC were relatively high. Clinical information was extracted from medical records. Tumor, node, metastasis staging was determined in accordance with the Union Internationale Contre le Cancer (International Union Against Cancer) staging system.[19] Tumor location was classified as the upper region (lung segments 1–3) or lower region (segments 4–10). Noninvasive adenocarcinoma (NAC) was defined as pure BAC without a desmoplastic reaction.

Survival rates of patients divided into three Brinkman index (B.I.) groups (B.I. 0, 1–500, and 500) were compared, and the distribution of NAC, IAC with a BAC component (IAC BAC) and IAC BAC in each B.I. group was analyzed. Correlations between B.I. and several pathologic prognostic factors (pathologic (p) stage, pT, pN, pleural invasion, lymphatic permeation (ly), vascular invasion (v), presence of papillary, acinar or solid component, necrosis, and modified scar grade (MSG)[20]) were examined: MSG is one of the prognostic factors for small lung adenocarcinoma, evaluated according to the structure and size of the central scar of adenocarcinoma, being a modification of the scar grade proposed by Shimosato et al.[16] When the p value was less than 0.10, the same examination was performed for tumors in the upper region and lower region, respectively.

Survival rates of each B.I. group were compared for adenocarcinoma with a BAC component (NAC and IAC BAC), IAC BAC, and IAC BAC, respectively. In IAC BAC and IAC BAC, prognostic factors that were correlated with B.I. were extracted.

The patients were followed up extensively, and the follow-up period ranged from 1 to 204 months, with a median of 84 months. Patients who died of causes other than lung adenocarcinoma were censored at the last follow-up. The 5- and 10-year survival rates were calculated by the Kaplan-Meier method. Correlations of B.I. with several pathologic factors were examined by the Mann-Whitney U test or Spearman coefficient test. When the p value was less than 0.05, differences were considered significant, and when between 0.05 and 0.10, we considered that there was a tendency for a difference.

TABLE 1. Relationship Between B.I. and Presence of a BAC Component and Invasion

Smoking Habit	B.I. 0 (n 97)	B.I. 1–500 (n 30)	B.I. > 500 (n 109)	Spearman Coefficient (ρ)
Histology				
NAC (n 28)	17 (61%)	4 (14%)	7 (25%)	0.0001
IAC BAC (n 139)	63 (46%)	20 (14%)	56 (40%)	
IAC BAC (n 69)	17 (25%)	6 (9%)	46 (66%)	

B.I., Brinkman index; BAC, bronchioloalveolar carcinoma; NAC, noninvasive adenocarcinoma; IAC BAC, invasive adenocarcinoma with a BAC component; IAC BAC, invasive adenocarcinoma without a BAC component.

RESULTS

The patients comprised 144 men and 92 women, ranging in age from 26 to 84 years with a median of 60 years. Among the 236 patients, 142 were classified as p stage IA, 8 as stage IB, 13 as stage IIA, 7 as stage IIB, 39 as stage IIIA, 20 as stage IIIB, and 7 as stage IV. Tumors were smaller than 20 mm in 112 cases and 20 to 30 mm in 124 cases. Lymph node metastasis was present in 77 cases (33%), ly in 117 cases (50%), v in 120 cases (51%), and pleural involvement (p2–3) in 49 cases (23%). Pleural involvement was defined as p0: no invasion of the visceral pleura, pl: invasion beyond the elastic framework of the visceral pleura, p2: exposure to the thoracic cavity, and p3: invasion to the parietal pleura, mediastinum, or diaphragm.

The ratio of smokers to nonsmokers was 1.4:1 (139:97 patients). Correlations between B.I. and presence of a BAC component and invasion are shown in Table 1. As the B.I. increased, NAC decreased and IAC BAC and IAC BAC increased (Table 1). The BAC component was

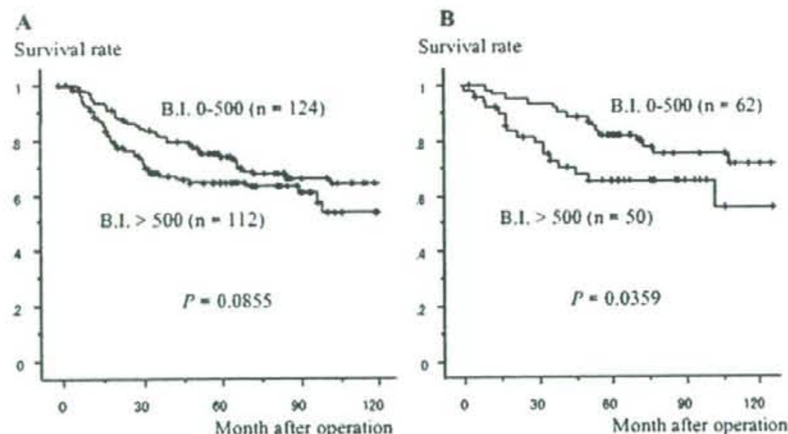

FIGURE 1. Survival curves for each B.I. group in patients with lung adenocarcinoma. *A*, Tumors 30 mm or less in diameter. *B*, Tumors 20 mm or less in diameter.

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the nonmucinous type (Clara/type II) in most cases (165/167) and mucinous type in two. The ratio of the number of tumors in the upper region to that in the lower region was 1.2:1 (129:107 tumors). A higher rate of carcinogenesis in the upper region to lower region was detected in smokers (1.4 times, 81:58 tumors), but almost equal in the two regions in nonsmokers (48:49 tumors).

The group with B.I. of more than 500 tended to have a worse outcome than the group with B.I. of less than or equal to 500 for adenocarcinomas 30 mm or less in diameter (p 0.0855, Figure 1 *A*), and had a significantly worse outcome for adenocarcinomas 20 mm or less in diameter (p 0.0359, Figure 1 *B*). The respective 5- and 10-year survival rates were 77.6 and 66.0% in the group with B.I. of 500 or less, and 66.3 and 59.6% in the group with B.I. of more than 500 for adenocarcinomas 30 mm or less in diameter. The respective 5- and 10-year survival rates were 81.5 and 73.6% in the group with B.I. of 500 or less, and 63.0 and 59.8% in the group with B.I. of more than 500 for adenocarcinomas 20 mm or less in diameter. Outcomes did not differ between men and women for adenocarcinomas 30 mm or less in diameter and 20 mm or less in diameter.

For adenocarcinoma as a whole, the correlations of B.I. with several pathologic prognostic factors we have reported

TABLE 2. Relationship Between B.I. and Clinicopathologic Prognostic Factors in Adenocarcinoma 30 mm or Less in Diameter

	No. of cases B.I. (0/1–500/>500)	5-yr Survival (%)	Mann-Whitney U Test p*	p† for Upper (U) and Lower (L) Region
Total	97/30/109	72.7		
p Stage				
Stage I	67/19/64	91.8	0.0761	U: ns
Stage II–IV	30/11/45	35.5		L: ns
pT				
T1	76/22/89	83.6	ns	
T2–4	21/8/20	33.3		
pN				
N0	71/19/69	79.6	ns	
N1–3	26/11/40	25.8		
Pleural invasion				
p 0–1	76/22/89	74.6	ns	
p 2–3	21/8/20	21.6		
Lymphatic permeation				
Negative	56/14/49	82.9	0.0486	U: 0.0848
Positive	34/17/66	41.1		L: ns
Vascular invasion				
Negative	53/13/50	75.6	0.0376	U: 0.0858
Positive	39/19/62	48.3		L: ns
Papillary component				
Absent	21/8/32	84.0	ns	
Present	76/22/77	68.7		
Acinar component				
Absent	49/15/48	79.5	ns	
Present	48/15/61	65.5		
Solid component				
Absent	73/18/52	78.5	0.0001	U: 0.0002
Present	24/12/57	62.7		L: 0.0692
Necrosis				
Absent	76/22/73	76.2	0.0544	U: ns
Present	21/8/36	61.9		L: ns
Modified scar grade				
Grade 1	17/4/8	100		
Grade 2	31/8/21	91.9	0.0002	U: 0.0001
Grade 3	28/10/39	65.4		L: ns
Grade 4	21/8/41	51.9		

*Mann-Whitney U test p value for relationship between B.I. and pathologic factors.
†Mann-Whitney U test p value for upper (U) region and lower (L) region.
ns: not significant or no tendency (p 0.1).
B.I.: Brinkman index.

