Original Article

Mitosis count and number of cancer cells in cases of primary pulmonary adenocarcinoma – correlations among phosphorylated histone 3, number of cancer cells, nuclear grade, pathologic features and prognosis

Running title: Mitosis count in lung adenocarcinoma

Abstract

Immunohistochemistry findings for the phosphorylated form of histone 3 (pHH3) have been shown to be a reliable mitosis-specific marker. We evaluated the correlation between pHH3-stained mitotic figures (PHMFs) and clinical outcome, and compared the results with findings for numbers of PHMFs and cancer cells. The primary tumor was obtained from 113 patients with pulmonary adenocarcinomas (≤2 cm maximum dimension). All specimens were stained with pHH3, then the number of cancer cells in each was determined. Cases with a cancer-cell index ≥1000 showed worse recurrence-free survival as compared to those with a value <1000 ($p<0.001$). Also, cases with a pHH3 index ≥0.27 showed worse recurrence-free survival as compared to <0.27 ($p=0.001$) and cases with a pHH3/cancer-cell index ≥0.001 showed worse recurrence-free survival as compared to <0.001 ($p=0.002$). Multivariate analysis demonstrated that pHH3/cancer-cell index was significantly correlated with prognosis, but not Ki-67 index. The number of cancer cells was also strongly correlated with progression of Noguchi's classification and WHO pathologic type. pHH3/cancer-cell index was correlated with prognosis, and those were useful for prognostic

evaluation of pulmonary adenocarcinoma patients. Furthermore, cancer cell number was correlated with Noguchi's classification and WHO pathologic type.

Keywords: nuclear grading, pulmonary adenocarcinoma, mitosis, histological classification, phosphorylated histone 3

Introduction

Based on histology findings, adenocarcinoma is the most frequent type of lung cancer encountered in most countries.[1] Histological grading of lung cancer is a significant indicator of prognosis, with several prognostic factors shown to correlate with patient outcome. In addition, increasing evidence suggests that lung adenocarcinoma morphologic characteristics, such as architectural and nuclear features, can stratify patients in regard to prognosis.[2–10] The newly proposed International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) International Multidisciplinary Classification of Lung Adenocarcinoma clearly emphasizes the prognostic significance of histologic subtypes in lung adenocarcinoma,[11] which has been validated by use of independent datasets.[12] Furthermore, to define comprehensive pathological prognostic marker sets, interest in the prognostic value of pathologic characteristics other than architecture is increasing. For example, evidences showing that mitotic count[13, 14] and Ki-67 labeling[15] are useful for predicting recurrence of stage I adenocarcinoma have been presented, as

well as findings indicating that nuclear area and major dimension are independent prognostic markers.[9]

Recently presented immunohistochemistry (IHC) results for phosphorylated histone 3 (pHH3), which is present during early prophase, have shown it to be a reliable mitosis-specific marker.[16, 17] pHH3 has also been found useful for assessment of proliferation index in gliomas,[18] meningiomas,[19] melanomas,[20] uterine smooth muscle tumors,[21] and pulmonary neuroendocrine carcinomas,[22] as well as to decrease inter-observer variability for detection of mitosis in melanomas.[20] pHH3 should be interpreted as positive only in cells with a staining pattern that highlights a mitotic figure.

Thus far, no study has been presented showing a correlation between pHH3-stained mitotic figures (PHMFs) and survival in patients with a pulmonary adenocarcinoma. In this study, we evaluated the correlation between PHMFs and clinical outcome, and compared the results with those for hematoxylin and eosin mitotic figures (HEMFs) and Ki-67 labeling index.

There are also no known studies regarding the correlation between number of cancer cells in a tumor and prognosis. Here, we counted the number of cancer cells in tumors and calculated tumor size, by which the number of cancer cells /mm^2 was calculated. We then evaluated the correlation of number of cancer cells with WHO classification, Noguchi's classification, and patient prognosis.

Material and methods

Patients

Primary tumors were obtained from consecutive 113 patients with pulmonary adenocarcinomas (2 cm maximum dimension) who were treated surgically during the period between January 2006 and December 2010. All patients underwent surgical resection at Dokkyo Medical University Hospital, Tochigi, Japan and informed consent for using materials for the study was obtained from each. Dokkyo Medical University Hospital Ethical Committee approved this study (#28147). Follow-up examinations were completed for all patients by September 2017.

