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Association of Ki67 Antigen With Mitotic Count, Tumor Size, and Necrosis in Neuroendocrine Tumors of Lung

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Abstract

Objectives: Lung cancer is the most prevalent cancer in the world and one of the five most common cancers in Iran. The level of Ki67 biomarker is related to any of the factors affecting the grading of neuroendocrine lung tumors, which is used as one of the grading criteria and a criterion for assessing and predicting survival and prognostic factors. The aim of this study was to investigate the relationship between Ki67 antigen incidence and mitotic count, necrosis, tumor size in the neuroendocrine tumors of the lung.

Materials and Methods: The samples of this analytical descriptive study, including the neuroendocrine tumors of the lung in paraffin blocks were chosen from the pathology archive of Imam Reza hospital collected during the last 10 years. Ki67 antigen immunohistochemical procedures, along with mitotic count and necrosis were performed by a collaborator pathologist. The collected data were analyzed by SPSS software and P-value less than 0.05 was considered a statistically significant level.

Results: The mean incidence of Ki67 antigen in the neuroendocrine tumors of the lung was 57.7± 6.5 and the lowest and highest incidence was 1% and 90%, respectively. In addition, the relationship between Ki67 antigen incidence and mitotic count and necrosis were significant while no significant relationship was observed between Ki67 antigen incidence and tumor size in typical carcinoid tumors.

Conclusions: Overall, the Ki67 antigen is regarded as a useful and essential tool for grading neuroendocrine lung tumors.

Keywords: Ki67, Neuroendocrine Lung Tumor, Metastasis, Tumor Grading

Introduction

Lung cancer is considered as one of the most common cancers worldwide and one of the five most prevalent cancers in Iran (1). Unfortunately, most patients are diagnosed in advanced stages and thus the survival rate of 5-year-old cancer is up to 15% (2). Pathologically, neuroendocrine tumors arise in many organs and share common pathological features although there is a specific system for staging and grading in each organ (3). Lung neuroendocrine tumors are classified into four categories according to the World Health Organization (WHO) classification (2004). This classification is based on morphology, mitotic counts, and necrosis and includes typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC), and small cell lung carcinoma (SCLC). However, there are diagnostic problems in this method due to common cytological features between AC and LCNEC, as well as LCNEC and SCLC and thus its efficacy in terms of prognostic value was rejected after several studies (4).

According to this classification, TC and AC diagnosis are based on the number of mitoses and the presence or absence of necrosis in hematoxylin-eosin-stained tissues, but mitotic counting is difficult and time-consuming and differentiating between pyknotic and mitotic nuclei can also be difficult. In addition, determining the presence or absence of necrosis can be complicated (5). The Ki67 antigen, as a nuclear Ag and only present in proliferative cells, is studied in these tumors while its clinical application is not well-defined yet. Further, its diagnostic use in separating TC and AC from the non-surgical samples of SCLC is regarded as its known role. This antigen is even used to separate the TCs from ACs. The application of new diagnostic techniques such as biochemistry-immunohistochemistry and molecular methods has a significant effect on the identification, diversity of histopathologic species, clinical behavior, and the prognosis of these tumors. Considering the biological behavior of the tumors based on their position and the degree of differentiation, the new classification of WHO is considered more beneficial in terms of both clinical and prognostic factors (6).

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Similarly, gastrointestinal neuroendocrine tumors are graded and classified according to the WHO method, as well as the European Neuroendocrine Tumor Society based on the incidence of Ki67 and the number of mitoses. Such a classification was proven to be effective for prognosis prediction (4).

As noted earlier, neuroendocrine tumors become metastatic due to their lack of symptom and delayed diagnosis. Previous studies investigated the relationship between the Ki67 biomarker and any of the factors affecting the grading of lung neuroendocrine cancer (Table 1).

Each classification has distinctly different treatment which makes an accurate initial diagnosis essential. In low-grade tumors, the surgical resection of the tumors is considered as the main treatment and can be curative as well. However, in an advanced disease such as SCLC, the medical treatment consists of somatostatin analogue therapy, targeted therapy, chemotherapy or peptide receptor radionuclide therapy which remains the mainstay of therapy. Prospective trials are needed to determine the other strategies that may be beneficial regarding these tumors (7,8).

