Int J Clin Exp Pathol 2014;7(4):1459-1468
www.ijcep.com /ISSN:1936-2625/IJCEP1402042

Original Article

Lepidic and micropapillary growth pattern and expression of Napsin A can stratify patients of stage I lung adenocarcinoma into different prognostic subgroup

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Received February 12, 2014; Accepted March 8, 2014; Epub March 15, 2014; Published April 1, 2014

Abstract: Histologic categories and related growth pattern proposed by IASLC/ATS/ERS classification has been reported to be prognostically important in lung adenocarcinoma. Thyroid transcription factor-1 (TTF1) and Napsin A have been investigated as potential prognostic parameters with conflicting results. A total of 211 cases with stage I lung adenocarcinoma were analyzed according to the IASLC/ATS/ERS classification with slight modifications. Expression levels of TTF1 and Napsin A were evaluated by immunohistochemistry. In univariate analyses, we found female sex (p=0.009), lepidic growth pattern (P=0.011) and lack of micropapillary pattern (P=0.048) were favorable predictor significantly associated with disease-free survival (DFS). Lack of mitosis (P=0.044) and Napsin A expression (P=0.031) were favorable predictors for overall survival (OS). Tumors with a maximum diameter ≤2 cm had both longer DFS (P=0.008) and OS (P=0.020). Negative TTF1 expression indicated increased risk of death, but failure in statistical significance (P=0.215). After multivariate analysis, histologic subtype, tumor size and gender were identified as independent predictor for DFS (RR: 0.343, 3.697, 0.494; P=0.006, 0.029, 0.019), no feature was found as an independent predictor for overall survival (P>0.05). To conclude, lepidic growth pattern, female sex and tumor size ≤2 cm are independent favorable predictors for tumor recurrence, tumors with more than 5% percentage of lepidic growth pattern will have a better prognosis than absence, in early-stage lung adenocarcinoma.

Keywords: Stage I, lung adenocarcinoma, IASLC/ATS/ERS classification, TTF1, Napsin A, prognosis

Introduction

Lung cancer is the leading cause of cancer mortality [1]. Adenocarcinoma is the most common histological subtype of lung cancer with heterogeneity. The 2004 World Health Organization classification of lung tumors is of limited clinical utility, as more than 90% of lung adenocarcinomas fall into mixed subtype with greatly varied clinical outcomes [2-4]. Approximately 30-40% patients will have a disease recurrence even among stage I cases [5].

Since the limitation of the 2004 WHO classification, a growing number of studies were raised trying to evaluate the clinical importance of histologic subtypes by assessing the presence and extent of histologic growth patterns, many histologic features have been demonstrated as prognostic factors, such as lepidic, solid and micropapillary pattern, nuclear features, tumor necrosis and so on [6-9]. Among these features, predictive potential of lepidic and micropapillary pattern were officially recognized in the new IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma, by introducing the new concept of AIS (adenocarcinoma with pure lepidic growth), MIA (minimally invasive adenocarcinoma of predominant lepidic growth with no more than 5 mm invasion), LPA (lepidic predominant adenocarcinoma) and adding micropapillary an a new histologic subtype.

Several studies [10, 11] have validated the correlation between adenocarcinoma subtypes base on IASLC/ATS/ERS classification and patients' outcome in cohort of North American and Australia respectively. For ethnic differences, Gu et al analyzed 292 stage I lung adenocarcinomas from Chinese patients [12]. Aim of the current study is to investigate the predictive