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previously[20] are shown in Table 2. In our previous study, a papillary, acinar or solid component, necrosis, and MSG were prognostic factors, and some conventional prognostic factors including p stage, pT, pN, pleural invasion, ly, and v. In the present study, correlations of B.I. with some of these factors including p stage, ly, v, presence of a solid component, necrosis, and MSG were detected. The correlations with ly, v, presence of a solid component, and MSG were strong for tumors in the upper region.

As we considered that carcinogenesis and progression might differ in each B.I. group, a further study was performed. Adenocarcinoma with a BAC component (NAC and IAC BAC) was slightly dominant in males and smokers; the male:female ratio was 1.7:1 (89:77 cases), and the numbers of tumors in the B.I. 0, 1 to 500, and more than 500 groups were 80, 24, and 63, respectively. However, when limited to NAC, the incidence of females and nonsmokers was high; the male:female ratio was 1:1.6 (11:17 cases) and the numbers of tumors in the respective B.I. groups were 17, 4, and 7, respectively. For adenocarcinomas with a BAC component (NAC and IAC BAC) 30 mm or less in diameter, the group with B.I. of more than 500 tended to have a worse outcome than the group with B.I. of less than or equal to 500 (p 0.0729, Figure 2*A*), and this was also the case for adenocarcinomas 20 mm or less in diameter (p 0.0661, Figure 2*B*). When limited to IAC BAC, no significant difference in outcome was detected between the group with B.I. of 500 or less and the group with B.I. of more than 500, but B.I. was correlated with the presence of a solid component and MSG, particularly in the upper region (Table 3).

IAC BAC was dominant in males and heavy smokers; the male:female ratio was 1:2.9 (18:51 cases) and the numbers of tumors in the B.I. 0, 1 to 500, and more than 500 groups were 17, 6, and 46, respectively. Although the survival rate did not differ significantly between the groups with B.I. of less than or equal to 500 and more than 500, B.I. and the presence of a solid component and necrosis were positively correlated, and B.I. and the presence of an acinar component showed a negative correlation, particularly in the upper region.

DISCUSSION

We reviewed 236 cases of peripheral lung adenocarcinoma 30 mm or less in diameter and examined some aspects of their carcinogenesis and progression pathologically. The relative risk for lung cancer among smokers compared with nonsmokers is reported to be 2 to 10 for adenocarcinoma.[3] Our results were similar, as smokers were dominant; nonsmoker:smoker ratio was 1:1.4. It is well known that lung cancer, especially squamous cell carcinoma and small cell carcinoma, occurs frequently in the upper region of the lung among smokers or individuals with asbestos exposure, because transairway carcinogens persist longer in the upper lobe owing to the lower ventilation rate and less efficient lymphatic clearance.[4–9] Our study showed that the carcinogenesis rate was higher in the upper region than in the lower region in smoker, suggesting that tobacco-smoking affected carcinogenesis of small adenocarcinoma in the upper region.

As the B.I. increased, NAC decreased and IAC BAC and IAC BAC increased, suggesting that NAC might progress to IAC BAC, and thereafter to IAC BAC, as a result of smoking.

The group with B.I. of more than 500 tended to have a worse outcome than the group with B.I. of less than or equal to 500. It has been reported that smoking is a poor prognostic factor in patients with lung adenocarcinoma[11] or stage I lung adenocarcinoma.[12] Shiba et al. reported that in patients with nonsmall, nonsquamous cell carcinoma, heavy smokers (30 pack-years) had a significantly worse prognosis than lighter smokers (0–30 pack-years) for overall stage and also stage I.[13] We only examined cases of definite adenocarcinoma, and found that the survival rate tended to be different between the group with B.I. of more than 500 and group with B.I. of 0 to 500 in adenocarcinomas 30 mm or less in diameter (p 0.0855), whereas it was significantly different for adenocarcinomas 20 mm or less in diameter (p 0.0359), suggesting that the degree of smoking affects the prognosis of lung adenocarcinoma. Several reasons why p values differed between adenocarcinomas 30 mm or less in diameter and those 20 mm or less in diameter were speculated that a prognostic

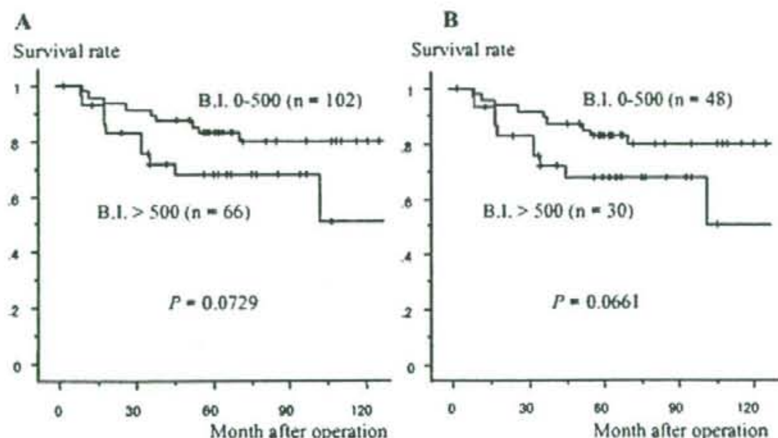

FIGURE 2. Survival curves for each B.I. group in patients with adenocarcinoma with a BAC component. *A*, Tumors 30 mm or less in diameter. *B*, Tumors 20 mm or less in diameter.

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TABLE 3. Relationship Between B.I. and Clinicopathologic Prognostic Factors in IAC BAC and IAC BAC

	IAC BAC No. of Case B.I. (0/1–500/>500)	Mann-Whitney U Test p*	p† for U and L Region	IAC BAC No. of Case B.I. (0/1–500/>500)	Mann-Whitney U Test p*	p† for U and L Region
Total (139)						
p Stage						
Stage I	40/12/32	ns		10/3/2	ns	
Stage II–IV	23/8/24			7/3/21		
pT						
T1	44/14/43	ns		15/4/39	ns	
T2–4	19/6/13			2/2/7		
pN						
N0	43/12/34	ns		11/3/28	ns	
N1–3	20/8/22			6/3/18		
Pleural invasion						
p 0–1	44/14/43	ns		15/4/39	ns	
p 2–3	19/6/13			2/2/7		
Lymphatic permeation						
Negative	34/8/21	ns		10/3/18	ns	
Positive	29/12/35			7/3/28		
Vascular invasion						
Negative	33/7/26	ns		6/1/16	ns	
Positive	30/13/30			11/5/30		
Papillary component						
Absent	8/4/10	ns		4/4/16	ns	
Present	55/16/46			13/2/30		
Acinar component						
Absent	31/11/22	ns		2/0/19	0.0011	U: 0.0032‡
Present	32/9/34			15/6/27		L: ns
Solid component						
Absent	47/12/30	0.0135	U: 0.0144	9/2/15	0.0964	U: ns
Present	16/8/26		L: ns	8/4/31		L: ns
Necrosis						
Absent	44/15/44	ns		15/3/22	0.0217	U: 0.0842
Present	19/5/12			2/3/24		L: ns
Modified scar grade						
Grade 1	—			0/0/1		
Grade 2	29/7/17	0.0518	U: ns	2/1/4	ns	
Grade 3	18/7/21		L: ns	10/3/18		
Grade 4	16/6/18			5/2/23		

*Mann-Whitney U test p value for relationship between B.I. and pathologic factors.
†Mann-Whitney U test p value for upper (U) region and lower (L) region.
‡Acinar component present frequently in low B.I. patients, particularly in upper region.
ns, not significant or no tendency (p 0.1); B.I., Brinkman index; BAC, bronchioloalveolar carcinoma; NAC, noninvasive adenocarcinoma; IAC BAC, invasive adenocarcinoma with a BAC component; IAC BAC, invasive adenocarcinoma without a BAC component.

difference was only detected in adenocarcinomas smaller than 20 mm, but was not significant for large and more progressed tumors, or that smoking was a mildly influential prognostic factor so that the p value did not constantly indicate significance.

