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Citation	Fukushima Journal of Medical Science. 58(1): 66-73
Issue Date	2012
URL	<a href="http://ir.fmu.ac.jp/dspace/handle/123456789/325">http://ir.fmu.ac.jp/dspace/handle/123456789/325</a>
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DOI	10.5387/fms.58.66
Text Version	publisher

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[Original Article]

## INTERPHASE CYTOGENETIC ANALYSIS OF LUNG ADENOCARCINOMAS WITH BRONCHIOLOALVEOLAR PATTERN

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(Received December 15, 2011, accepted April 6, 2012)

**Abstract** : Aneuploidy has been suggested as a marker for stratification of many neoplasms but its potential usefulness in adenocarcinoma (ADC) with bronchioloalveolar (BAC) pattern has not been well defined. We examined paraffin-embedded tissue sections from 28 cases of ADC with BAC pattern as well as 7 benign lung lesions and 9 normal lung tissue samples for chromosomal aneuploidy by in situ hybridization using digoxigenin-labelled probes for chromosomes 1 and X.

Of the 28 ADC with BAC pattern, 17 (61%) were diploid and 11 (39%) were aneuploid. Of the 17 diploid cases, 7 (41%) were male and 10 (59%) were female and of the 11 aneuploid cases, 2 (18%) were male, 9 (82%) were female. Regarding the cell type, 24 (86%) were adenocarcinomas in situ (AIS) so called BAC and minimally invasive ADC (MIA), and 4 cases (14%) were invasive ADC. Of the 12 cases each of AIS and MIA, 9 (75%) and 8 (67%) had diploid pattern respectively. Of the 4 invasive ADC cases, all had aneuploid pattern. Seventeen cases (71%) with T1 tumor size ( $>0$  mm  $\sim$   $\leq 30$  mm), had diploid and 4 cases (100%) with T2 tumor size ( $>30$  mm  $\leq 70$  mm) had aneuploid pattern. Statistical analyses showed that nuclear diploidy was significantly correlated with AIS and MIA tumor types while aneuploidy correlated with invasive ADC type ( $P=0.025$ ). Also a significant correlation was found between ploidy and tumor size ( $P=0.033$ ).

In conclusion, these findings suggest that DNA ploidy analysis provides useful information for the assessment of cellular kinetics and reflect histopathological subtypes in ADC with BAC pattern that are destined to behave aggressively.

**Key words** : Interphase cytogenetics, ploidy analysis, lung adenocarcinoma with bronchioloalveolar pattern

### INTRODUCTION

Bronchioloalveolar carcinomas (BAC) comprise a distinct category of lung ADC with an incidence rate as high as 24% of all lung cancers<sup>1)</sup> Although BAC is a low-grade malignancy and generally has a favorable prognosis, its biological behavior is variable and the postoperative outcome is unpredictable. The definition of BAC, so as adenocarcinoma in situ (AIS) based on the WHO classification is more restricted. It excludes cases with stromal,

vascular or pleural invasion<sup>2)</sup>.

Tumors showing these features are classified as ADC of mixed type with predominant BAC pattern. The current definition<sup>3)</sup> has a high clinical significance. The main reason for this revision was that resected localized BAC shows a 100% 5-year survival. The presence of multifocal tumor and aerogenous spread are usually indicative of poor prognosis. Many studies have shown that the growth rate differs markedly among tumors and that it closely correlates with prognosis. Hence, it is

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unclear which factors contribute most to the heterogeneity in the growth potential among tumors. Nuclear ploidy analysis has been shown to be useful for assessment of biological behavior of tumors<sup>4</sup>. Several approaches can be used to perform ploidy analysis such as propidium iodide (PI), karyotype analysis and flow cytometry<sup>5-7</sup>.

Karyotype analysis, however, requires tissue culture and may result in a selective growth of wrong cell populations. Flow cytometry has been successfully used to assess nuclear ploidy in a variety of tumors<sup>8</sup>. These methods have their own problems as they may include undesired cell populations and they lack the advantage of simultaneous assessment of tissue morphology.