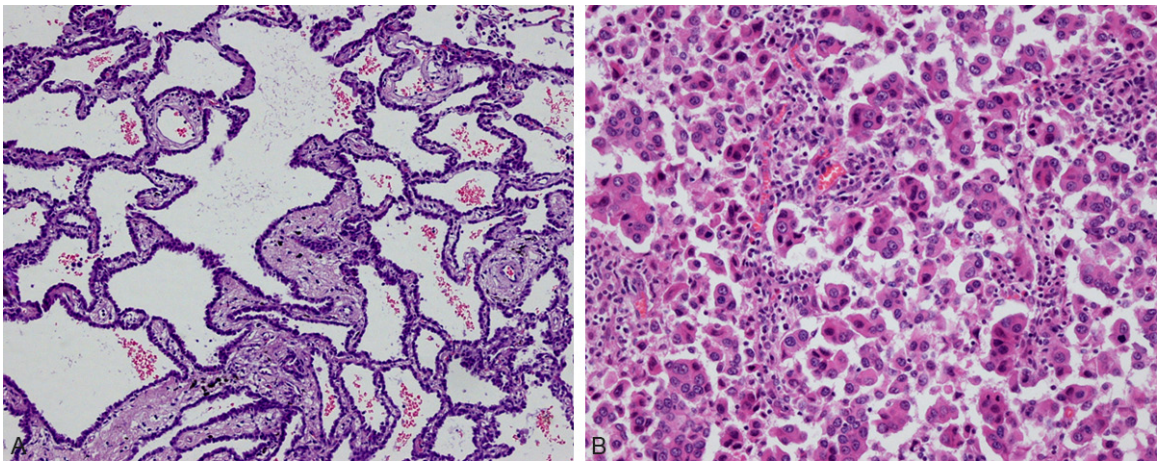

Figure 1. A. Lepidic pattern: consist of a proliferation type II pneumocytes and Clara cells along the surface alveolar walls with no evidence of stromal, vascular, or pleural invasion (100×). B. Micropapillary pattern: small papillary clusters composed of glandular cells with peripheral nuclei growing in airspace without fibrovascular cores (200×).

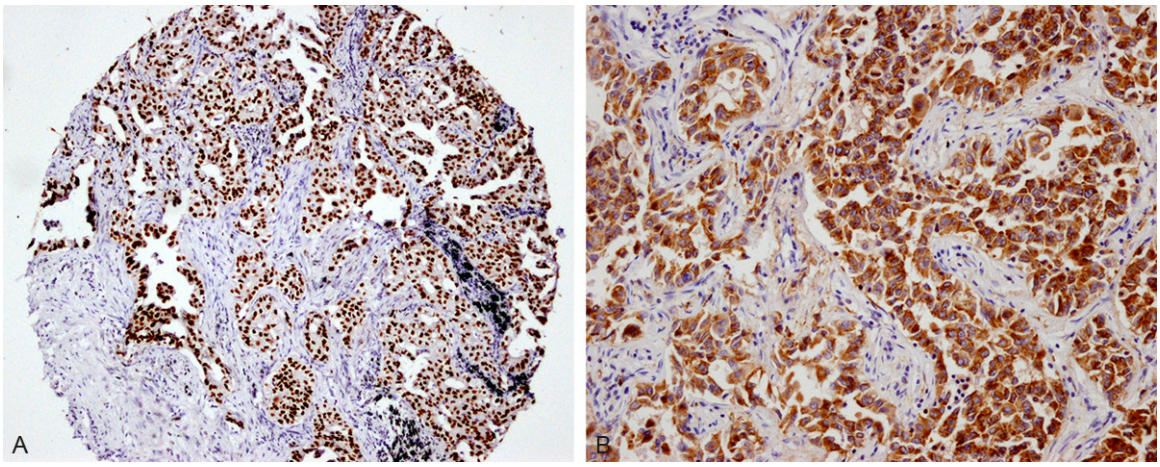

Figure 2. Immunohistochemical analysis using tissue microarray is shown. A. Representative section of Thyroid transcription factor1 (TTF1)-positive lung adenocarcinoma (100×). B. Representative section of Napsin A-positive lung adenocarcinoma (200×).

importance of histologic distribution introduced by the IASLC/ATS/ERS classification, in a Chinese cohort of stage I lung adenocarcinoma by performing a detailed semiquantitative assessment of growth pattern (lepidic and micropapillary); Validating the prognostic utility of TTF-1 and Napsin A expression using the current uniform cohort of early-stage lung adenocarcinomas, and their association with IASLC/ATS/ERS classification.

