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Clinical relevance and accuracy of p63 and TTF-1 for better approach of small cell lung carcinoma *versus* poorly differentiated nonkeratinizing squamous cell carcinoma

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Abstract

Lung cancer high mortality rate remains a major problem, despite the actual progress in its early detection and therapeutic design. Since lung cancer' treatment requires separation of tumors in small cell carcinoma and non-small cell carcinoma, the histopathological diagnosis focuses on this basic distinction, while immunohistochemistry contributes considerably to confirm the diagnosis accuracy. In order to check the assumption that p63 is a useful marker for squamous cellular differentiation, we used two antibodies: anti-p63 and anti-thyroid transcription factor-1 (TTF-1), based on their immunoexpression to differentiate small cell lung carcinoma (SCLC) from poorly differentiated nonkeratinizing squamous cell carcinoma (SCC). Our study included 48 cases of lung carcinoma (lung biopsies and wedge resection formalin-fixed and paraffin-embedded). The 48 cases included 23 SCLCs and 25 poorly differentiated nonkeratinizing SCC. The expressions of p63 and TTF-1, respectively, proved to be useful in distinguishing SCLC from poorly differentiated nonkeratinizing SCC, on surgical and biopsic sections. The p63 positivity and TTF-1 negative expression consequently indicated a poorly differentiated nonkeratinizing SCC, while the opposite immunostaining pattern was flagged in SCLC. Our results are useful for a targeted therapy, as long as they point out a significant role in marking of the correct diagnosis of lung tumors.

Keywords: p63, TTF-1, small cell lung carcinoma, poorly differentiated nonkeratinizing squamous cell carcinoma.

Introduction

The histopathological differentiation between small cell lung carcinoma (SCLC) and poorly differentiated non-keratinizing squamous cell carcinoma (SCC) is important for the therapeutic conduct, which frequently is not always easy to be performed, based only on the morphological features of tissue samplings. There are circumstances when differentiation of SCLC from poorly differentiated non-keratinizing SCC can be difficult [1, 2]. The therapeutic conduct must be guided depending on the most relevant markers, which offer a certain diagnosis [3].

Also, the therapeutic approach must be personalized and carefully adapted, reported to the etiopathogenic and genotypic aspects [4].

We have to take into account the following aspects: all the present comorbidities, associated pathologies, the medical and family history and the heredo-collateral antecedents [5].

Also, the aspects concerning the potential adverse events, quality of life and the vulnerabilities, must be carefully approached [6]. The risk and resilience factors must be known and evaluated and the therapeutic approach must be ethical with the lowest number of adverse events encountered [5].

The thyroid transcription factor-1 (TTF-1) is a specific tissue transcription factor that mediates the cell differentiation in lungs, thyroid and brain. A TTF-1 expression was described in 81–100% of SCLC [4].

p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells, but its expression was not so often used for this purpose. The immunohistochemical (IHC) studies on lung neoplasia describe p63 expression consistent in poorly differentiated nonkeratinizing SCC but not in SCLC [7].

The aim of our study is to demonstrate that p63 is a reliable, highly specific and sensitive marker in squamous differentiation during oncogenesis. Furthermore, because p63 immunoreactivity in adult epithelia is normally restricted to progenitor cells, we will appreciate if it will represent a novel biomarker that assist in accurate discrimination of SCC from lung adenocarcinoma.

Materials and Methods

Our study included 48 cases of lung carcinoma (lung biopsies and wedge resection, formalin fixed and paraffin

embedded). The 48 cases included 23 SCLCs and 25 poorly differentiated nonkeratinizing SCCs.

The samples were taken from bronchial biopsies, obtained *via* fiberoptic bronchoscopy from patients with lesions that are clinically suspected to have bronchial dysplasia or bronchial carcinoma in the beginning or in an advanced stage.

The surgical fragments were obtained from patients diagnosed with primitive lung tumors, incidentally discovered, in the absence of symptoms that could suggest the presence of the illness, on which exploratory lobectomy, pneumectomy or thoracotomy were applied together with biopsy sampling.

The immunohistochemistry was performed on 4 μm sections by using anti-p63 and anti-TTF-1 antibodies in the EnVision–Horseradish peroxidase (HRP) visualization system.