Considering the above-mentioned explanations, diagnostic problems were found in the WHO classification for neuroendocrine lung tumors. Furthermore, such problems were observed in studies conducted on identifying Ki67 abilities, optimizing patient's control and management, determining the biological behavior of the tumor and selecting the best treatment, as well as considering the failure to perform similar studies in East Azerbaijan province. It is worth mentioning that Ki67 may be the only available information on the status of cell proliferation in small biopsy specimens, which provides a final decision for the patient's condition. Accordingly, this study aimed to evaluate the relationship between Ki67 antigen and the number of mitoses, the size of the tumor, and the amount of necrosis in lung neuroendocrine tumors.

Materials and Methods

The current analytical descriptive study was started after the approval by the Research Committee of Tabriz University of Medical Sciences and after obtaining a license from the University Ethics Committee. The samples were taken from the pathology archive of the Pathology Department of Imam Reza Hospital of Tabriz. These samples were visited during the last ten years (2008-2018) and their pathology reports were lung neuroendocrine tumors thus their tissue specimens in a paraffin block were suitable for immunohistochemistry testing in terms of tissue quality and quantity. Additionally, information about the examination of the samples was extracted from previous pathological reports, and the associate pathologist had no access to this information. It should be noted that staining and histopathologic examination were conducted in of one-sided (blind) form and the samples were identified as a code. Then, a microscope slide containing 3-µm sections were prepared from the paraffin blocks of lung neuroendocrine tumors. After hematoxylin-eosin staining, the samples were prepared for staining regarding Ki67 marker using a standard immunohistochemistry method.

Sample Size

The target population encompassed all patients with varying degrees and stages of lung neuroendocrine tumor and the research community included all the archival specimens of patients in the Pathology Department of Imam Reza hospital of Tabriz obtained during 2008-2018. Given the rareness of this tumor and the estimation at the beginning of the study, 120 samples were eligible and were included in the study although some of the samples were excluded from the study due to not having the required criteria.

Inclusion Criteria

Only those samples who had complete pathological information with a definitive diagnosis of neuroendocrine tumors in previous reports.

Exclusion Criteria

The lack of access to samples for pathological and immunohistochemically reanalysis, insufficient data in patient's files and records, non-neuro endocrine lung cancer which was confirmed by the histological assay.

Immunohistochemically Staining

The samples of these patients were stained by immunohistochemical methods and the rate of this biological marker was measured, followed by analyzing the results of staining based on the clinical and pathologic

Table 1. The WHO Classification of KI67 Staining Criteria by Mitotic Count

	Valid Case	Mitosis/10 HPF	Ki67 for quantiles	Ki67 Roc Value	Necrosis
WHO 2004	384				
TC	110	1	<3	<4	Absent
AC	81	>1 - <10	3 - <25	4 - <20	Focal
LCNEC	90	10 - 55	25 - <80	20 - <65	Diffuse
SCLC	103	>55	≥80	≥65	

TC: Typical carcinoid; AC: Atypical carcinoid; LCNEC: Large cell neuroendocrine carcinoma; SCLC: Small cell lung carcinoma; HPF: High power fields.

variables. First, the records of patients who underwent surgery for removing lung neuroendocrine tumors were investigated and clinical and pathological data including age, sex, mass size, and risk factors, extracted from these records, along with the pathologic records of the hematoxylin-eosin blocks and slides were extracted from the pathology archives. Then, the second pathologist in the laboratory reviewed the above-mentioned data and confirmed the diagnosis. Finally, the preparation process was performed for the immunohistochemistry test.