Materials and methods

Patient selection

We reviewed electronic clinical records database to identify all the cases of primary pulmonary adenocarcinoma who underwent a radical surgery at Cancer Hospital/Institute of the Chinese Academy of Medical Sciences (CAMS) during 2003-2006. Patients with a single nodule and stage I postoperative evaluation, a minimum of 1 hematoxylin and eosin (H&E)-stained histologic slide per centimeter of the greatest tumor dimension available for histologic analysis were included in our study, those received neoadjuvant therapy, died during perioperation or died of other diseases were excluded. Clinicopathologic information was collected including age at diagnosis, gender, smoking status (current, former, or never), tumor stage and tumor size.

Table 1. Clinicopathological Characteristics distribution according to expression of TTF1 and Napsin A

Variables	No. of patients	TTF1		*P*-value	No. of patients	Napsin A		*P*-value
		Negative (-)	Positive (+)			Negative (-)	Positive (+)	
Age				1.000*				0.021*
≤62	72	5 (6.9)	67 (93.1)		74	3 (4.1)	71 (95.9)	
>62	63	5 (7.9)	58 (92.1)		65	11 (61.9)	54 (83.1)	
Gender				0.747*				0.581*
Male	76	5 (6.6)	71 (93.4)		78	9 (11.5)	69 (88.5)	
Female	59	5 (8.5)	54 (91.5)		61	5 (8.2)	56 (91.8)	
Smoking status				0.281				0.074
Never smokers	85	4 (4.7)	81 (95.3)		88	5 (5.7)	83 (94.3)	
Former smokers	19	2 (10.5)	17 (89.5)		19	3 (15.8)	16 (84.2)	
Current smoker	31	4 (12.9)	27 (87.1)		32	6 (18.8)	26 (81.3)	
Tumor size				0.118*				0.522*
≤2 cm	29	0 (0)	29 (100)		32	2 (6.3)	30 (93.8)	
>2 cm	106	10 (9.4)	96 (90.6)		107	12 (11.2)	95 (88.8)	
Histologic subgroup (IASLC/ATS/ERS)				0.706				0.500
Adenocarcinoma in situ (AIS)	0	0	0		0	0	0	
Minimally invasive adenocarcinoma (MIA)	1	0 (0)	1 (100)		1	0 (0)	1 (100)	
Invasive adenocarcinoma								
with lepidic component	26	1 (3.8)	25 (96.2)		25	1 (4.0)	24 (96.0)	
without lepidic component	108	9 (8.3)	99 (91.7)		113	13 (11.5)	100 (88.5)	
Micropapillary component (percentage)				1.000*				0.598*
≤30%	126	10 (7.9)	116 (92.1)		130	14 (10.8)	116 (89.2)	
>30%	9	0 (0)	9 (100)		9	0 (0)	9 (100)	
Mitosis count (per 10 HPF)				0.706*				0.009*
0	0	0	0		0	0	0	
1-5	102	7 (6.9)	95 (93.1)		103	6 (5.8)	97 (94.2)	
>6	33	3 (9.1)	30 (90.9)		36	8 (22.2)	28 (77.8)	

*Fisher's exact test.

In all, 240 cases were considered as stage I according to the 6th edition Union for International Cancer Control/American Joint Committee on Cancer TNM classification and 29 cases were excluded after adjusting to the 7th edition [13, 14].

Histopathologic analysis

All available hematoxylin and eosin (H&E)-stained tumor slides (mean, 3.1 slides/case; range, 1-7 slides/case) were independently reviewed by 2 separate pathologists (XY and DML) who were blinded to clinical outcomes (using an leica DM3000 microscope with a standard eyepiece measuring 22 mm in diameter). Extent of lepidic and micropapillary pattern were semiquantitatively recorded by percentage present in the entire set of tumor slides in 5% increments.

All 211 cases were stratified into four groups according to the IASLC/ATS/ERS classification and percentage of lepidic component: 1) adenocarcinoma in situ, AIS (100% lepidic component); 2) minimally invasive adenocarcinoma, MIA (in all of these cases lepidic component >80%); 3) adenocarcinoma with lepidic pattern (at least 5% enumerable lepidic component), and 4) adenocarcinoma without lepidic pattern (none or less than 5%). Only 14 cases (6.6%) were considered as micropapillary predominant invasive adenocarcinoma after adjusting for the new IASLC/ATS/ERS classification, whereas 98 (46.4%) tumors contained micropapillary pattern of less quantity than the predominants, we repeated the process of dividing all cases into different groups base on the percentage of micropapillary component by setting a threshold level continuously from 5% (the minimally evaluable percentage) to 95% (the maximum), found that only when the threshold level is 30% did the micropapillary component affects survival function significantly. We also investigated mitotic count per 10 HPF which have been reported as significant histological