Several studies have shown that in situ hybridization using chromosome specific probes is a reproducible and valid method of detecting numerical chromosome aberrations and assessing nuclear ploidy in routinely processed, paraffin embedded tissue sections<sup>9,10</sup>. The method had been also referred to as "interphase cytogenetics."<sup>11,12</sup>. Furthermore, we had a collection of 28 cases of ADC with BAC pattern available which provided materials for this study. Difficulties in ADC subclassification arise from the fact that ADC are highly heterogeneous histologically. Using an in situ hybridization method on serial tissue sections from lung ADC with BAC pattern, we performed DNA ploidy analysis to provide information for the assessment of cellular kinetics, T subset factors and histopathological aspect in ADC with BAC pattern.

## MATERIALS AND METHODS

### *Tissue Preparation*

We investigated paraffin-embedded tissue sections from surgically completely resected 28 cases of histologically classified as AIS (commonly called BAC), minimally invasive ADC (MIA) and invasive ADC. In term of MIA and invasive ADC, we subclassified mucinous and non-mucinos type according to the latest WHO classification<sup>2</sup>. As for BAC, MIA and invasive ADC of non-mucinous type, they were histologically classified by subtypes as clara cell, bronchial surface epithelial (BSE) and type II cell types. In addition, 9 normal lung autopsy tissue samples and surgically resected 7 benign lesions ; Mild atypia of alveolar epithelial cell (1), goblet cell hyperplasia (1), sarcoidosis (1), tuberculosis (1), adenoma (1), and alveolar wall thickening with fibrosis (2) were analyzed. Furthermore, TNM staging was

used according to the revised systems for lung cancer. T component as tumor size was examined to determine relevance to nuclear ploidy findings. We classified T1 ( $>0$  mm  $\sim$   $\leq 30$  mm), T2 ( $>30$  mm  $\leq 70$  mm) according to TNM staging system for lung cancer<sup>13</sup>. The tissues had been fixed in 10% formalin and embedded in paraffin by standard methods. Serial paraffin sections of 6  $\mu$ m thicknesses were mounted on silane-coated glass slides and incubated in an oven at 60°C overnight. A section from each tissue was stained with hematoxylin and eosin and reviewed to verify the diagnosis.

### *In Situ Hybridization*

We examined nuclear ploidy in histological sections of ADC with BAC pattern using probes specific for chromosomes 1 and X (Boehringer Mannheim GmbH, Germany). The probes were labelled in vitro in the presence of digoxigenin-11-dUTP by nick translation of the plasmids pUC 1.77 (isolated from human satellite III DNA) for chromosome 1 and pDMX1 for chromosome X under stringent conditions, the probes hybridize in situ to the pericentromeric region of chromosome 1, centromeric region of chromosome X. The probes fragment length distribution showed a maximum of 200-500 bases. Sheep antidigoxigenin (Fab fragments) labelled with peroxidase (Boehringer Mannheim) was employed to detect digoxigenin. In situ hybridization was performed according to a protocol optimized for routine paraffin sections. We incorporated microwave pretreatment of the paraffin sections to enhance signal detectability by in situ hybridization. Hybridization was considered to be satisfactory when more than 90% of cells contained signals. The sections were prehybridized for 15 minutes at 80°C with 50 ml of a mixture of 60% formamide in SSC containing 10% dextran sulphate, 50 ng/ $\mu$ l herring sperm DNA, and 50 ng/ $\mu$ l baker's yeast. After washes in SSC, the sections were hybridized with the same prehybridization mixture but containing 1 ng/ $\mu$ l of the digoxigenin labelled DNA probe to either chromosome 1 or X for 10 minutes at 80°C overnight at 37°C in a moist chamber. The slides were then stringently washed with a solution of 60% formamide in SSC and 0.05% Tween-20 and TBS to remove unbound probes. Following hybridization, the sections were incubated with 1:20 normal sheep serum for 30 minutes to block non-specific binding sites and 1:50 peroxidase labelled sheep anti-digoxigenin for 1 hour at room temperature, washed with TBS and reacted with 0.05% diaminobenzidine and 0.01% H<sub>2</sub>O<sub>2</sub> in TBS to visualize the

signals.

#### *Evaluation of Hybridized Sections*

Hybridization was considered adequate when more than 90% of cells contained signals. Hybridized sections were assessed essentially as described previously<sup>4</sup>. Criteria for counting signals were (1) overlapping interphase nuclei were excluded; (2) signals of nearly similar size were counted; (3) split spots were counted as 1 signal; and (4) weak signals were excluded. The inter observer variability in assessing the signals was estimated at less than 8%. The number of signals present in 100 nuclei from the tumor cells per section was counted. The percentage of nuclei having no, 1, 2, 3, and more than three signals for each chromosome was determined.