Immunohistochemistry Study on These Samples With KI67 Antibody

First, the 3-μm sections of the fixed samples were prepared and then to fix the tissues on the slide, it was stored at 60°C for 24 hours. The slides were then placed in the xylene after 24 hours for deparaffinization. The subsequent tissue dehydration was performed by alcohol as well. Next, the slides were placed in peroxidase for 10 minutes after washing in deionized water. After the peroxidase step, the slides were once more washed with deionized water and placed in a citrate buffer solution to activate the antigens, and finally, boiled in the microwave for 20 minutes. Then, the slides were washed again in the deionized water and the surrounding of the related tissue was marked with the immunohistochemistry mark and the corresponding antibody was poured over the slides after cooling. Before pouring the antibody, all tissues were blocked with the serum for 5 minutes. After placing the slides in a humid and dark chamber for 60 minutes, they were rinsed twice with tris-buffered saline (TBS) buffer, then the corresponding secondary antibody (Envision kit) was added over the tissues, and they were incubated for 45 minutes. After the desired time, the slides were washed in the TBS buffer, followed by adding the chromogen (dye) to the slides. Next, they were placed in the humid and dark chamber for 10 minutes, then rinsed with deionized water and placed in hematoxylin for 5 minutes in order to stain the nucleus. Afterward, the slides were washed with deionized water, dehydrated by alcohol, made transparency by xylene, and finally, assembled, followed by performing cell counting on 100 cells in 10 regions with strong staining and recording the percentage of positive cells (Labeling Index). Then, the "expert eye count" method was used to count the brown-stained cells in the nucleus, which, according to previous studies, has comparable results that are similar to the result of Gold standard "computer assist count".

Ten separate fields with a magnification of 40x were counted and considered in terms of the number of positive cells for the antibody in the areas full of cells and then the results of Ki67 staining and mitotic count were analyzed according to the WHO calcification (4), the details of which are provided in Table 1. To ensure the accuracy of the test, the experiments were conducted in two steps by two testers, and in the case of disagreement, the samples were re-evaluated and recorded upon the agreement of both parties, and the sample was considered positive in the case of positive antigen.

Data Analysis Method

The obtained data were statistically analyzed by descriptive statistics (mean ± SE), frequency and percentage, along with mean difference test for independent groups and quantitative variables employing SPSS software (version 17). Furthermore, Fisher exact test, as well as Pearson and chi-square tests were used for comparing qualitative variables. Similarly, the McNemar test was utilized in the case of non-normal distribution. The independent *t* test was also applied to compare the quantitative variable. In this study, $P<0.05$ was considered statistically significant.

Results

A total of 120 samples were considered in all archival specimens of patients with neuroendocrine lung tumor in different stages in the Pathology Department of Imam Reza hospital. The mean ± standard deviation of patients' age was 56.23±14.30. The youngest and oldest patients were 33 and 84 years old. The histogram of the age distribution of the patient's sample is illustrated in Figure 1.

Among the 120 collected samples from the archives of neuroendocrine lung tumors, 78 (65%) cases were for males and 42 (35%) of them were for females. Moreover, the frequency of the examined tumors was 30% (36 patients), 5% (6 patients), and 65% (78 patients) for TC tumor, AC tumor, and SCLC, respectively.

As regards gender, the frequency of lung neuroendocrine tumors is shown in Figure 2.

Likewise, the frequency of the observed necrosis was 30% (n=36), 40% (n=48), and 30% (n=36) for absent, focal, and diffuse necrosis, respectively. Additionally, the mean ± standard deviation of Ki67 antigen incidence in neuroendocrine tumors was 57.7±6.5% with a slight staining of 1% and maximum staining of 90% (Figure 3).

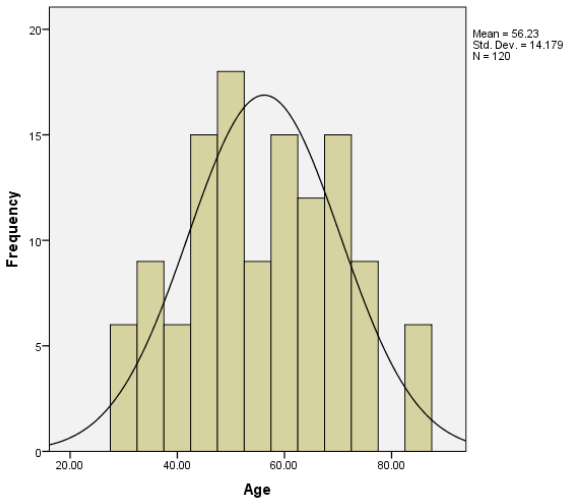

Figure 1. Patients Age Histogram.