Table 2. Univariate analysis of association between clinicopathologic factors and 5-year overall survival (OS) and disease-free survival (DFS)

Variables	No. of patients	5-year OS rates (%)	*P*-value	5-year DFS rates (%)	*P*-value
Age			0.267		0.167
≤62	109	93.2		84.2	
>62	102	87.6		75.3	
Gender			0.542		0.009
Male	115	90.0		74.2	
Female	96	91.8		86.6	
Smoking status			0.726		0.574
Never smokers	133	92.3		79.8	
Former smokers	20	89.1		74.2	
Current smokers	50	90.0		82.6	
Tumor size (diameter)			0.020		0.008
≤2 cm (T1a)	46	100		95.4	
>2 cm (T1b)	165	88.1		75.9	
Histologic subgroup (IASLC/ATS/ERS)			0.315		0.011
Adenocarcinoma in situ (AIS)	4	90.9		100	
Minimally invasive adenocarcinoma (MIA)	26	88.4		95.2	
Invasive adenocarcinoma					
with lepidic component	46	91.8		86.6	
without lepidic component	135	77.9		74.5	
Micropapillary component (percentage)			0.112		0.048
≤30%	197	91.8		80.9	
>30%	14	77.9		70.1	
Mitosis count (per 10 HPF)			0.044		0.180
0	6	100		100	
1-5	162	93.2		79.7	
>6	43	80.1		78.0	

prognostic factors for lung adenocarcinoma. Discrepancies between the 2 pathologists were later examined on a multiple-headed microscope and consensus was made after further discussion.

Tissue microarray and immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue blocks were available with sufficient tumor tissue for tissue microarray construction in 164 patients. In brief, 2 representative tumor areas were marked on H&E-stained slides, and cylindrical 1.0-mm tissue cores were arrayed from the corresponding paraffin blocks into a recipient block by Tissue Microarrayer (Beecher Instruments corp. America), resulting in three tissue microarray blocks. Finally, 140 cases had adequate cores available for immunohistochemical analysis on all of three TMA blocks.

4 um-thick sections from the microarray blocks were deparaffinized with xylene and rehydrated. Antigens were retrieved by pressure cooking for 100 seconds in citrate buffer (pH 6.0). Sections were incubated with primary monoclonal antibodies against TTF1 (working solution, Dako) and Napsin A (clone KCG1.1; 1:100; Abcam, Cambridge, UK) for 1 hour at room temperature. Followed by a incubation with the Rabbit Anti-Goat Immunoglobulins/HRP (working solution, Dako) secondary antibody for 10 minutes at room temperature. Antibodies were visualized using the Dako EnVision kit (Dako) with diaminobenzidine as chromogen. Finally sections were counterstained with hematoxylin.

Table 3. Univariate analysis of association between molecular markers and 5-year overall survival (OS) and disease-free survival (DFS)

Variables	No. of patients	5-year OS rates (%)	*P*-value	5-year DFS rates (%)	*P*-value
In all available patients					
Expression score of TTF1	135		0.215		0.121
0	10	75.0		65.6	
1	37	82.9		71.5	
2	58	87.7		81.1	
3	30	100		87.7	
Expression score of Napsin A	139		0.031		0.507
0	14	70.7		60.6	
1	24	82.9		79.2	
2	63	90.6		81.2	
3	38	95.8		84.1	
In patients of stage IA without lepidic component			0.035		0.544
Expression score of Napsin A	113				
0	13	68.4		57.0	
1	18	77.8		77.8	
2	54	90.8		80.0	
3	28	94.1		78.8	

TTF1 positivity was scored upon observation of sole nuclear staining; Napsin A positivity was assessed by granular cytoplasmic staining. Expression was evaluated based on the intensity of immunostaining by scores (0, absent; 1, weak; 2, moderate; 3, strong).