We examined the correlations of B.I. with several pathologic prognostic factors for adenocarcinoma as a whole, and found that ly, v, presence of a solid component, necrosis, and MSG were correlated. Because these correlations were strong in the upper region of the lung, adenocarcinoma might progress and dedifferentiate predominantly in the upper region as a result of smoking. In adenocarcinoma with a BAC component, the group with B.I. of more than 500 tended to have a poorer outcome than the group with B.I. of less than or equal to 500, and in IAC BAC, B.I. correlated with MSG and the presence of a solid component, suggesting that the degree of smoking affects tumor progression in adenocarcinoma with a BAC component. In IAC BAC, which is considered to be de novo carcinoma or a progressed form of adenocarcinoma with a BAC component, outcome did not differ between the group with B.I. of 500 or less and the group with B.I. of more than 500. Nevertheless, a detailed examination revealed that an acinar component was predominant in the group with B.I. of 500 or less, whereas a solid

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component and necrosis was predominant in the group with B.I. of more than 500. We considered that the mechanism of progression might differ according to the degree of smoking, and that smokers tended to have a dedifferentiated component. Previously, it has been suggested that lung adenocarcinoma in smokers is progressed or has special characteristics on the basis of immunohistochemical or molecular biological examinations, exhibiting a high Ki-67 labeling index,[13] a high frequency of K-*ras*[21–24] or p53 mutation,[23,24] a low frequency of epidermal growth factor receptor (EGFR) mutation,[22] a high incidence of loss of heterozygosity,[25] and a high frequency of p53 mutation with transversion pattern.[26]

In conclusion, small adenocarcinoma in smokers seems to occur frequently in upper region, have more frequent invasive features, and be much more progressed and dedifferentiated than that in nonsmokers, predominantly in the upper region of the lung. Tobacco-smoking may have an effect on the carcinogenesis and multistep progression of peripheral lung adenocarcinoma 30 mm or less in diameter, particularly in the upper region.

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Original Paper

Expression profile of early lung adenocarcinoma: identification of *MRP3* as a molecular marker for early progression

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Abstract

Early lung adenocarcinoma is well-recognized as a small-sized non-invasive adenocarcinoma or localized non-mucinous bronchioloalveolar carcinoma (LNMBAC); however, the molecular events associated with these early lesions are not clear. To determine the genes involved in tumorigenesis at the early stage of lung adenocarcinoma, we compared the mRNA expression profiles of LNMBAC and normal lungs with an oligonucleotide array. Immunohistochemical analyses were performed to confirm the expression of detected genes. We identified 183 differentially expressed genes, of which 15 were up-regulated and 168 down-regulated. Among them, most up-regulated genes, such as *AQP3* and *Claudin-4*, were expressed in both adenocarcinoma cells and type II alveolar pneumocytes, corresponding to the histological similarity between these cell types. However, multidrug resistant protein 3 (MRP3) was only expressed on tumour cell membranes and not in type II alveolar pneumocytes, as confirmed by immunohistochemistry. Moreover, the number of MRP3-positive cells significantly increased from AAH (the precursor lesion of lung adenocarcinoma) to LNMBAC. We conclude that MRP3 could be a novel molecular marker for LNMBAC, whose expression increases during the early progression of tumourigenesis.
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Keywords: lung adenocarcinoma; localized non-mucinous BAC (LNMBAC); expression profile; microarray; MRP3

Introduction

Lung cancer is the leading cause of death in western countries and Japan. Among the four major histological types, adenocarcinoma is the most common, with the number of afflicted patients increasing in recent years. As lung adenocarcinomas are usually detected late in disease progression and are recalcitrant to morphological and molecular analyses, owing to their histological and cytological heterogeneity, the molecular mechanisms of adenocarcinoma tumourgenesis remain relatively unknown, especially in the early stage [1].

Introduction of high-resolution computed tomography (HRCT) to lung cancer diagnosis has made it possible to detect atypical adenomatous hyperplasia (AAH), a presumed precursor lesion or peripheral small-sized adenocarcinoma that can hardly be detected by X-ray examination [2–6]. Early lung adenocarcinoma is a novel concept and category derived from the clinicopathological analysis of small peripheral lung adenocarcinomas. Thus, this category is different from early cancer, which usually indicates stage I lung adenocarcinomas of the TMN classification. Noguchi *et al* histopathologically examined small adenocarcinomas (<2 cm) and subdivided them into two groups; those having a replacing growth structure and those having a non-replacing and destructive structure [7]. The former group includes three types: type A, a localized bronchioloalveolar carcinoma (LBAC); type B, comprised of LBAC with alveolar collapse; and type C, LBAC with active fibroblastic change. Because type A and type B adenocarcinomas, which are localized non-mucinous BAC (LNMBAC) in the World Health Organization (WHO) classification, have

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no interstitial invasion, no lymph node metastasis, and their 5 year survival rates are 100%, they are categorized as early lung adenocarcinomas.

According to recent studies, the progression of lung adenocarcinoma is considered to be caused by sequential molecular events like the adenoma–carcinoma sequence of colon cancer [8]. Studying LNMBAC could be a great help in elucidating the first step of the sequential molecular events underlying tumourigenesis. Although several studies have reported molecular events involving K-ras or P53 for small adenocarcinoma or AAH, most of the molecular mechanisms of the early stage remain unclear [9–11]. Understanding the probability and interval of clinical progression from AAH to overt malignancy, as well as from early to advanced adenocarcinoma, would also be very useful for clinical management. Novel tumour markers for early adenocarcinoma may help to diagnose LNMBAC more objectively.

The microarray technique is a recently developed and powerful tool for examining the expression of a massive number of genes at one time and has proved useful in the characterization of cancers [12–14]. In the present study, to determine molecular events and to find genes that characterize early lung adenocarcinoma, we compared the gene expression of LNMBAC and normal lungs with the oligonucleotide microarray. We identified a gene set that distinguished the tumour from the normal lung and specifically found that MRP3 expression increased during the early progression of tumourigenesis, suggesting its utility as a novel molecular marker for early lung adenocarcinoma.

Materials and methods

Tissue samples

Ten lung adenocarcinomas and 10 normal lungs were obtained from patients who underwent surgical resection at National Cancer Centre Hospital, Japan, and the study was approved by the National Cancer Centre ethics review board. Of the samples, four were paired tumours and corresponding non-cancerous lung tissue from the same patients. First, small samples for molecular analysis were obtained from surgically resected specimens and immediately cut into small pieces, snap-frozen in liquid nitrogen and stored until use. The the rest of the whole resected specimens were fixed routinely in 10% formalin, cut serially into 5–7 mm slices, and macroscopically examined. From the section including the largest diameter of the tumour, all the tumour tissue as well as the surrounding lung tissue was removed and embedded in paraffin, and then cut into 4 μm sections. Haematoxylin and eosin (H&E) and elastica and Van Gieson (EVG) staining was performed. All tumours were <3 cm in diameter (range 0.7–2.7 cm) and histologically diagnosed as LNMBAC without active fibroblastic change by two individual pathologists (Figure 1 and Table 1). For immunohistochemical analysis, 15 normal lungs, 10 hyperplasias, 15 AAHs and 12 LNMBACs were analysed. Sections were prepared from formalin-fixed, paraffin-embedded tissues of surgically resected samples.