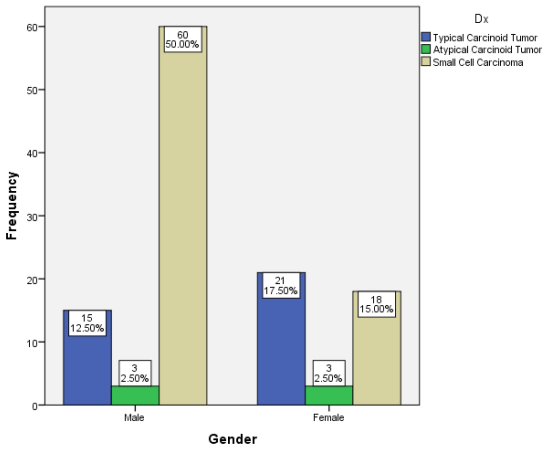

Figure 2. Prevalence of Neuro-endocrine Tumors by Gender Classification.

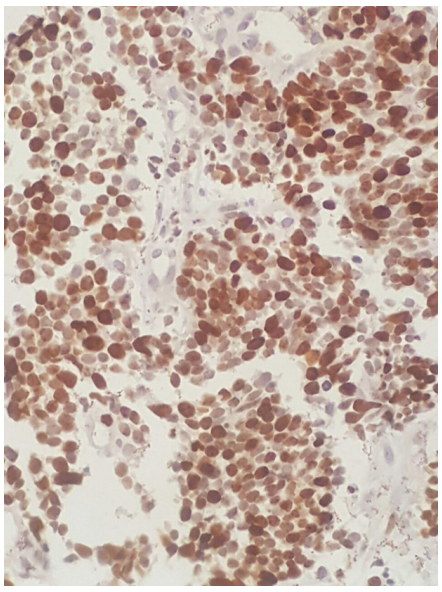

Figure 3. Ki 67 Staining in Small Cell Lung Carcinoma.

Based on the findings, the mean Ki67 staining prevalence of TCT, AC, and SCLC was 30%, 5%, and 65% of all studied samples, respectively. The frequency of Ki67 index cut-off was calculated 6% as well.

The correlation between the percentage of Ki67 staining and the mitotic count was significant ($P=0.002$ & r=0.993). In addition, the correlation between the percentage of Ki67 staining and necrosis rate was significant ($P=0.001$ & r=0.817). However, there was no significant relationship between the percentage of Ki67 staining and the size of the tumor in TCT ($P=0.352$).

Table 2 demonstrates the results of the correlation between Ki67 antigen staining and mitotic count, necrosis, and tumor size.

Table 2. Association of Ki67 Antigen Staining With Mitotic Counts, Necrosis Ratio, and Tumor Size

	Ki67	Mitotic Count	Necrosis Ratio	Tumor Size
Ki67	-	0.002	0.001	0.352
Mitotic count	0.002	-	0.001	0.563
Necrosis	0.001	0.001	-	0.563
Tumor size	0.352	0.001	0.001	-

Discussion

In the process of identifying congressional prognosis, grading is a very necessary tool for estimating tumor behavior. Further, the strength of the grading tools is determined by the rate of result validation in their repeatability (3). The tumor marker can be considered as a molecule that determines the probability of cancer or provides information about the possible origin and cancer behavior such as metastasis and its expansion, along with the possibility of cancer recurrence. Numerous factors and biomarkers play a role in the incidence and development of various types of tumors. Among these factors and biomarkers, proliferative Ki67 factor is one of the most well-known proteins associated with the cell cycle which is used in diagnostic histopathology. Jaworska et al introduced the monoclonal antibody obtained from the mouse. This antibody identifies Ki67, as a nuclear antigen expressed just in proliferating cells (9). The nature and exact composition of the antigen are unknown, but it probably appears in certain stages of the cell cycle. It is believed that the number of Ki67 stained cells represents the number of cells in the cycle or fraction growth. Furthermore, it appears that Ki67, which indicates cell proliferation, positivity begins at the midpoint of G1 while Ki67 expression is negative in early G1 phase and G0. Then, its expression increases during the cycle in a way that the cells of the G2-M phase are extremely positive (10). The other variables of cell proliferation such as thymidine labeling and flow cytometry, are now widely used as a marker for cell proliferation (11). Similarly, the Ki67 antigen level is a marker for assessing the amount of cell proliferation and is also an indicator for determining the risk of tumor metastasis and its prognosis.