#### *Statistical Analysis*

Correlation of nuclear ploidy findings with histopathological features was determined by Fisher's exact test. The degree of significance was set at  $p < 0.05$ .

## RESULTS

#### *Comparison of Diploid and Aneuploid Findings with histopathological factors*

The mean percentage of aneuploid nuclei having  $\leq 2$  or  $> 2$  signals per 100 tumor cells in each section was determined. The mean percentage of aneuploid nuclei for each tissue was determined. Nuclear ploidy was shown in histological sections of normal lung and ADC with BAC pattern. Representative areas from tissue sections of normal lung tissue, benign lesions and ADC with BAC patterns were shown in Figure 1 with positive staining for chromosome 1 or X.

Figure 1A shows normal lung alveolar epithelial cells with 1 to 2 signals per nucleus and thus a diploid pattern. Panel B shows alveolar wall thickening with fibrosis with diploid pattern. Panel C shows non-mucinous AIS with BAC pattern, histologically type II cell type, with 1 to 2 signals per nucleus in most cells and thus a diploid pattern. Panel D shows aneuploid pattern as clara cell type in non-mucinous invasive ADC. Panel E shows BSE cell type from MIA with 1 to 2 signals per nucleus in most cells and thus a diploid pattern. Panel F shows invasive ADC with mucinous cell type and multiple signals per nucleus thus aneuploid pattern. Histopathologically, nine cases of diploid AIS,

6 cases were BSE subtype (67%). Of the 7 case of MIA with non-mucinous type from diploid pattern, 5 cases were clara cell type. As for 11 cases with aneuploid pattern, 3 cases of AIS were type II or clara cell subtype. But in MIA and invasive ADC, only clara cell subtype was identified. Of the 4 cases of mucinous ADC, 3 were aneuploid pattern.

#### *Histopathological and Nuclear Ploidy Findings*

Table 1 summarized the histopathological data and nuclear ploidy findings in lung ADC with BAC pattern. The patients had an average age of 62.7 years old and 19 (68%) cases were females and 9 (32%) were males. Smoking factor was recorded in 2 males (40 packs/year) and 2 females (13 packs/year). The remaining cases were non-smokers. Of the 28 ADC with BAC pattern, 17 (61%) were diploid and 11 (39%) were aneuploid. Of the 17 diploid cases, 7 (41%) were male and 10 (59%) were female. Of the 11 aneuploid cases, 2 (18%) were male, 9 (82%) were female. Regarding the cell type, 24 (86%) cases were AIS (43%) and MIA (43%) and 4 cases (14%) were invasive ADC. Of the 12 cases each of AIS and MIA, 9 (75%) and 8 (67%) had diploid pattern respectively. Of the 9 non-mucinous MIA, 7 (78%) had diploid and 2 (22%) had aneuploid pattern. All of the 4 invasive mucinous and non-mucinous ADC cases, had aneuploid pattern. There was a marked difference in the rate of aneuploidy and diploidy between AIS and MIA vs. invasive ADC ( $p = 0.025$ ). Proportion of diploid pattern between AIS and MIA showed same extent but invasive ADC was increasingly aneuploid. The 3 patients who died of the disease were aneuploid pattern. The 9 normal lung tissue samples and 7 benign lesions were classified as diploid pattern.

Seventeen cases (71%) with T1 factor (tumor size  $> 0$  mm  $\sim \leq 30$  mm) had diploid and 4 cases (100%) with T2 factor (tumor size  $> 30$  mm  $\leq 70$  mm) had aneuploid pattern. The frequency of aneuploidy was elevated in ADC larger than 30 mm tumor size (T2). The case with the largest tumor diameter measuring 57 mm tumor was aneuploid. T1 tumor size was correlated with AIS cell type of diploid pattern. But T2 tumor size was coincided with MIA and invasive ADC of aneuploid pattern. There was a significant correlation of diploid pattern with T1 tumor size but aneuploid pattern indicated T2 tumor size ( $p = 0.033$ ).