Microarray analysis

For gene expression analysis, we used the GeneChip human Genome HG-U95Av2 oligonucleotide microarray (Affymetrix, Santa Clara, CA, USA). Target cRNA for microarray hybridization was prepared as follows. Total RNA was isolated using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. From 5 μg total RNA, double-stranded cDNA with a T7 promoter tag was generated using the Superscript Choice system (Invitrogen), and biotin-labelled cRNA was synthesized from the double-stranded cDNA by *in vitro* transcription, using a BioArray RNA transcript-labelling kit (Enzo Diagnostics, Farmingdale, NY, USA). The cRNA was purified using RNeasy columns; 20 μg biotin-labelled cRNA was fragmented at 94 °C for 35 min in 40 μl 1× RNA fragmentation buffer (40 mM Tris/acetate, pH 8.1, 100 mM K acetate and 30 mM Mg acetate) and used for microarray hybridization. Hybridization, washing, staining, and scanning were carried out according to the manufacturer's instructions. Briefly, 10 μg biotin-labelled and fragmented cRNA was hybridized to the microarray in 200 μl 1× 4-morpholinepropanesulphonic acid (MES) hybridization buffer (100 mM MES/Na-MES, pH 6.6, 890 mM NaCl, 20 mM EDTA, 0.01% Tween 20) containing 0.1 mg/ml herring sperm DNA (Promega, Madison, WI, USA) and 0.5 mg/ml acetylated BSA (Invitrogen) at 45 °C for 16 h with rotation. Subsequently,

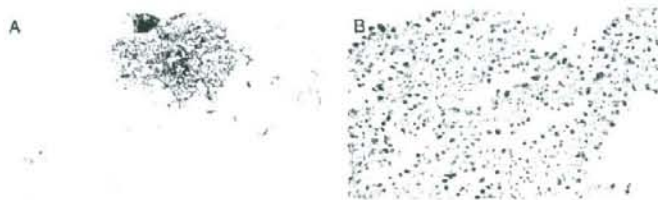

Figure 1. Histology of early lung adenocarcinoma (LNMBAC). The boundary between the tumour and the surrounding normal lung is indistinct. Each tumour cell resembles a type II pneumocyte and grows by replacing alveolar lining cells (H&E original magnification, ×400)

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the microarrays were washed with non-stringent wash buffer (60 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 894 mM NaCl, 6 mM EDTA and 0.01% Tween 20) at 25 °C and then with stringent wash buffer (100 mM MES/Na-MES, pH 6.6 26 mM NaCl and 0.01% Tween 20) at 50 °C, stained with streptavidin–phycoerythrin (Molecular Probes) and biotinylated antistreptavidin (Vector Laboratories, Burlingame, CA, USA) and scanned with a GeneArray scanner (Hewlett-Packard, Santa Clara, CA, USA).

Data analysis

Using the Microarray Suite 4.0 software package (Affymetrix), scanned images were transformed into signals called the average difference, which represented the mean difference of signal intensities between match and mismatch probe pairs. The hybridization intensities were normalized to 1000 across all samples. Data analyses were done using Excel (Microsoft), GeneSpring (Silicon Genetics, Redwood City, CA, USA) and Cluster (Stanford) and visualized using Tree and View (Stanford).

RT–PCR

From the total RNA samples, template cDNA was synthesized with an oligo(dT) primer and Super Script Choice System (Invitrogen). PCR was performed with AmpliTaq Gold and the GeneAmp 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The primer sets for MRP3 were designed as 5′-CTGCTAAACCCTGACCCTCTGCGG-3′ (forward) and 5′-TCCAGCAGCTGCTGCACCACCATC-3′ (reverse). PCR conditions were as follows: 1 cycle at 94 °C for 10 min, then 30 cycles at 95 °C for 15 s, 60 °C for 1 min and 75 °C for 30 °C, followed by a final 75 °C extension for 10 min. For standardization of the amount of RNA, expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was quantified for each sample.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections by an immunoperoxidase method, as described previously [15]. Antibodies used for immunohistochemical analysis were MRP3 (M3II-21; Signet Laboratories Inc., MA, USA) at a dilution of 1 : 100, TTF-1 (Neomarkers, Fremont, CA, USA) at a dilution of 1 : 200, AQP3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 200, and Claudin-4 (Zymed Laboratories, South San Francisco, CA, USA) at a dilution of 1 : 200.

Staining evaluation

Staining was evaluated by two independent observers. The positivity index was expressed as the percentage of positive cells in each lesion.

Results

Gene expression profiling of LNMBAC

To identify genes that characterized early lung adenocarcinoma, we compared expression profiles between 10 LNMBACs and 10 normal lungs, using the oligonucleotide array. We applied two independent types of supervised analysis.

The first analysis proceeded using the following criteria. (a) Genes whose average differences (mean difference in signal intensity between perfect match and mismatch probe pairs) were >1000 in at least 5/20 samples. (b) t-Test with significance set at $p < 0.05$ to identify genes expressed differently between tumours and normal tissue. (c) Multiple testing corrections (Young and permutation test) applied by Gene Spring software. Multiple testing correction adjusts the individual p value for each gene to keep the overall error rate to less than or equal to the specific p value cut-off. Using these criteria, we selected 183 differentially expressed genes from 12 696 probe sets, 15 genes that were up-regulated in tumours (Table 2) and 168 down-regulated genes (Supplementary Table 1, available at: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2383.html). To validate that these genes could actually distinguish early adenocarcinomas from normal lungs, we applied hierarchical clustering to classify the same sample sets, using the 183 selected genes (Figure 2).

The second analysis proceeded using the following criteria. (a) Presence in tumours (i.e. exactly

Table 1. Clinicopathological feature of surgically resected specimens

Patient No.	Gender	Age	Stage	T	N	M	Histological differentiation	Vascular invasion	Lympho duct invasion	Smoking		Brinkman index
K186	F	61	IA	1	0	0	Well	−	−	+	Current	375
K187	M	55	IA	1	0	0	Well	−	−	+	Current	700
K230	F	66	IA	1	0	0	Well	−	−	−	Never	
K228	F	69	IA	1	0	0	Well	−	−	+	Current	165
K236	F	73	IA	1	0	0	Well	−	−	−	Never	
K265	M	76	IA	1	0	0	Well	−	−	+	Former	450
K269	M	52	IA	1	0	0	Well	−	−	+	Current	640
K274	M	59	IA	1	0	0	Well	−	−	+	Former	360
K303	M	65	IA	1	0	0	Well	−	−	−	Never	

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Table 2. List of genes up-regulated in LNMBAC compared to normal lungs

Symbol	Affymetrix probe No.	p Value	Locus	Gene description
ABCC3	1930_at	0.016	17q22	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
WFDC2	33933_at	0.001	20q12−q13.2	WAP four-disulphide core domain 2
AQP3	39248_at	0.003	9p13	Aquaporin 3
ADAM8	40712_at	0.021	10q26.3	A disintegrin and metalloproteinase domain 8
CACNB1	36557_at	0.024	17q21−q22	Calcium channel, voltage-dependent, β 1 subunit
MST1R	1317_at	0.028	3p21.3	Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)
BENE	33331_at	0.032	2q13	BENE protein
CLDN4	35276_at	0.001	7q11.23	Claudin 4
KIAA0907	33885_at	0.026	1q21.2	KIAA0907 protein
KIAA0657	35780_at	0.013	2q36.3	KIAA0657 protein
GCN5L2	38628_at	0.003	17q21	GCN5 general control of amino-acid synthesis 5-like 2 (yeast)
CHC1	1196_at	0.009	1p36.1	Chromosome condensation 1
LGALS3BP	37754_at	0.003	17q25	Lectin, galactoside-binding, soluble, 3 binding protein
ITPR3	37343_at	0.032	6p21	Inositol 1,4,5-triphosphate receptor, type 3
KIAA0076	36084_at	0.032	6p21.1	KIAA0076 gene product