The IHC study included a monoclonal anti-human p63 antibody (p63 clone, DAKO, USA, 1/150 dilution), The samples were pre-treated through boiling for 20 minutes in a pH 9 solution, at 95–99^{0}C, and incubated with primary antibody for 30 minutes. 3,3'-Diaminobenzidine (DAB) was used to visualize the reaction, then counterstained with Mayer's Hematoxylin. We obtained a final reaction product with brown nuclear localization. For the negative control of the reaction, the primary antibody was replaced with a buffer solution, and for the positive control, we used different sections from a p63-positive oral squamous carcinoma.

For TTF-1 immunostaining (8G7G3/1 clone, DAKO, USA), the samples were heated, pre-treated in a citrate buffer solution (pH 6), for 20 minutes, in a microwave, and then treated with 3% hydrogen peroxide (H_2O_2), for 10 minutes, to block endogenous peroxidase.

The sections were incubated for 30 minutes with anti-TTF-1 primary antibody (1/100 dilution), and then treated for 30 minutes with secondary-UltraVision™ antibody (HRP Polymer), followed by the visualization of the reaction with DAB and nuclear counterstaining with Mayer's Hematoxylin. For the positive control, we included a positive TTF-1 thyroid carcinoma, and for the negative control, we left out the primary antibody.

We considered TTF-1 and p63 with nuclear staining pattern, and for diagnostic purposes, we ignored any cytoplasmic reactivity. The TTF-1 nuclear immune reactivity was quantitatively quantified as 0 degree (absent), 1 degree (minimal), 2 degrees (moderate) or 3 degrees (intense), based on staining features of most of the tumors. Based on Wu *et al.* criteria [1], we take into consideration:

- p63: positive score when intense immunostaining was present in ≥50% of the tumor cells; the rest of the cases had a negative score.
- TTF-1: positive score when the staining was present in ≥5% of the tumor cells; the rest had a negative score.

Statistical analysis

All analyses were carried out using Statistical Package for the Social Sciences (SPSS) software (version 17.0, Chicago, IL, USA) and Microsoft Excel. The reported *p*-values are two-sided. $P<0.05$ was considered statistically significant.

Results

Lung carcinoma with small cells

All 23 cases of SCLC were p63 negative or rarely ambiguous for p63. The intense and convincing TTF-1 immunoreaction of back up bronchial cell nuclei served as an internal positive control.

We detected TTF-1 in 20 (87%) out of the 23 cases of SCLC (Figure 1, a and b). Immunostaining with TTF-1 had a nuclear localization, and varied from intense to weak (Figure 1, c and d). In one case of SCLC, the tissue section showed negative reaction for both markers (p63 and TTF-1). Positive TTF-1 immunostaining was noticed in benign columnar bronchial cell.

In one case, initially diagnosed with SCLC intermediary cells type in Hematoxylin–Eosin (HE) routinely staining (Figure 2a), the tumor cells were p63 positive, with nuclear expression showing interest to 60–80% of the cells (Figure 2b) and negative TTF-1, suggesting a diagnosis of poorly differentiated nonkeratinizing SCC.

The revision of HE-stained samples, alongside with IHC profile for p63+/TTF-1 panel of antibodies allowed this case to be reclassified as poorly differentiated non-keratinizing SCC.

Carcinoma with poorly differentiated nonkeratinizing squamous cells

All 25 cases of poorly differentiated nonkeratinizing SCC were p63+/TTF-1 (Table 1). All poorly differentiated nonkeratinizing SCC were TTF-1 negative.

Table 1 – ***Immunostaining with p63 and TTF-1***

Studied cases	*p63*	*TTF-1*
SCLC (*n*=23 cases)	0 (0%) positive	20 (87%) positive
Poorly differentiated nonkeratinizing SCC (*n*=25 cases)	25 (100%) positive	0 (0%) positive
The initial diagnosis changed into SCLC (one case)	positive	negative

SCLC: Small cell lung carcinoma; SCC: Squamous cell carcinoma; TTF-1: Thyroid transcription factor-1; *n*: No. of cases.

p63 is firmly expressed in all the sections we have examined, all sections showed p63 immunoreaction with strong staining intensity and a high percentage (50–100%) of positively-stained tumor cells (Figure 2c).

These cells are easily differentiated from the cells in SCLC. In rare cases of p63+ SCC, we had occasionally signaled p63- anaplastic focal spot; these tumors did not show cytomorphological features that suggest SCLC, the sample sections from p63- areas having IHC p63-/TTF-1 profile (non-suggestive for poorly differentiated non-keratinizing SCC or SCLC diagnosis).

Discussions

The differential diagnosis between SCLC and poorly differentiated nonkeratinizing SCC has major therapeutic importance. SCLC is treated through chemotherapy, with the exception of rare surgical resection cases for early peripheral lesions [7].