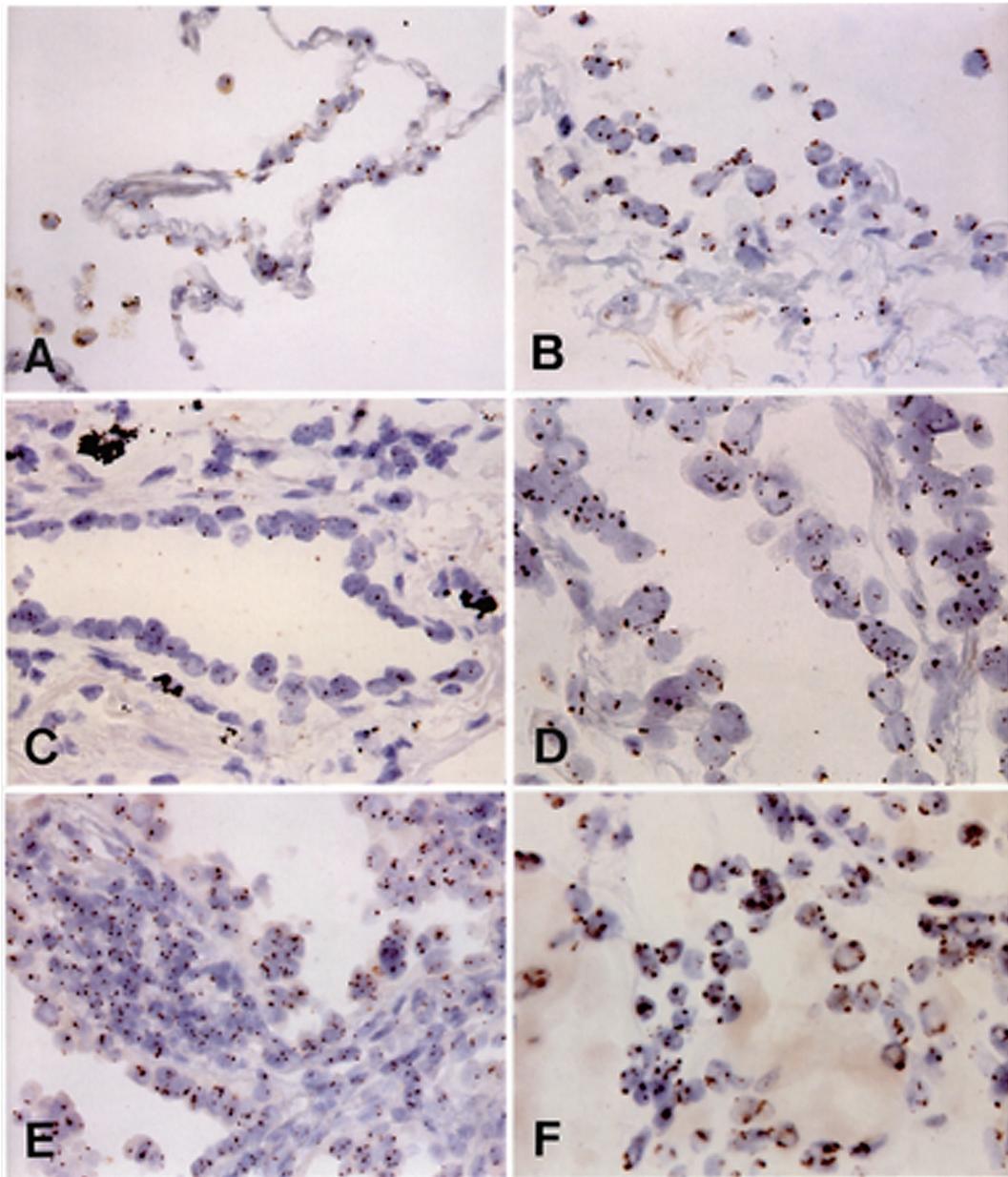


Fig. 1. Nuclear ploidy findings of normal lung tissue, benign lesions and ADC with BAC pattern. (A) Normal lung alveolar epithelial cells showed 1 to 2 signals per nucleus and thus a diploid pattern. (B) Alveolar wall thickening with fibrosis showed a diploid pattern. (C) Non-mucinous BAC, histologically classified as type II cell type, showed 1 to 2 signals per nucleus in most cells and thus a diploid pattern. (D) Aneuploid pattern classified as clara cell type in non-mucinous invasive ADC. (E) BSE cell type from BAC with 1 to 2 signals per nucleus in most cells and thus a diploid pattern. (F) Invasive ADC with mucinous cell type showed multiple signals per nucleus and thus aneuploid pattern.