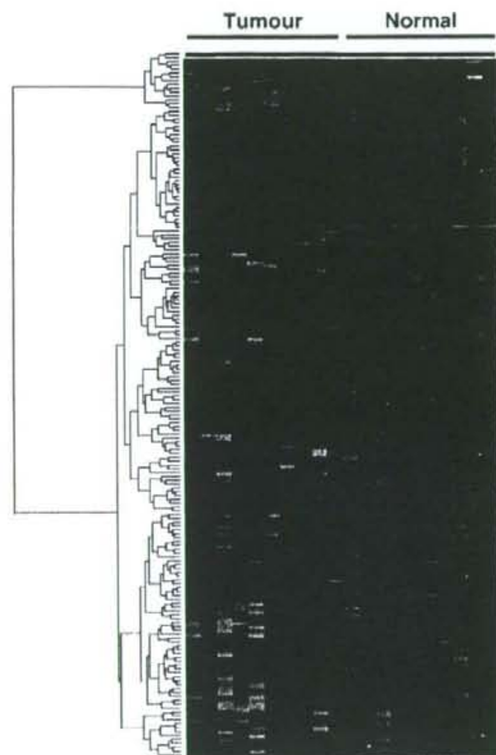

Figure 2. Expression profiling of LNMBAC. Hierarchial clustering was performed, in which 183 differentially expressed genes clearly differentiated LNMBACs from normal lungs. The normalized expression index of each transcript is indicated by a coloured bar. Up-regulated genes were expressed as red and down-regulated genes as green

expressed in the samples). (b) Average difference of >1000. (c) A > three-fold increase in average difference when compared to 10 normal sample mixtures in at least 5/10 samples. Using these criteria, we selected 140 genes that were up-regulated by more than three-fold (Supplementary Table 2, available at: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2383.html).

Genes expressed in both type II alveolar pneumocytes and LNMBAC

For the up-regulated genes listed in Table 2 and Supplementary Table 2, we selected interesting genes and performed immunohistochemical analysis on LNMBACs. We also examined AAH, hyperplasia and normal regions from the surrounding lung.

We examined *TTF-1*, *AQP3* and *Claudin-4* (Figure 3). We also analysed *MST1R* (*RON*), *MUC1*, *CD24*, *HNF1b*, *ARFGEF1*, *GRB7*, *AQP5* and *NQO1* (data not shown); those genes were specifically expressed in tumour cells and could not be detected in the interstitial areas in carcinoma tissue. In surrounding regions, normal type II pneumocytes were also positive in every case, whereas type I cells were negative. The staining intensity of TTF-1 in tumour cells was the same as in normal type II pneumocytes; however, that of AQP3 and Claudin-4 in tumour cells was stronger than in normal type II pneumocytes. In hyperplasia and AAH, these genes were also positive in the type II pneumocytes and atypical cells that morphologically resembled type II pneumocytes, respectively (data not shown). Therefore, these genes were considered to be related to features of type II pneumocytes.

MRP3 expression in LNMBAC

Of the genes listed in Table 2 and Supplemenatry Table 2, *MRP3* was the only one that was up-regulated more than three-fold in all 10 samples as well as being undetectable in the normal lung. (Figure 4) To confirm the mRNA level of *MRP3*, we applied RT–PCR (Figure 5). In all four LNMBACs examined, *MRP3* was up-regulated when compared to the corresponding normal lung tissue, where expression was almost undetectable.

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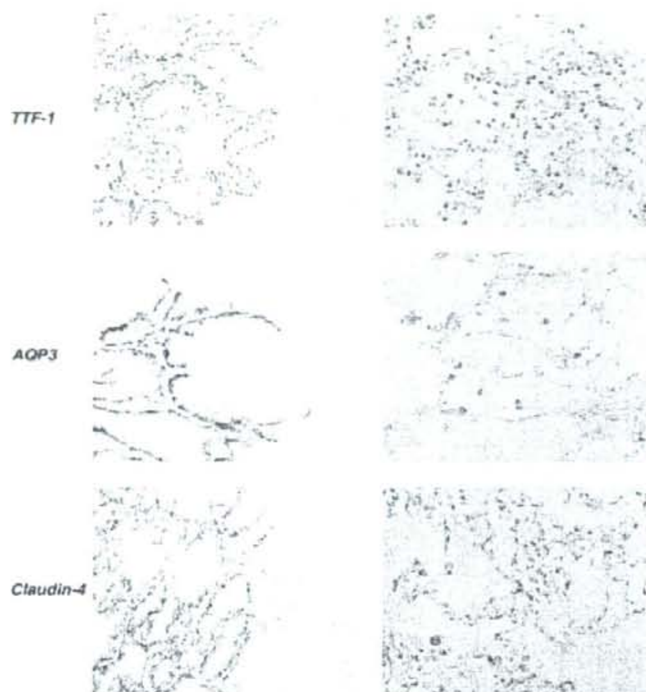

Figure 3. Immunohistochemical analysis of *TTF-1* (top), *AQP3* (middle) and *Claudin-4* (bottom) on LNMBAC (left) and normal lung (right). These genes were specifically expressed in tumour cells and could not be detected in interstitial areas in carcinoma tissue. In surrounding normal regions, histologically normal type II pneumocytes were also positive in every case, whereas type I cells were negative. The staining intensity of TTF-1 in tumour cells and normal type II pneumocytes was the same, while that of AQP3 and Claudin-4 was stronger in tumour cells

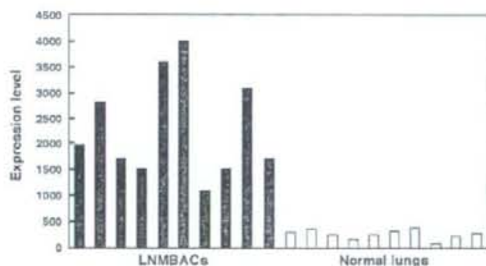

Figure 4. Expression signal of *MRP3* as measured by oligonucleotide microarray. Each bar expresses raw average difference data. All black bars representing the expression signal in LNMBACs indicate a range of about 1000–4000, whereas all white bars representing signals from normal lungs indicate undetectable expression

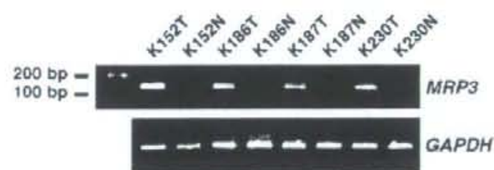

Figure 5. *MRP3* expression in LNMBAC. RT–PCR was performed to confirm the mRNA level of *MRP3* with four paired samples. The expression level of *MRP3* in LNMBAC was up-regulated when compared to that in the corresponding normal lung tissue

To determine whether *MRP3* was also expressed at the protein level, we employed a monoclonal antibody against MRP3 in an immunohistochemical study (Figure 6) and counted positively-stained cells in each lesion (Figure 7). We examined LNMBAC, the surrounding normal lung, hyperplasia and AAH. The immunohistochemical study demonstrated strong expression of MRP3 on cell membranes in LNMBACs, and most LNMBAC samples expressed MRP3 in more than 50% of tumour cells. In normal lung and hyperplasia, including type II pneumocytes, we could not detect MRP3 protein. MRP3 protein expression in AAH varied among samples; however, most samples did not express or scarcely expressed MRP3. The percentage of positive cells increased stepwise according to the progression of lung adenocarcinoma.