The SCLC differentiation from poorly differentiated nonkeratinizing SCC can be difficult to analyses by using only the morphological test.

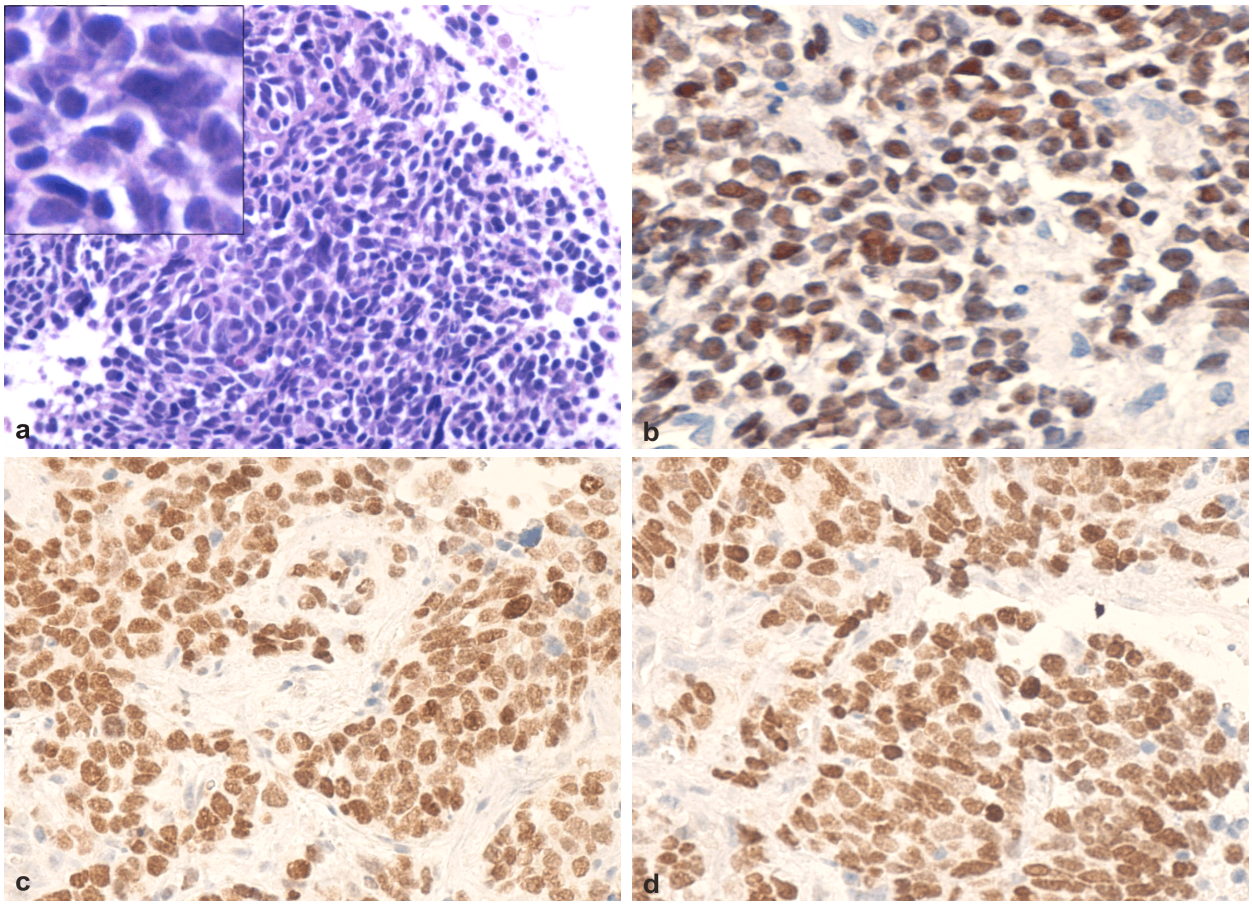

Figure 1 – ***SCLC: (a) HE staining, ×200/×400 (inset); (b–d) Anti-TTF-1 antibody positive immunomarking, ×200 – central group of positive tumoral cells (d). SCLC: Small cell lung carcinoma; HE: Hematoxylin–Eosin; TTF-1: Thyroid transcription factor-1.***