## DISCUSSION

It is widely known that ploidy changes may play a role in the pathogenesis and the development of many different clinicopathological features of the neoplasms, but the actual mechanisms have not exactly determined and may be different in accordance with the organs and the cyto- and histopathological subtypes<sup>14,15</sup>. We performed a comparative

study on the presence or the absence of the chromosomal aneuploidy in 28 cases of ADC with BAC pattern, 7 cases of benign lung diseases and 9 normal lung tissue samples. BAC is a unique pattern of lung cancer which expresses subtle but distinctive features based on the age of the patient, sex ratio, cyto- and histopathology of the tumor, growth rate, progression, and prognosis. The mean age of patients with BAC is younger than that of patients

Table 1. Comparison between nuclear ploidy pattern in lung ADC with BAC pattern

Feature	No. of cases	Ploidy pattern	
		Aneuploid	Diploid
Total	28	11	17
Mean age (yr)	62.7	64	61.8
Histological type*			
AIS	12	3	9
MIA	12		
Mucinous	3	2	1
Non-mucinous	9	2	7
Invasive ADC	4		
Mucinous	1	1	0
Non-mucinous	3	3	0
T factor**			
T1	24	7	17
T2	4	4	0

\*AIS and MIA vs. invasive ADC ( $P=0.025$ )\*\* T1 vs. T2 ( $P=0.033$ )

ADC; adenocarcinoma BAC; bronchioloalveolar carcinoma AIS; adenocarcinoma in situ

MIA; Minimally invasive ADC T factor; Tumor size T1 &gt;0 mm ~ ≤30 mm

T2 &gt;30 mm ~ ≥70 mm

with non-BAC lung carcinoma by 5 years<sup>16,17</sup>. Our findings disclosed that the incidence of BAC was predominant in female but other studies showed various male to female ratios<sup>18,19</sup>. Regarding the multifocal growth potential, the growth of mucinous subtype is more rapid than that of the sclerotic subtype and BAC cases exhibited a 20% incidence of dedifferentiation into patterns of poorly differentiated ADC<sup>19</sup>. Ploidy analysis or analysis of the elevated DNA content of the tumor cells can be one of the powerful biological markers to differentiated BAC from the non-BAC counterpart<sup>20</sup>. It may also be helpful to know the pathologic role of BAC played in the development and progression of lung ADC<sup>21-24</sup>. In addition, correlative study between BAC aneuploidy and many different distinctive features of the neoplasm including its epidemiology, gross and microscopic pathology, progression and prognosis may give clues to obtain more profound understanding of the malignant potential of lung tumors<sup>25</sup>. The method using in situ hybridization has some advantages in the ploidy analysis in oncology. It can readily be applied to the archival formalin fixed paraffin-embedded materials because of the minimal physico-chemical damages to the tumor cell DNA. The discernible visualization results are also suitable for the aim of our study to make proper evaluation of the correlation between the tumor

growth and other associated features. The in situ hybridization technique is applicable to numerous different tumors from the different organs including the gastrointestinal tract the hepatobiliary tract, the urinary tract<sup>26</sup>, the gynecological tract such as the uterus<sup>27</sup>, the breast<sup>28</sup>, the central nervous system<sup>29</sup> and it enables us to make comparative studies on the tumorigenesis as a whole.

Our study revealed difference in the occurrence of aneuploidy between subtypes of ADC with BAC pattern and benign tumors or normal lungs. Of the ADC with BAC pattern, 11 were aneuploid and 17 were diploid. One benign lung disease with mild atypia of alveolar epithelial cell was aneuploid and 15 benign and normal lungs were diploid. These results suggest the possibility that aneuploidy may reflect T subset factors and histopathological aspect of subtypes of ADC with BAC pattern. More detailed study of ours focusing on the distribution of aneuploidy among the cases of ADC with BAC pattern disclosed the fact that there may be some interesting correlation between the occurrence of aneuploidy and several pathologic features such as T factor from TNM subset, histopathologic subtypes including AIS, non-mucinous MIA or invasive ADC and mucinous subtypes of ADC with BAC pattern. Also ploidy findings reflected histopathologic subset of BSE type, Clara cell type, and type II cell