Tissue specimens and pathologic information

Resected specimens were fixed in 10% to 15% neutral buffered formalin at room temperature, then embedded in paraffin for histology examinations. Sections (2 μm thick), including the largest cut surface of the tumor, were made, then stained with hematoxylin and eosin (H&E), and examined under light microscopy. Tumors were classified according to the criteria of the WHO International Histological classification of Tumours[23] as well as histological criteria proposed by Noguchi and colleague[24], which classified small-sized lung adenocarcinomas histologically. Staging was evaluated according to the International Union Against Cancer TNM Classification of Malignant Tumors (seventh edition)[25].

Immunohistochemical examinations

After all H&E-stained slides were reviewed, 1 representative block from each case was selected. Next, 4-μm sections were cut from the selected blocks, then deparaffinized in xylene and dehydrated in graded alcohol solutions. A standard avidin–biotin complex peroxidase technique was used for immunohistochemical staining of primary antibodies against pHH3

(Ser10, polyclonal, dilution 1:2000) and an anti-Ki-67 antibody (clone MIB-1, Immunotech, Westbrook, ME; dilution 1:100). For immunohistochemical staining, 4-μm-thick sections were deparaffinized. Heat-induced epitope retrieval was performed using Target Retrieval Solution High pH (DAKO, Carpenteria, CA) for pHH3 and Target Retrieval Solution (DAKO) for Ki-67. The sections were then stained using a Ventana Discovery XT automated immunohistochemical staining device (Ventana, Tucson, AZ), according to the manufacturer's guidelines. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain.

Histologic evaluations and nuclear grading criteria

Mitoses were counted using 1 representative H&E- and pHH3-stained slide for each case under an Olympus BX53 microscope (Olympus, Tokyo, Japan). In this manner, the number of mitoses in each tumor specimen was determined. We also counted the number of cancer cells in the tumor specimens using a NanoZoomer® Digital Pathology device (Hamamatsu Photonics, Japan). All counts were performed by viewing the monitor screen with a ×40 objective and ×10 video ocular. When examining the fields, all

tumor cells were selected, while necrotic and inflammatory areas were avoided, though overlapping nuclei were omitted. The nuclear profile measurement area was assessed by tracing the nuclear membrane with the computer mouse (Figure 1 a). We also count square-meter of tumor by this virtual slide system (Figure 1 b).

We also determined the Ki-67 index of 1000 of the counted tumor nuclei, which was expressed as the percentage of Ki-67-positive nuclei as compared with all counted tumor nuclei.

We previously reported that the nuclear grading system is a useful parameter for determining the prognosis of patients with small pulmonary adenocarcinomas.[9] In this study, we chose fields where large nuclei were frequently observed in the largest cut surface of each tumor. Necrotic and inflammatory areas were avoided, and overlapping nuclei were omitted. Any tumor cell was judged to be positive when its nuclear area and nuclear dimension were 5 and 3 times larger, respectively, than the corresponding values for small lymphocytes (figure 2). Trained observers (I.Y, K.M, and K.H) reviewed each slide in detail using low magnification (×40) and selected a few areas that included tumor cells with large nuclei. The number

of tumor cells judged to be positive was then counted and a field was judged as a positive field if it contained more than 5 positive cells. The case was judged as a positive case when more than 3 such positive fields were observed.

Statistical analysis

Associations between clinicopathologic variables and histologic findings were analyzed using Fisher's exact test for categorical variables and Wilcoxon's test for continuous variables. Analyses of the correlations of clinicopathologic features with nuclear size and pHH3 mitosis were performed using an F test, Student's t test, and Tukey's test. Receiver operating characteristic (ROC) curve analysis was done to estimate an optimal cutoff level of each variable. Patients were followed from the time of surgery to documented recurrence, and censored on the date of death or last follow-up if no recurrence was noted. Data for patients lost to follow-up were also censored. Overall survival (OS) and recurrence-free survival (RFS) curves were calculated using the Kaplan-Meier method and non-parametric group comparisons were performed using a log-rank test. Multivariate

analyses were performed using Cox proportional hazards regression. Relationships among the number of HEMFs, PHMFs, and Ki-67 index were investigated using Pearson's correlation coefficient test. A p value less than 0.05 was regarded as significant. We examined features shown to be significant in univariate analysis findings using a multivariate model. Independent staging factors for pulmonary adenocarcinomas were evaluated by multivariate analysis for nuclear grading. All analyses were performed using the SPSS statistical software package (version 23.0; SPSS, Chicago, IL, USA, 2016).

Results

Clinical findings

The clinicopathologic features of the cases examined are listed in Table 1. Mean observation periods were both 5.7 years, ranged from 0.5 to 9.1 years. The clinicopathologic features of the recurrence cases examined are listed in Supplementary Table 1.