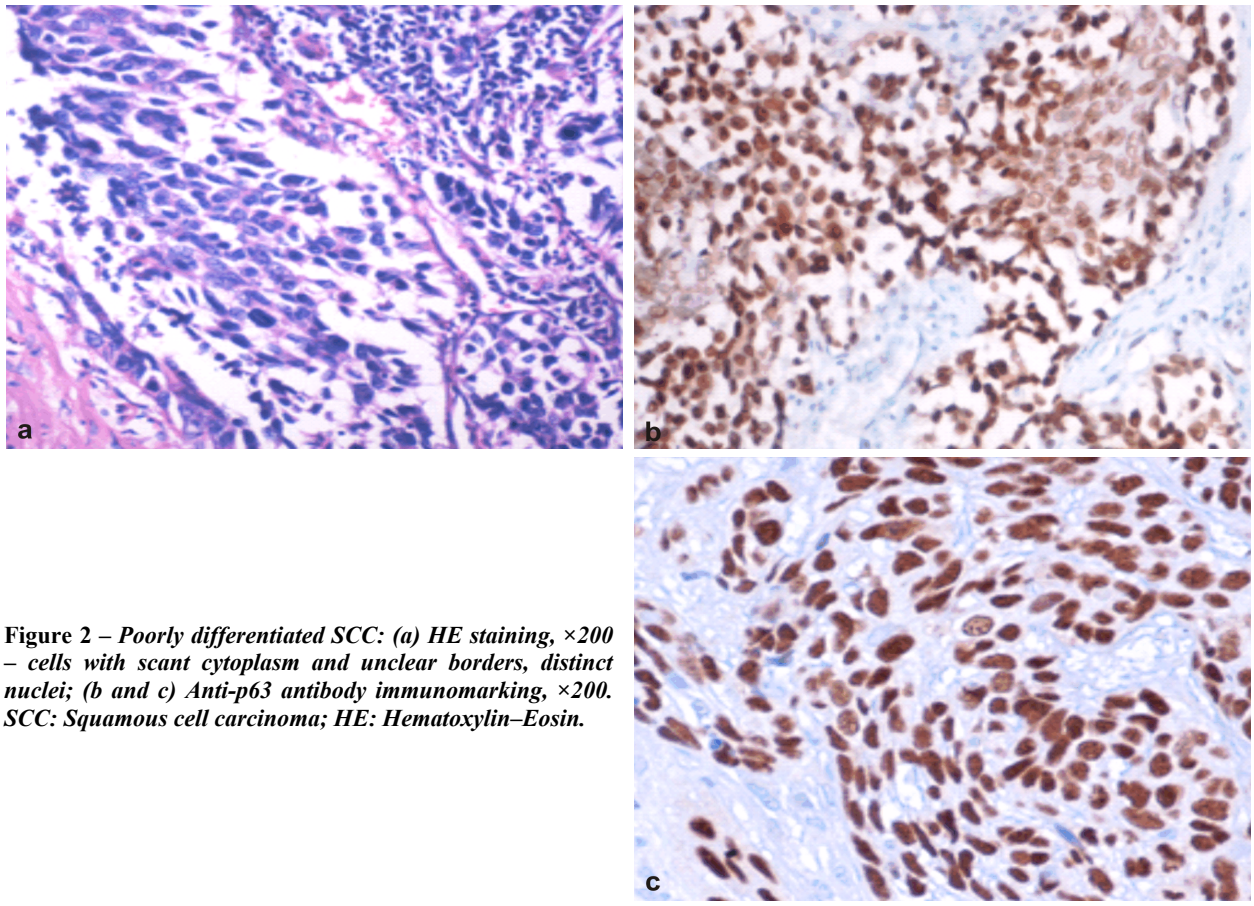

Figure 2 – ***Poorly differentiated SCC: (a) HE staining, ×200 – cells with scant cytoplasm and unclear borders, distinct nuclei; (b and c) Anti-p63 antibody immunomarking, ×200. SCC: Squamous cell carcinoma; HE: Hematoxylin–Eosin.***

Even though, to confirm the diagnosis for SCLC, neuroendocrine (NE) markers can be used, their use can be limited by possible crushing artifacts, by reduced cytoplasm of tumor cells, and a low sensitivity in SCLC [8].

Differentiating the basaloid SCC from the SCLC NE tumor, Sturm *et al.* described the TTF-1 utility and of cytokeratins (CKs) 1, 5, 10 and 14 (detected with the 34βE12 antibody), the authors showing that TTF-1 and 34βE12, associated with specific NE markers, make a panel of antibodies useful to distinguish these tumors. Still, the reduced cytoplasm in SCLC limits the use of cytoplasmic markers, such as CKs and, more so, in SCLC they are positive only in certain keratins [9–11].