Statistical analysis

Statistical analysis was performed by Statistical Package for Social Sciences (SPSS) Version 17.0. Relationships between clinicopathologic parameters and immune marker were evaluated using the chi-square test. Survival curves were estimated using the Kaplan-Meier method. The log-rank test was used to compare survival curves. Univariate and multivariate analyses using Cox regression were undertaken to assess the clinicopathologic variant as an independent prognostic factor for survival. Multivariate analysis was used for variables that were found to be significant in the univariate analysis. All of the statistical tests were 2 tailed and *P* value less than 0.05 was considered as statistically significant.

Results

Clinicopathological characteristics

The median age of all 211 patients was 62 years old (range: 32-82 years), with 115 patients (54.5%) being male and 96 (45.5%) being female. Most of the patients were non-smokers (n=141, 66.8%), whereas 50 (23.7%) patients were current smokers and 20 (9.5%) were former smokers.

According to the 2004 WHO classification, most cases were evaluated as mixed subtype (n=179; 85.0%). Of the 32 cases (15.0%) that had a single growth pattern, acinar was the most common pattern (n=20; 9.5%), then the nonmucinous BAC (n=4; 1.9%) and solid with mucin (n=4; 1.9%), followed by papillary (n=2; 0.9%). In addition, 1 (0.4%) case was signet ring adenocarcinoma and 1 (0.4%) was pure micropapillary pattern could not be classified by 2004 WHO classification.

According to the IASLC/ATS/ERS classification, there were 4 (1.9%) case of adenocarcinoma in situ (AIS), 26 (12.3%) cases of minimally invasive adenocarcinoma (MIA), the rest being invasive adenocarcinoma (n=181; 85.8%). According to the criteria mentioned in materials and methods previously, 46 (21.8%) cases were invasive adenocarcinoma with lepidic pattern and 135 (64%) were invasive adenocarcinoma without lepidic pattern. Micropapillary pattern was observed in 112 (53%) patients. Representative pictures of lepidic and micropapillary pattern were shown in **Figure 1**.

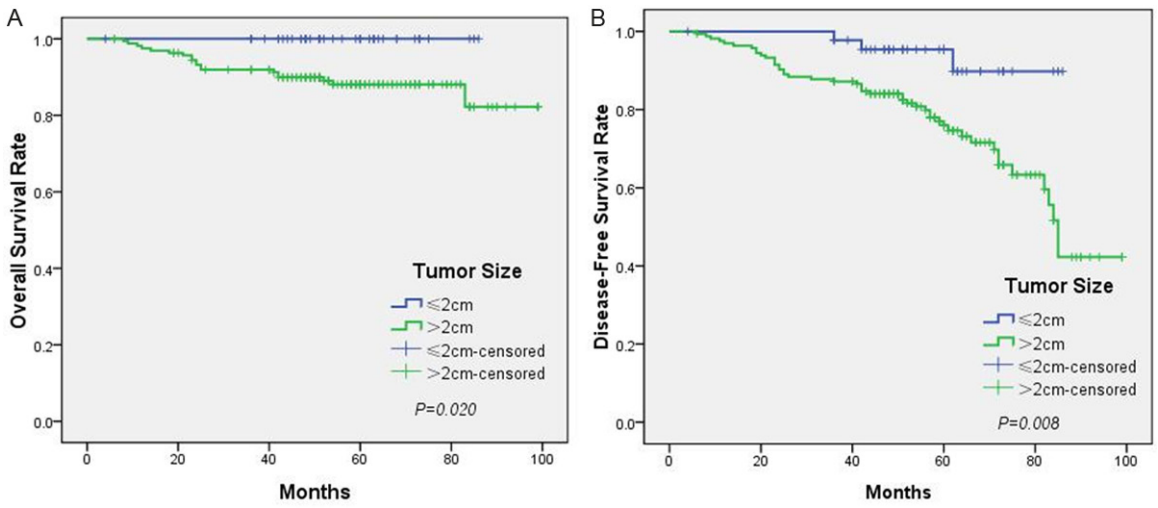

Figure 3. A. Five-year overall survival (OS) for different tumor size. B. Five-year disease-free survival (DFS) for different tumor size.