type. The frequency of aneuploidy was markedly elevated in the ADC more than T2 subset. Seven cases with ADC above T1 were aneuploid and 17 cases with tumors smaller than 30 mm as T1 were diploid. Four patients with tumors more than T2 were aneuploid. The case with the largest tumor diameter measuring 57 mm same as T2 was aneuploid. T1 with less than 30 mm in tumor size was correlated with AIS cell type. But T2 above 30 mm in tumor size was coincided with MIA and invasive ADC. Two patients who died of the disease had aneuploid pattern of mucinous ADC with T2. These findings suggest that DNA ploidy analysis provides useful information for the assessment of cellular kinetics and reflects T factors with ADC of BAC pattern<sup>30</sup>. A positive correlation between aneuploidy and mucinous BAC was identified; 3 cases with mucinous ADC with BAC pattern were aneuploid and 1 case was diploid. Also, 8 cases with non-mucinous ADC with BAC pattern were aneuploid and 16 cases were diploid. Thus, a positive correlation of aneuploidy with mucinous ADC with BAC pattern growth and with dedifferentiation capability of non-mucinous ADC with BAC pattern into mucinous counterpart can be suggested. BAC with different cytopathological features showed different aneuploidy ratios. The aneuploid BSE was far less frequent than diploid BSE type. Diploid BSE type was frequently observed in BAC. On the contrary, MIA and invasive ADC were not detected. Such difference may be related to the growth potential of ADC with BAC pattern which is derived from different precursor-progenitor cells<sup>31</sup>.

It may also be due to the different growth processes via the metaplasia among the basal epithelium, clara cells and type II pneumocytes. Aneuploidy was more frequently observed in ADC with BAC pattern of more advanced clinical stages than low stages. Of the 24 cases of T1 factor, 17 case with diploid pattern, 7 cases with aneuploid pattern. The factors of larger tumor size, mucinous subtype, MIA and invasive ADC subtype were closely related to aneuploid DNA elevation. There were no statistically significant correlation between aneuploidy and gender, age, cancer staging or grading but aneuploidy promoted the occurrence of early distant metastasis while the diploid type was associated with late (after 3 years) local tumor recurrence<sup>32,33</sup>.

The results of our retrospective study suggest a potential that aneuploidy DNA elevation ADC with BAC pattern may be one of the efficient markers of malignancy<sup>34</sup>. Filderman *et al.*<sup>14</sup> analyzed DNA con-

tent and proliferative S-phase fraction of stage I (T1N0M0 and T2N0M0) subset non-small cell lung carcinoma of the lung using flow cytometry and reached the conclusion that the measurement of the DNA content and the tumor proliferative fraction in T1N0M0 subset NSCC may provide prognostic information and that it may help identify a subset of patients at high risk for tumor relapse. Miyamoto *et al.*<sup>25</sup> evaluated prognostic significance of the DNA ploidy, Ras, and p21 expression by the stages of the lung carcinoma and they found DNA content as malignant potential indicator for ADC suggesting the needs for the multivariate combinatorial evaluation using the DNA ploidy study and other biological markers to lung cancer<sup>35</sup>.

Recently, mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) are frequently detected in lung ADC with BAC differentiation. 70 ADC tumors with BAC components were screened for EGFR mutations within exon 18-21. Chromosomal imbalances and EGFR mutations were detected in 24% of BAC. Also, mutated EGFR demonstrated gains in chromosome 7p,16p and 20q and losses in 8p<sup>36-38</sup>. Presence of EGFR mutations seems to be linked to a variety of chromosomal imbalances and DNA ploidy evaluation. Additional studies are clearly warranted. Our study revealed that ploidy change may reflect T factors and histopathological subtypes of ADC with BAC pattern. Based on ploidy analysis, AIS and MIA may be regarded as having a similar behavior. A finding in our study is that determination of diploidy in ADC with BAC pattern reflects smaller tumor size as T1 and suggest AIS thus favorable patients prognosis. But aneuploidy in ADC with BAC pattern suggests the possibility of MIA or invasive ADC, therefore, a need for careful follow up to detect early metastasis.

In conclusion, our findings suggest that nuclear aneuploidy may be an indicator of carcinogenesis in the lung, and detection of aneuploidy by interphase cytogenetics using chromosome specific probes may be useful in identifying ADC with BAC subclassification that are destined to behave aggressively.

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