Discussion

In this paper we present gene expression profiling of LNMBAC and identify a distinct gene set that distinguishes tumours from normal lung tissue; these

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Figure 6. Immunohistochemical evaluation of *MRP3* expression in LNMBAC. Immunohistochemistry demonstrated negative expression in normal lung (A) and hyperplasia (B). Staining was also rarely detectable in AAH (C). *MRP3* expression was observed on the cell membrane in LNMBAC (D) and another case of LNMBAC (E and F). Original magnifications: ×40 (D), ×100 (E) and ×200 (F)

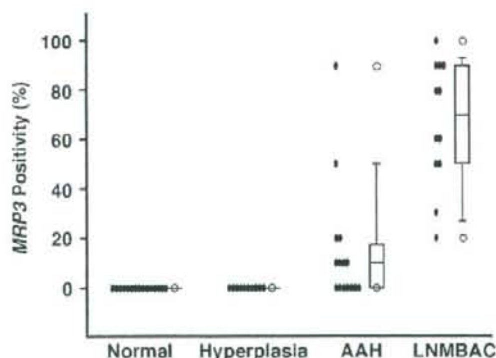

Figure 7. Relationship between immunohistochemical expression of MRP3 and LNMBAC. Scattergram and box plot show the relationship between *MRP3* immunostaining and normal lung, hyperplasia, AAH and LNMBAC. The box encompasses the 25th to 75th percentiles of results obtained, with the 50th percentile representing the median. The 5th and 95th percentiles are shown as white circles, and below and above the circles are 10th and 90th percentile whisker caps, respectively. The percentage of positive cells increased according to the stepwise progression of lung adenocarcinoma

genes particularly characterize LNMBAC. Of the up-regulated genes, we identified *MRP3* as a novel molecular marker for LNMBAC. We also identified novel type II pneumocyte lineage tumour markers.

MRP3 was identified as an up-regulated gene by two independent supervised analyses. The most remarkable feature of *MRP3* was that its expression was detected in tumour cells but not in normal type II pneumocytes. *MRP3* expression increased concomitantly with the stepwise progression from the precursor lesion of lung adenocarcinoma to AAH to LNMBAC. Moreover, *MRP3* was not detected in type II pneumocyte hyperplasia. The border between AAH and LNMBAC is unclear and sometimes varies by pathologist. This diagnostic difficulty generates severe confusion for clinical treatment; the establishment of standard management is of top priority for clinical oncologists. *MRP3* could therefore be a novel objective tumour marker for histological diagnosis and an important marker for lung cancer in the future. Our findings also suggest that *MRP3* might function as a primary factor in carcinogenesis. *MRP3* was found to be an anti-cancer drug-resistant gene in a lung cancer cell line [16]. *MRP3* belongs to the ABC transporter

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membrane protein family, and some anti-cancer drugs induce *MRP3* expression to obtain a resistant phenotype [17–21]. However, none of the samples used in this study had received any anti-cancer drug therapy before analysis; therefore the observed *MRP3* expression was not induced by anti-cancer drugs. There is widely accepted view that BAC is less chemosensitive than other non-small cell lung carcinomas; however, this is not clearly supported by scientific evidence [22]. The hypothesis that observed *MRP3* expression in LNMBAC might produce congenital drug resistance would need further study. Recent studies have revealed that *ABCG2/BRCP*, a member of the ABC transporter family, can generate a cancer stem cell phenotype in addition to drug resistance [23,24]. If cancer stem cells also play a role in lung adenocarcinogenesis, then *MRP3* expression might be involved.

Well-established type II pneumocyte markers, such as *KL-6* (*MUC1*) and *SP-D*, are already used in clinical practice to determine the activity of interstitial pneumonia [25]. Upon expression profiling of LNMBAC, we identified several novel type II pneumocyte markers, such as *AQP3, RON* and *Claudin-4*, and it is expected that these molecules might also serve as markers for lung pathophysiology. Additionally, these markers were also expressed by tumour cells in LNMBAC; thus, they could be type II pneumocyte-lineage tumour markers. We postulate that there are two main reasons why many novel type II pneumocyte lineage tumour markers were identified by this simple comparison of expression profiles. The first reason is that LNMBACs have a relatively smaller interstitial area when compared to advanced adenocarcinomas; therefore, the expression profiles mainly represent tumour cell expression. The second reason is that LNMBACs are composed of monotonous tumour cells that pathologically resemble normal type II pneumocytes. Thus, while normal lungs are composed of a mixture of type I and type II pneumocytes and bronchiolar epithelium, the comparison of expression profiles of LMNBACs and normal lungs reflected the difference in the number of 'normal and neoplastic' type II pneumocytes. The molecular signature corresponds to the histological features; the tumour cells in LNMBACs also mimic the characteristics of type II pneumocytes at the molecular level. These findings support the hypothesis that some type II pneumocytes may be progenitor cells for adenocarcinomas of the BAC type. Immunohistological analysis also revealed that some type II pneumocyte lineage markers, such as *AQP3* and *Claudin-4*, might be over-expressed by tumour cells and may have some carcinogenic function.

In summary, we analysed the gene expression profile of LNMBAC and identified novel type II lineage tumour markers in addition to a novel tumour marker, *MRP3*, whose induction reflected the stepwise progression of lung adenocarcinogenesis. This study serves as a step toward better diagnosis and treatment of LNMBAC.

Acknowledgements

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Supplementary material

Supplementary material may be found at the web address: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2383.html

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ORIGINAL ARTICLES

Cytological Features of Signet-Ring Cell Carcinoma of the Lung:

Comparison With the Goblet-Cell-Type Adenocarcinoma of the Lung

Koji Tsuta, M.D.,[1] Yasuo Shibuki, C.T., I.A.C.,[1] Naoki Maezawa, C.T., I.A.C.,[1] Naobumi Tochigi, M.D.,[2] Akiko Miyagi Maeshima, M.D.,[1] Yuko Sasajima, M.D.,[1] Hisao Asamura, M.D.,[3] and Yoshihiro Matsuno, M.D.[1*]

Signet-ring cell carcinoma (SRCC) and goblet-cell-type adenocarcinoma (GCA) are mucin-producing lung adenocarcinomas. Primary SRCC shows an aggressive clinical course, whereas GCA shows infrequent distant metastasis, but more frequent intrapulmonary metastases resembling lobar pneumonia. To distinguish SRCC from GCA, this study investigated the respective cytological features of these lesions. We selected 10 cases each of SRCC and GCA from the archival imprint smears. We assessed them for the following 10 cytological features. Necrosis/debris was observed in 60% of the SRCC and 90% of the GCA. A mucinous background was observed in 10% of the SRCC and 90% of the GCA. Significant inflammation was observed in none of the SRCC and 80% of the GCA. Stromal cluster was observed in 30% of the SRCC and 70% of the GCA. Nuclear overlapping was observed in 50% of the SRCC and in all of the GCA. Single tumor cells were observed in 80% of the SRCC and 10% of the GCA. Honeycomb-like cluster was observed in none of the SRCC and 80% of the GCA. Prominent nucleolus was observed in 50% of the SRCC and 40% of the GCA. Nuclear membrane irregularity was observed in 70% of SRCC and 60% of the GCA. Nuclear pleomorphism was observed in all of the SRCC and none of the GCA. The cytological features of SRCC were the presence of single tumor cells and nuclear pleomorphism, whereas that of GCA were the presence of abundant mucin and significant inflammation in the background, and a honeycomb-like cluster. Diagn. Cytopathol. 2009;37:159–163. © 2009 Wiley-Liss, Inc.