Immunohistochemical expression of TTF1 and Napsin A

For tissue cores falling off during the process of immunohistochemistry, 135 and 139 cases of TTF1 and Napsin A respectively had an evaluable result. The staining results for two immune markers were illustrated in **Figure 2**. For TTF1, there are 10 (7.4%) cases scored 0, 37 (27.4%) had a score of 1, 58 (43.0%) had a score of 2, and 30 (22.2%) had a score of 3, positive rate was 92.6% (n=125). As to Napsin A, 14 (10.1%) cases had a score of 0, 24 (17.3%) had a score of 1, 63 (45.3%) had a score of 2, and 38 (27.3%) had a score of 3, positive rate was 89.9% (n=125). There was no significant difference between clinicopathological features and expression of TTF1. Expression of Napsin A was higher in patients younger than median age (p=0.021) and patients with less mitotic counts (p=0.009). Clinicopathological Characteristics distribution according to expression (positive vs. negative) of TTF1 and Napsin A were summarized in **Table 1**.

Survival analysis

By the end of 2011, cut-off point of follow-up in this study, 168 (79.6%) patients were still alive without any progression (recurrence or metastasis), 31 (14.7%) were alive with varying degrees of progression and 18 (8.5%) patients had died of lung adenocarcinoma. Median clinical follow-up was 57 months (range from 4 to 99 months).

Univariate analysis of association between clinicopathologic factors and DSF/OS was shown in **Table 2**. Prognostic value of TTF1 and Napsin A expression was shown in **Table 3**. Female (P=0.009), higher percentage of lepidic pattern (P=0.011), lower percentage of micropapillary component (P=0.048) were associated with longer DFS. Lower mitosis count/10 HPF (P=0.044) and higher expression score of Napsin A (P=0.031) indicated a longer OS, even in patients of adenocarcinoma without lepidic pattern (poor prognostic histologic subgroup), Napsin A expression was an good prognostic factor of overall survival (P=0.035). Patients with a tumor ≤2 cm showed both better DFS (P=0.008) and OS (P=0.020). Survival curves were shown in **Figures 3**-**5**.

In multivariate analysis, gender, tumor size and histologic subtype base on new classification with slight modifications were identified as independent clinical outcome indicator in disease-free survival. For overall survival, no independent prognostic factor was found (**Table 4**).

Discussion

Results of the current study support that, the proposed IASLC/ATS/ERS international multidisciplinary classification is more useful than the 2004 WHO classification in stratifying lung adenocarcinoma into histologic subtypes with significantly different prognosis, in a cohort of 211 resected stage I cases. This confirmation coincides with many other studies reported recently [10-12].

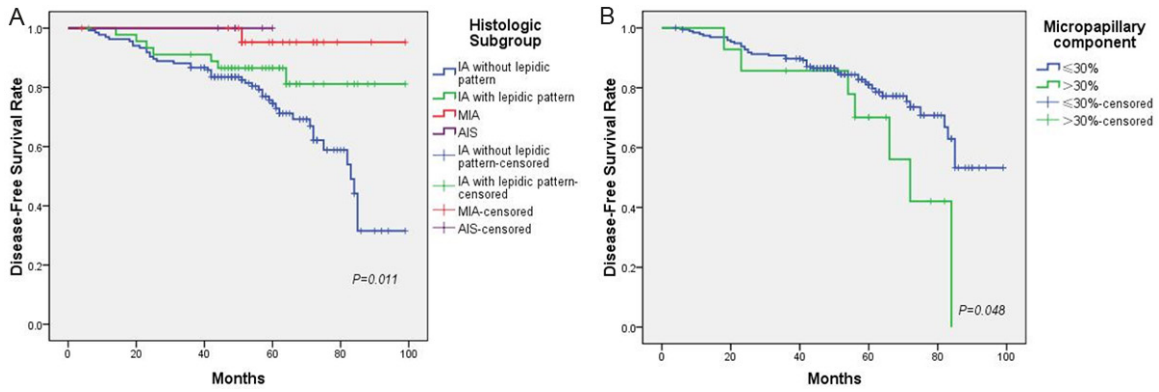

Figure 4. A. Five-year disease-free survival (DFS) for different histologic subgroup. B. Five-year disease-free survival (DFS) for different percentage of micropapillary growth pattern.