The average age of the patients in the study was 56.23 with a range of 33-84. In the study conducted by Clay et al, the average age of the patients was 60.5 with a range of 21-83. Apparently, the cause of the 12-year-old difference in the low range of our study is related to the late detection of neuroendocrine tumors in Iran (12). In another study by Rindi et al, 62.5% of lung neuroendocrine tumors included ACT, but this rate was 5% in our study, which seems to be due to differences in the risk factors of this type of tumor in the American and Asian societies (4).

In our study, the correlation between Ki67staining and mitotic count and necrosis rate was reported significant. This correlation with necrosis rate was stronger than the correlation with the mitotic count although, based on the results of Yachida et al, this correlation with mitotic count was stronger ($P=0.002$) in their study. This difference

seems to be due to the difference in the prevalence rate of ACT in these two studies, as well as the preparation of the biomedical samples of this type of tumor and the presence of artifacts which affect the mitotic count (13).

Skov et al (5) also found that the Ki67 proliferation index had a significant relationship with mitotic count ($P=0.021$). Moreover, Dhall et al (14) reported a significant correlation between Ki67 staining and mitotic count and tumor prognosis ($P=0.005$).

In a study conducted by Strosberg et al, a significant correlation was observed between mitotic count and Ki67 antigen staining rate in the biopsy samples of the neuroendocrine tumor. They further concluded that the combination of these two grading methods can be effective in estimating the survival rate and early detection of metastases (15). Likewise, Clay et al examined the correlation between Ki67 antigen and neuroendocrine tumors in a way that the amount of Ki67 index cut-off was estimated between 4 and 5.4%, but it was 6% in the present study. It seems that this difference is related to the limited number of samples in our study and the absence of control samples (12).

Additionally, Walts et al indicated that the Ki67 antigens had a significant correlation ($P<0.001$) with detecting and determining the grade of lung neuroendocrine tumor. In this study, the correlation between histopathologic findings (the number of mitoses and necrosis) and Ki67 staining rate was not significant, but in our study, this correlation was significant which seems to be due to the difference between the applied methods in both studies (16).

In a study conducted by Grimaldi et al, the amount of Ki67 staining rate was significantly associated with mitotic count ($P=0.044$), necrosis ($P=0.003$), and tumor size ($P=0.012$). However, the findings of our study demonstrated that the correlation between Ki67 antigen staining rate and mitotic count and necrosis was significant but there was no significant correlation with tumor size. This difference regarding tumor size is probably attributed to the low number of gross tumor samples and the low sample size in our study (17).

Considering the results of this study, Ki67 antigen staining is regarded as a suitable tool for determining the grade of lung neuroendocrine tumor with high sensitivity and specificity, which is consistent with the result of the study by Zahel et al (18) in which the percentage of Ki67 antigen staining was above 80%. In our study, the mean Ki67 antigen staining was 57.7%. This difference is due to the low number of samples in our study compared with the study by Zahel et al that included 200 samples.

Each classification has distinctly different treatment thus making an accurate initial diagnosis is essential (17). According to the need for careful determination of the pathology diagnosis of these tumors, a triple classification system based on Ki67 staining, mitotic count, and necrosis can be an important contribution to the treatment of the patients.

Jahchan et al identified tricyclic antidepressants and related molecules as the potent inducers of cell death in small cell carcinoma cells through the activation of stress pathways (18).

Based on these results and the problems arisen in the investigation of mitotic count and necrosis including a mistake in distinguishing the mitoses from pyknotic nuclei and covering the mitoses by the artefacts in hematoxylin-eosin slides, it seems that Ki67 staining helps us to make a quicker and more precise diagnosis of these tumors.

Limitations

The main limitation for this study was the small size of the sample which was due to the low incidence of neuroendocrine lung tumors. This shortcoming was compensated through lengthening the period of the study (10 years). Future researchers are recommended to conduct other studies including larger sample size. Furthermore, they can prospectively investigate and followed up the patients by an oncologist in order to evaluate the relationship between prognosis and the Ki67 index.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The study was approved by the Ethics Committee of Tabriz University of Medical Sciences (Code of Ethics: (IR.TBZMED.REC.94/3-5/3).

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