Key Words: imprint smear; lung adenocarcinoma; signet-ring cell carcinoma; mucinous-bronchiolo-alveolar carcinoma

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Signet-ring cell carcinoma (SRCC), a unique subtype of mucin-producing adenocarcinoma, is characterized by abundant intracellular mucin accumulation and a crescentic nucleus displaced toward one end of the cell which may arise in various organs, including the stomach, colon, urinary bladder, prostate, and breast.[1-5]

We previously reported the clinicopathological and histological characteristics of primary lung carcinoma containing SRCC components. When the SRCC component occupied 50% or more of the lesion, the age of occurrence was younger, and blood vessel invasion, lymph vessel invasion, and lymph node metastasis were significantly more frequent, and the pathological stage was higher than in carcinomas whose SRCC component occupied less than 50% of the lesion, and in the non-SRCC. Furthermore, the 5-year survival rate of patients with an SRCC component occupying 50% or more of the lesion was significantly lower (28.4%) than that of patients with an SRCC component occupying less than 50% of the lesion (50.0%), or those with a non-SRCC lesion (52.7%).[6]

Goblet-cell-type adenocarcinoma (GCA) is characterized by displacement of the nucleus to the base of tall columnar cells that contain an abundance of cytoplasmic mucin and by growth along the alveolar walls. Most GCA lesions are classified as mucinous bronchiolo-alveolar-carcinoma (BAC) in the latest WHO classification.[7] GCA is associated with less frequent lymph node and/or distant metastasis than the conventional types of adenocarcinoma, but with more frequent intrapulmonary

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metastasis, showing lobar pneumonia-like clinical features.[8]

We previously analyzed the immunohistochemical differences between SRCC and GCA and compared these findings with those of normal tissue with 18 antigen expression patterns. These findings indicated that SRCC clustered as alveolar lining cells, whereas GCA clustered as gastric foveolar cells and bronchial goblet cells.[9]

Although SRCC and GCA are both mucin-producing primary lung carcinomas, each lesion exhibits distinct clinical features and immunohistochemical characteristics. The distinction of SRCC from GCA is critical because there are major differences in the clinical manifestations. The cytologic features of SRCC of the lung are not yet fully understood, because of the rarity of primary SRCC of the lung, which has incidence ranging from 0.14 to 1.9% of all lung carcinomas.[10,11] In this study, we elucidated the cytologic features of SRCC in the lung and compared these findings with those of GCA.

Materials and Methods

Cases and Tumor Specimens

We selected 10 cases each of SRCC and GCA from the archival imprint smears. Imprint smears were obtained from the maximum fresh cut surface of surgically resected tumor specimens, fixed routinely in 95% ethanol and then stained using the Papanicolaou technique. The resected specimens were then fixed in an inflated state by transbronchial infusion and/or injection of 10% buffered formalin. All tissue blocks containing tumor tissue were embedded in paraffin. Consecutive 5-μm sections were cut and then stained with hematoxylin–eosin and alcian blue–periodic acid-Schiff.

All histologic specimens were carefully evaluated by two or three certificated pathologists. None of these cases had undergone any preoperative chemotherapy or radiotherapy. The research was approved by the Institutional Review Board and an informed consent was obtained from each patient enrolled in this study.

Cytologic Examination

Imprint smear slides, three to four in each case, were examined by three observers (K. T., Y. S., and N. M.) and assessed for the following parameters without knowledge of the clinicopathologic data for each patient.

Background. The parameters included the presence or absence of (1) necrosis and/or debris, (2) mucinous background (lump of mucin or entire background), (3) significant inflammation (moderate to severe inflammatory cell infiltration), and (4) stromal cluster (myxomatous stroma with spindle cells).

Table I. The Cytological Features of SRCC and GCA

	SRCC		*GCA*		
	(−)	*(+)*	*(−)*	*(+)*	*P* value
Necrosis and/or debris	5	5	1	9	0.141
Mucinous background	9	1	1	9	<0.001
Significant inflammation	10	0	2	8	0.001
Stromal cluster	7	3	3	7	0.179
Nuclear overlapping	4	6	0	10	0.087
Single tumor cells	2	8	9	1	0.001
Honeycomb-like cluster	10	0	2	8	0.001
Prominent nucleolus	5	5	6	4	1.000
Nuclear membrane irregularity	3	7	4	6	1.000
Nuclear pleomorphism	0	10	10	0	<0.001

SRCC, signet ring cell carcinoma; GCA, goblet cell adenocarcinoma.

Shape of the Tumor Cluster. Additional parameters included the presence or absence of (5) nuclear overlapping in more than three layers, (6) single tumor cells, and (7) a honeycomb-like cluster.

Cytomorphological Features. The parameters included the presence or absence of (8) prominent nucleolus, (9) nuclear membrane irregularity, and (10) nuclear pleomorphism (more than 3-fold variation in nuclear size).

Disagreements in judgment were resolved by means of a joint review of the slides using a multiheaded microscope.

Statistical Analysis

Statistical analysis was performed using SPSS 12.0 for Windows (SPSS, Chicago, IL). Chi-square or Fisher's exact tests were used and a P value of 0.05 or less was regarded as significant.

Results

Background

The cytological features of SRCC and GCA are shown in Table I. The presence of necrosis and/or debris was observed in 5 of 10 SRCC (50%) (Fig. 1A) and in 9 of 10 GCA (90%) (Fig. 1B). A mucinous background was observed in 1 of 10 SRCC (10%) and 9 of 10 GCA (90%). Significant inflammation was observed in none of the SRCC and 8 of 10 GCA (80%). In GCA, the main component of the inflammatory cells consisted of polymorphonuclear leukocytes. However, in SRCC the accompanying inflammatory cells consisted of mainly a few lymphocytes. A stromal cluster was found in 3 of 10 SRCC (30%) and in 7 of 10 GCA (70%).

Shape of the Cluster

Clusters with nuclear overlapping in more than three layers were observed in 6 of 10 SRCC (60%) and all GCA (100%). The presence of single tumor cells

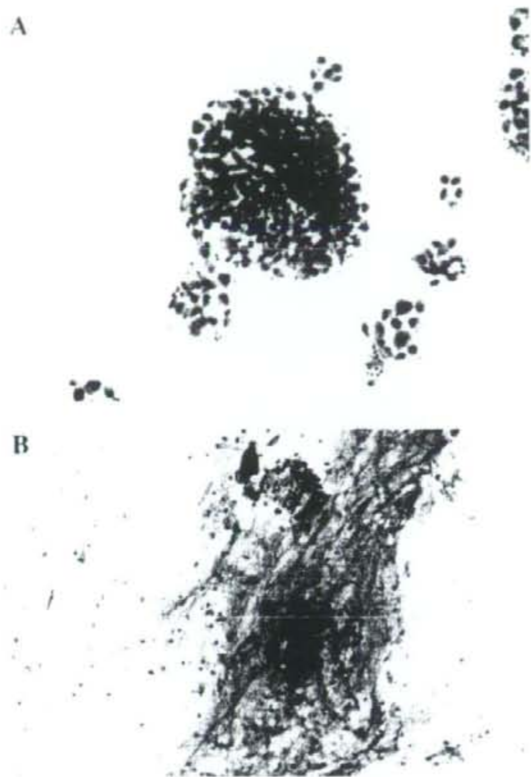

Fig. 1. A: As a cytological feature of SRCC, the tumor cluster showed a slightly loose cohesive cluster with a clear background. Papanicolaou stain, ×10. **B:** As cytological features of GCA, the tumor cluster showed tightly packed clusters with abundant mucin and inflammatory background. Papanicolaou stain, ×10. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