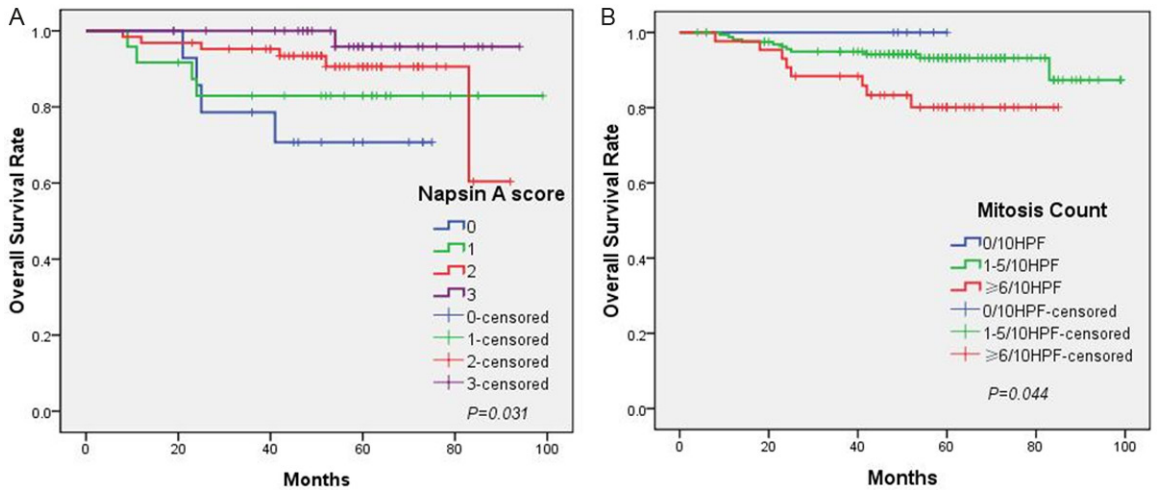

Figure 5. A. Five-year overall survival (OS) for different Napsin A expression. B. Five-year overall survival (OS) for different mitotic count.

Either in univariate or in multivariate analysis, our classification of the four histologic subgroups was significantly associated with 5-year DFS, AIS and MIA had 100% or approximate 100% 5-year DFS, supporting the advantages of the new IASLC/ATS/ERS classification in predicting clinical outcome and helpful use in selecting individual therapy. The conclusions are in line with Russell's work [10]. However, limited by the small numbers of micropapillary predominant and solid predominant adenocarcinoma, we did not follow the newly proposed classification strictly in our study. We just divided the invasive adenocarcinomas into two groups according to the present of lepidic pattern, analyses showed that, comparing with adenocarcinomas with lepidic pattern, those without lepidic pattern had a shorter DFS, even the percentage of lepidic pattern was no more than 10%, indicating that tumors lack of lepidic pattern had an elevated risk of relapse or metastasis. These findings certified lepidic pattern as a strong histologic predictor indirectly and indicate that, perhaps the simplified criteria would be suitable for stage I adenocarcinomas with the advantage of easy operation and little discrepancy between different pathologists. This assumption has not been elucidated before, however further studies should be implemented for more evidence and validation.

Analyses of the sample data were performed on tumor size and mitotic count as well. Tumor size had significant impacts on both 5-year DFS and OS, mitotic count associate with 5-year OS

Table 4. Multivariate analysis of overall survival (OS) and disease-free survival (DFS)

Variables	DFS	*P*-value	OS	*P*-value
	RR (95% CI)		RR (95% CI)	
Gender (male vs. female)	0.494 (0.274-0.891)	0.019	-	-
Histologic subgroup (non-lepidic vs. any percentage of lepidic)	0.343 (0.160-0.732)	0.006	-	-
Micropapillary component (>30% vs. ≤30%)	1.617 (0.719-3.635)	0.245	-	-
Mitosis count (≥1/10 HPF vs. none)	-	-	2.018 (0.688-5.916)	0.201
Tumor size (diameter >2 cm vs. ≤2 cm)	3.697 (1.142-11.964)	0.029	234754.032 (0.000-∞)	0.958
Napsin A (negative vs. positive)	-	-	0.339 (0.099-1.157)	0.084