Correlation between nuclear grade and prognosis

Kaplan-Meier survival curve analysis showed that the mean RFS of patients whose nuclear grade was positive was 8.6 years (95% confidence interval(CI), 7.62 - 9.50), while that of those with nuclear grade negative findings was 10.8 years (95% CI,10.6 - 11.6). Thus, a nuclear grade positive finding was significantly correlated with recurrence ($p<0.01$) (Figure 3 a). Also Kaplan-Meier survival curve analysis showed that the mean OS of patients whose nuclear grade was positive was 7.3 years (95% CI, 6.66 - 7.94), while that of those with nuclear grade negative findings was 8.4 years (95% CI, 7.84 - 9.04). Thus, a nuclear grade positive finding was significantly correlated with shorter survival (p=0.037) (Supplementary Figure 1 a).

Correlation between cancer cell index and prognosis

We counted the number of pHH3-positive cells among all cancer cells in a total of 113 slides (Figure 1). The mean number of pHH3-positive cells per slide was 32.8 (range 0 to 234).

In small-sized adenocarcinomas, there were few positive cells, thus we analyzed all tumor cells in whole specimens and found that number of cancer cells ranged from 3670 to 178,568 per tumor, with an average of

67,605 (Figure 2). We defined cancer cell index as the number of cancer cells within 1 mm^2. ROC curve analysis showed a cut-off value of 992 /mm^2 (area under the curve (AUC)=0.697, 95%CI=0.576-0.817). In general, cases with a cancer cell index of ≥1000 had worse RFS as compared to those with a value <1000 (mean RFS 5.42 years, 95% CI 3.80 - 7.04vs. 10.6 years, 95% CI 10.1 - 11. 2, p<0.001) (Figure 3 b). Also cases with a cancer cell index of ≥1000 had worse OS as compared to those with a value <1000 (mean OS 5.95 years, 95% CI 4.75 - 7.15vs. 8.14 years, 95% CI 7.72 - 8.65, p<0.001) (Supplementary Figure 1 b).

Correlation between pHH3/cancer cell index and prognosis

We defined pHH3 index as the number of pHH3 positive cells within 1 mm^2. ROC curve analysis revealed a cut-off value of 0.27 /mm^2 (AUC=0.725, 95%CI=0.595-0.855). Cases with a pHH3 index of ≥0.27 had worse RFS as compared to those with a value <0.27 (mean RFS 7.73 years, 95% CI 6.64 - 8.81 vs. 10.8 years, 95% CI 10.3 - 11.4, p<0.001) (Figure 3 c). Also cases with a pHH3 index of ≥0.27 had worse OS as compared to those with a value <0.27 (mean OS 6.95 years, 95% CI 6.92 - 7.70 vs. 8.46 years, 95% CI 7.95 - 8.97, p=0.001) (Supplementary Figure 1 c).

We then defined the cut-off number for pHH3 positive cells/cancer cells as the pHH3/cancer cell index. ROC curve analysis revealed a cut-off value of 0.00092 (AUC=0.661, 95%CI=0.531-0.792). Cases with a pHH3/cancer cell index of ≥0.001 had worse RFS as compared to those with a value <0.001 (mean RFS 5.86 years, 95% CI 3.77 - 7.96 vs. 9.92 years, 95% CI 9.24 - 10.6, p<0.001) (Figure 3 d). Also cases with a pHH3/cancer cell index of ≥0.001 had worse OS as compared to those with a value <0.001 (mean OS 5.74 years, 95% CI 4.29 - 7.19 vs. 7.97 years, 95% CI 7.49 - 8.45, p=0.002) (Supplementary Figure 1 d).

Furthermore, ROC curve analysis of Ki-67 index revealed a cut-off value of 11.3 (AUC=0.676, 95%CI=0.566-0.786). Cases with a Ki-67 index of ≥11.3 had worse RFS as compared to those with a value <11.3 (mean RFS 7.97 years, 95% CI 6.66- 9.29 vs. 10.3 years, 95% CI 9.61-11.03, p=0.005) (Figure 3 e). Also Cases with a Ki-67 index of ≥11.3 had worse OS as compared to those with a value <11.3 (mean OS 7.15 years, 95% CI 6.33 - 7.97 vs. 8.08 years, 95% CI 7.52- 8.63, p<0.001) (Supplementary Figure 1 e).