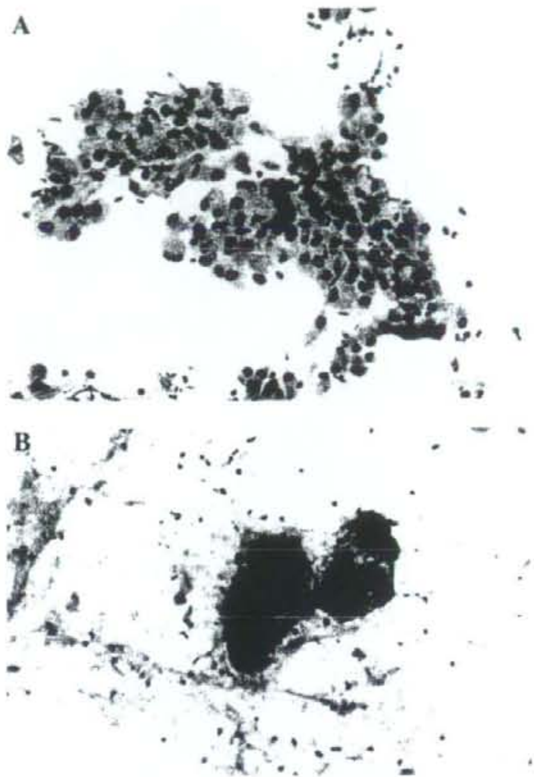

Fig. 2. A: As cytological features of SRCC, the tumor nest is partly composed of varying amounts of granular intracellular mucin accumulation and a crescentic nucleus displaced toward one end of the cell. Papanicolaou stain, ×20. **B:** As cytological features of GCA, the tumor nest is composed of abundant intracellular mucin accumulation and clear cell borders with honeycomb-like cluster. Papanicolaou stain, ×20. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

was observed in 8 of 10 SRCC (80%) (Fig. 2A) and 1 of 10 GCA (10%). A honeycomb-like cluster was observed in none of SRCC and in 8 of 10 GCA (80%) (Fig. 2B).

Cytomorphological Features

The presence of a prominent nucleolus was observed in 5 of 10 SRCC (50%) and 4 of 10 GCA (40%). The presence of nuclear membrane irregularity was observed in 7 of 10 SRCC (70%) and 6 of 10 GCA (60%). The presence of nuclear pleomorphism was observed in all SRCC (Fig. 3A) and none of the GCA (Fig. 3B).

The tumor cluster of SRCC was composed of varying amounts of mucin-containing cells. Only a few clusters were observed in which all cells consisted of mucin-containing cells. The mucin in the cytoplasm of SRCC appeared granular to pinkish. The tumor cluster of GCA consisted almost completely of mucin-containing tall columnar cells. Mucin in the cytoplasm of GCA appeared clear to yellowish.

Statistical Analysis

The statistical analysis demonstrated the cytological features of SRCC to include the presence of single tumor cells and nuclear pleomorphism. In addition, the cytological features of GCA included the presence of a mucinous background and significant inflammation, with the formation of honeycomb-like cluster.

Discussion

In this study, we elucidated and compared the cytological features of SRCC and GCA. SRCC and GCA each showed the respective characteristic cytological features. The cytological features of SRCC were the presence of single tumor cells, nuclear membrane irregularity with nuclear pleomorphism, a less mucinous background, and

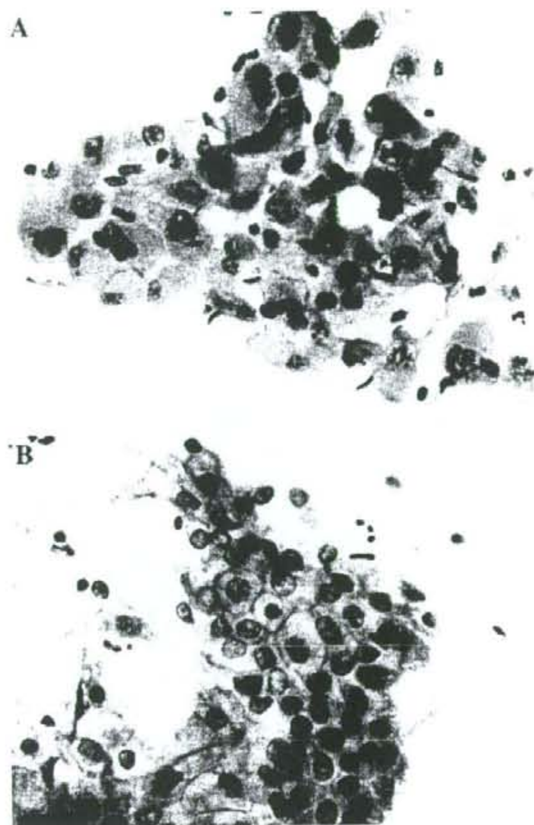

Fig. 3. **A:** As cytological features of SRCC, the tumor cells showed distinctive nuclear pleomorphism and prominent nucleoli. Papanicolaou stain, ×40. **B:** As a cytological feature of GCA, the tumor cells showed nuclear membrane irregularity without nuclear pleomorphism. Papanicolaou stain, ×40. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

less inflammatory cell infiltration. The cytological features of GCA were a tightly packed three-dimensional cluster with a honeycomb-like arrangement, nuclear membrane irregularity without nuclear pleomorphism, a mucinous background, and significant inflammation.

Almost all GCA showed a dirty background, thus demonstrating the presence of necrosis and/or debris, and significant inflammation. However, only half of SRCC showed a dirty background. In some organs, a dirty background indicates an invasive carcinoma. In general, GCA is known to show less invasive growth than SRCC. These differences may explain the biological reaction to excessive mucin in the background. Although, both SRCC and GCA produce and retain mucin in the cytoplasm, SRCC tends to retain mucin only in the cytoplasm, whereas GCA tends to not only retain mucin, but also to secrete mucin.

Both SRCC and GCA demonstrated nuclear membrane irregularity. However, nuclear pleomorphism was exclusively observed in SRCC. In lung adenocarcinoma, nuclear pleomorphism is known to be a predictor of invasiveness.[12,13] These findings may be related to the characteristic features of SRCC, which favor invasion of the parenchyma and promote distant metastasis.[6] Furthermore, we demonstrated that nuclear pleomorphism served as one of the criteria to differentiate SRCC and GCA.

We observed differences in the formation of tumor clusters between SRCC and GCA. Cell–cell adhesion is important for the maintenance of tissue architecture and the alteration of biologic behavior of tumor cells. Recently, Moon et al.[14] reported that SRCC of the lung showed reduced E-cadherin and β-catenin expression. E-cadherin and β-catenin complexes have been suggested to play critical roles in cancer development and progression.[15,16] The presence of single cells or a loose cohesive cluster at the periphery in the SRCC cluster may reflect the aforementioned phenomenon.

A honeycomb-like cluster was exclusively observed in GCA. An overlapping cluster and distinctive cell borders may contribute to the honeycomb-like appearance. We previously reported that nuclear overlapping in more than three layers and more than a 3-fold variation in nuclear size were found to be independent predictive factors for invasion in small-sized lung adenocarcinomas with a nonmucinous BAC component.[12] These findings indicate that the cytological features of nuclear overlapping should not be used to judge invasion in mucin-producing lung adenocarcinoma.

In GCA, most cells are mucin-containing cells, whereas SRCC lesions contain various amounts of coexisting nonmucinous cells. We previously reported that pure SRCC is rare and the average proportion of the SRCC component has been reported to be 41.4%.[6] The cytological findings obtained in this study are probably reflected in this observation.

The second edition of the WHO Histological Typing of Lung Tumors (1981) described BAC as "an adenocarcinoma in which cylindrical tumor cells grow upon the walls of preexisting alveoli." Much attention was focused on the recognition of the growth pattern (lepidic growth or not), but not on the tumor cell phenotype.[17]

During the last three decades, a number of studies have addressed issues related to the diagnosis of BAC by cytological methods.[12,13,18-25] However, most of these studies were performed before the application of the criteria described in the 1999 WHO third edition[26]; therefore, most of the studies analyzing these cytologic features lumped GCA together with nonmucinous BAC, while few reports elucidated the cytologic features of GCA. Thus, these previous descriptions of the cytological features do not always apply to GCA.