Note: - is variables not included in the analysis.

in a borderline significance (P=0.044). Our result demonstrate tumor size no more than 2 cm is an independent prognostic factor of disease-free survival. Obviously, tumor size of 2 cm is a useful cut-off point, this is consistent with Noguchi's proposals [15], as well as supports the 7th UICC/AJCC criteria on TNM status, which separate T1 into T1a and T1b in accordance with a 2 cm cut-off point of tumor size. Noguchi and colleagues also pointed out that mitoses >5/10 HPF would be a worse predictor for patients' survival, our study get the same detection but with marginal significance in univariate analysis and no significance in multivariate analysis.

As to the immunohistochemical analysis, we estimated two familiar markers for clinical diagnostic use in NSCLC, thyroid transcription factor 1 (TTF1) and Napsin A. There was no significant difference between clinicopathological features and expression of TTF1. Expression of Napsin A has been found higher in patients younger than median age than in older group (p=0.021), clinical significance was indeterminate and require further verification. TTF1 has been considered as a potential prognostic indicator in NSCLC with conflicting results [16, 17]. When refer to survival analysis of our study, strong TTF1 expression is found to be an decreased risk factor of 5-year DFS and OS, the positive trend is consistent with most of the previous studies [16], but fail in statistical significance (P=0.215). It may due to a large number of cases (n=71, 33.6%) primarily brought into the histologic analysis were abandoned or lost for various reasons during the procedure of immunohistochemical analysis, such as insufficient of tumor tissue or destroyed tissue block.

According to univariate analysis of our data, higher expression of Napsin A indicated a statistical longer OS. Similarly, Lee et al [18]. We also validated that, even in patients of adenocarcinoma without lepidic pattern (histologic subgroup of poorer prognosis), predictive efficacy of Napsin A expression still exist in overall survival analysis (P=0.035). To our knowledge, the current study is the first attempt to further stratify patients with poor prognosis primarily determined by histologic classify, by way of examining Napsin A expression. Despite high expression of Napsin A was suggested to be possibly useful for detecting tumorigenic potential of rat lung hyperplasia [19], so far mechanism of effect performed by Napsin A in stage I lung adenocarcinomas is not clear, further studies are necessary to investigate the role of Napsin A in the oncogenesis of lung adenocarcinoma.

With respect to prognosis significance of other clinical features, we found that patients' gender was significantly associate with 5-year DFS, and female was an independently favorable predictor for stage I patients after adjusting to multivariate analysis. Koga and co-workers also stated that female patients had a significant longer survival than male patients [20]. There was no significant difference in survival rate between different smoking status. Since in our sample, only 70 patients (former, n=20; current, n-50) had a history of smoking, and most of them were male (n=61).

Like all research, our study had some limitations. First, we did no strictly comply with the new IASLC/ATS/ERS classification. In order to ensure that there were sufficient numbers of patient in each histologic subgroup for stable analysis, more detailed classification base on acinar, papillary and solid pattern were not performed in the invasive adenocarcinoma subgroup. Second, for the same reason, though prognostic potential of micropapillary pattern

was estimated, subgroups were too rough to offer more useful information. Third, limited by a small sample size, only 66.4% cases (n=140) were available to conduct immunohistochemistry analysis, it may result in an underestimate of the predictive efficacy of TTF1 and Napsin A. Further study might consider a large sample and more detailed research design.

Despite these limitations, our study supports the advantage of the new IASLC/ATS/ERS classification in predicting clinical outcome. Our findings offer implications for patients of early-stage lung adenocarcinoma that tumors with more than 5 percentage of lepidic patterns will have a better prognosis than absence. Furthermore, female sex, tumor size ≤2 cm, low percentage of micropapillary pattern, lack of mitosis and high Napsin A expression are markers of favorable prognosis.

Acknowledgements

This work was supported by National Science Foundation of China (N0. 30770932) and The Capital Medical Development Foundation (No. 2011-4002-01).

Disclosure of conflict of interest

None.

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