Correlations between pHH3/cancer cell index and Noguchi's classification or

WHO classification

Univariate analysis demonstrated that pHH3/cancer cell index, cancer cell index, pHH3 index, Ki-67 index, nuclear grade, WHO classification, pathological stage, lymphatic invasion and vascular invasion were strongly correlated with patient RFS ($p<0.05$) (Table 2). Also univariate analysis demonstrated that pHH3/cancer cell index, cancer cell index, pHH3 index, nuclear grade, WHO classification, pathological stage and lymphatic invasion were strongly correlated with patient OS ($p<0.05$) (Supplementary Table 2).

Multivariate analysis demonstrated that pHH3/cancer cell index was strongly correlated with patient RFS ($p<0.05$). Furthermore, Cox regression analysis showed that pHH3/cancer cell index had a hazard ratio of 3.505 ($p=0.008$) (Table 2). Also multivariate analysis demonstrated that pHH3/cancer cell index and WHO classification were strongly correlated with patient OS ($p<0.05$). Furthermore, Cox regression analysis showed that pHH3/cancer cell index had a hazard ratio of 2.445 ($p=0.048$) (Supplementary Table 2).

We analyzed the correlation between number of cancer cells and Noguchi's classification, and noted a greater number of cancer cells as the classification increased (Figure 4 a). We divided the patients into the Noguch-1 group, which was composed of those classified as Noguchi A and B, and Noguchi-2, composed of Noguchi C, D, E, and F patients. Cancer cell index showed a strong correlation with this grouping (average: 537.6/mm^2 in Noguchi-1, 848.0/mm^2 in Noguchi-2, $P<0.05$). We also analyzed the correlation between number of cancer cells and WHO classification, and found that the classification became worse with a greater number of cancer cells (Figure 4 b). In addition, we divided patients based on WHO classification into 2 groups, those with an adenocarcinoma *in situ* or minimally invasive adenocarcinoma (WHO-1), and cases of invasive carcinoma including lepidic, papillary, acinar, and solid predominant adenocarcinomas (WHO-2). Our results showed that cancer cell index was strongly correlated with this grouping (average: WHO-1, 620.7/mm^2; WHO-2, 872.8/mm^2; $p<0.05$)

Discussion

In this curated series of resected pulmonary adenocarcinoma cases, pHH3/cancer cell index were independent prognostic factors related to OS and RFS. If prospectively validated, our results may have important implications for adjuvant management and follow-up of patients undergoing resection of lung adenocarcinoma.

Mitotic figures are indicators of tumor proliferation ability, with higher growth rate related to higher grade of malignancy and elevated risk of recurrence. Histopathologically, assessment of mitotic figures is important and performed for patients with a variety of diseases. In cases of astrocytoma, WHO recommends that mitotic count is useful for pathological grading.[26] Similarly, mitotic count is important for gastrointestinal stromal tumor cases[27] and it has been pointed out that it correlates with recurrence and patient prognosis.

Mitotic count for lung cancer has been examined in several studies. Ki-67 labeling index was found to be a statistically significant prognostic factor for disease-free survival of patients with non-small cell lung cancer who underwent a video-assisted thoracoscopic segmentectomy procedure.[15] Also, Kadota et al. demonstrated a significant association of mitotic count

with histology grade, Ki-67 proliferative index, and recurrence-free probability, and concluded that mitotic count was the single most important predictor of recurrence in patients with stage 1 lung adenocarcinoma.[14] In another study, Duhig et al. suggested that mitotic index is the only independent prognostic marker for patients who have undergone resection for stage I lung adenocarcinoma.[28]

In our study, we counted mitotic figures using the pHH3 stain. Recently, immunohistochemistry findings for pHH3, which is present during early prophase, have shown it to be a reliable mitosis-specific marker[16, 17] and also useful for assessment of proliferation index in gliomas,[18] meningiomas,[19] melanomas,[20] and uterine smooth muscle tumors,[21] with decreased inter-observer variability for detecting mitosis in melanomas.[20] pHH3 should be interpreted as positive only in cells with a staining pattern that highlights a mitotic figure. Counting mitotic figures with the assistance of pHH3 immunostaining is more sensitive for detecting mitotic figures than the traditional method of counting H&E-stained mitotic figures, which was reported by Tsuta et al. regarding lung neuroendocrine tumors.[22] In the present study, pHH3 index was strongly correlated with patient prognosis,

as those with a value ≥0.27 had worse OS as compared to those with a value <0.27 (5-year survival 69.5% vs. 92.7%, p=0.001). In the present study, we counted the number of cancer cells in individual tumor specimens. Although there are no previous reports regarding the number of cancer cells in lung adenocarcinoma tumors, we considered that a higher number of cancer cells would be associated with worse prognosis, thus cancer cell index would also be strongly correlated with prognosis. Our results showed that patients with a cancer cell index ≥1000 had worse OS as compared to those with an index <1000 (5-year survival 52.5% vs. 89.3%, p<0.001). Also, in analysis of the correlation between number of cancer cells and Noguchi's classification, the classification increased as the number of cancer cells increased. After dividing our patients into two groups based on Noguchi's classification (Noguchi-1: Noguchi A, B; Noguchi-2: Noguchi C, D, E, F), cancer cell index was shown to be strongly correlated with that grouping (average: Noguchi-1, 537.6/mm^2; Noguchi-2, 848.0/mm^2; P<0.05). We also analyzed the correlation between number of cancer cells and WHO classification, which revealed that the classification worsened as the number of cancer cells increased. Following division of our patients into two groups based on WHO

classification (WHO-1: adenocarcinoma *in situ*, minimally invasive adenocarcinoma; WHO-2: invasive carcinoma including lepidic, papillary, acinar, solid predominant adenocarcinomas), cancer cell index was strongly correlated with this grouping (average: WHO-1, 620.7/mm^2; WHO-2, 872.8/mm^2; $p<0.05$).

We analyzed the pHH3/cancer cell index in the present study and found a strong relationship between it and OS (log-rank test, P=0.048). Thus, when the ratio of pHH3-positive cells to cancer cells is greater than 1:1000, patient prognosis is poor. In addition, multivariate analysis showed that high pHH3/cancer cell index was significant risk factors for death.

In conclusion, our findings stress the importance of pHH3/cancer cell index for estimating malignancy of small-sized adenocarcinomas in the lung. Application of pHH3/cancer cell index along with a pure histological classification such as the WHO or Noguchi classification may make more precise prediction of the biological behavior of small-sized adenocarcinomas as compared to histological classification.

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Conflict of interest

None declared.

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Figure Legends

Figure 1. Histology findings of small adenocarcinoma.

We counted the number of cancer cells (yellow-lined nucleus) in each tumor specimen using a NanoZoomer® Digital Pathology device (Hamamatsu Photonics, Japan).

Figure 2. Histological findings of representative small pulmonary adenocarcinomas judged to be nuclear grade positive (a, b, c) or nuclear grade negative (d, e, f).

a) Hematoxylin and eosin stain, original magnification ×200. Solid-type adenocarcinoma with nuclear grade positive cells.

b) Ki-67 staining of tumor shown in 1-a, original magnification ×200. A large number of Ki-67-positive cells can be seen.

c) PHH3 staining of tumor shown in 1-a, original magnification ×200. Three pHH3-positive cells can be seen.

d) Hematoxylin and eosin stain, original magnification ×100. Lepidic-type adenocarcinoma with nuclear grade negative cells.

e) Ki-67 staining of tumor shown in 1-d, original magnification ×100. Only a few Ki-67-positive cells can be seen.

f) PHH3 staining of tumor shown in 1-d, original magnification ×100. One pHH3-positive cell can be seen.

Figure 3. Recurrence-free survival curves for all patients.

(a) Recurrence-free survival curves for patients with nuclear grade positive and negative ($p<0.01$). (b) A cancer cell index (number of cancer cells/mm2) value of 1000 was used as the cutoff ($p<0.001$). (c) A pHH3 index (number of pHH3 cells/mm^2) value of 0.27 was used as the cutoff ($p<0.001$). (d) A pHH3/cancer cell index (number of pHH3 positive cells among all cancer cells) value of 0.001 was used as the cutoff ($p<0.001$). (e) A Ki-67 index value of 0.125 was used as the cutoff ($p=0.005$).

Figure 4. Box plot of cancer cells in all patients classified according to (a) Noguchi's and (b) WHO pathologic classification.

Supplementary Figures Legends

Supplementary Figure 1. Overall survival curves for all patients.

(a) Overall survival curves for patients with nuclear grade positive and negative ($p=0.037$). (b) A cancer cell index (number of cancer cells/mm2) value of 1000 was used as the cutoff ($p<0.001$). (c) A pHH3 index (number of pHH3 cells/mm^2) value of 0.27 was used as the cutoff ($p=0.001$). (d) A pHH3/cancer cell index (number of pHH3 positive cells among all cancer cells) value of 0.001 was used as the cutoff ($p=0.002$). (e) A Ki-67 index value of 0.125 was used as the cutoff ($p<0.001$).

Supplementary Table Legends

Supplementary Table 1. Recurrent patients characteristics

Supplementary Table 2. Cox regression analysis findings of overall survival