P63 is a marker of squamous differentiation, the over-expression of this gene is constantly identified in lung SCC through genic expression profile through IHC [12, 13]. P63 expression was IHC detected in over 80% of most studies, though there are tumor areas and even negative, well-differentiated p63. It was stated that p63 plays a critical role in maintaining the integrity of the squamous epithelium and other epithelia, and it is typically expressed in SCC and in other neoplastic lesions [14, 15].

Up to present, p63 expression has been shown in basal layer of the squamous epithelium, urothelium, prostate basal cells, mammary epithelium myoepithelial cells, and those in the basal back up cells of ciliated bronchial epithelium [16]. The presence of p63 in bronchial cells back up can explain the capacity of bronchial epithelium to suffer squamous metaplasia or squamous differentiation during oncogenesis.

P63 have an important role in SCC from the level of the head and neck too, and its expression is elevated in these tumors. Tumors derived from non-squamous epithelia that can suffer squamous metaplasia, such as endometrial carcinoma, endocervicals, thyroidian or with urothelial origin, can be p63+, while tumors with renal and colonic origin, but also Barrett metaplastic epithelium (which in rare exceptions does not differentiate squamously) are p63 [1, 17].

TTF-1 is a homeodomain of 38 kDa from the family of *NKX-2* gene, which is expressed in thyroid, lungs and diencephalon, and can play an important role in cell morphogenesis and differentiation of thyroid and lungs [18]. Recent studies have described differences of TTF-1 expression between histological types of lung cancer, showing TTF-1 reaction in 89–100% of SCLC, 62.5–90% of adenocarcinoma and absent/low expression in SCC (0–27%) and large cell carcinoma (0–25%) [1, 19].

In order to improve diagnostic accuracy, the use of an antibody panel is recommended. Studies show that, for lung adenocarcinomas, TTF-1 and napsin A have proven to be not only diagnostic markers but also prognostic markers [20].

In small biopsies, in order to separate poorly differentiated nonkeratinizing SCCs from SCLCs, the use of an antibody panel that includes TTF-1, p63, high-molecular weight keratin (HMWK) (34βE12), and cluster of differentiation (CD) 56 is recommended [21].

In our study, the p63k/TTF-1 antibody panel proved to be useful in distinguishing SCLC from poorly differentiated nonkeratinizing SCC, on surgical and biopsic sections. P63 expression, alongside negative TTF-1 reaction, indicated consistent poorly differentiated non-keratinizing SCC, and we took notice of the opposite staining pattern in SCLC. Even though TTF-1+/p63 can be seen in lung adenocarcinoma too, the characteristic cytomorphological tumor features allow the distinction between adenocarcinoma and SCLC [22].

In line with other studies, the relevant markers have a great impact and must be further investigated [4, 6].

Wang *et al.* described a focal p63 expression in 65% of lung adenocarcinoma and extended p63 immunostaining in 100% of SCC [23].

Taking into consideration the variable immunoreactivity of p63 in lung adenocarcinomas, current studies suggest replacing it with p40, a superior marker, in order to better differentiate squamous carcinomas from adenocarcinomas of the lung [24].

In well-differentiated SCC, the centrally localized cells, with squamous maturation or keratinization, were usually p63+ (these cells differentiating easily from SCLC cells). In some p63+ SCCs was noted occasional p63- anaplastic foci. These tumors did not show cytomorphological features that suggest SCLC, the sample sections from p63- areas having a p63-/TTF-1- IHC profile (non-suggestive for poorly differentiated nonkeratinizing SCC or SCLC diagnosis). Aspects similar to Wang's description were noted in our study, too.

Conde *et al.* confirms differentiated IHC p63+ expression in SCC in comparison to adenocarcinoma ($p<0.0001$), and p63+ immunoreaction in 50% of the 20 large cell carcinoma cases (being reclassified as SCC); all p63+ cases (34%) were diagnosed as SCC [21].

The p63 enhancement and overexpression are extremely frequent in SCC, and are associated with a better survival [22]. Pelosi *et al.* do not find any connection between p63 expression and survival, but presented a p63 expression in >80% of tumor cells in SCC, noting a prolonged survival in patients with the enhancement of *p63* gene and a lower death risk in those with intense p63+ tumors [25–27].

The therapeutic approach must be carefully ethical adapted, personalized and reported to the markers together with the associated comorbidities [4, 14, 27].

Conclusions

Our study sustain the role of p63 and TTF-1 antibody panel as useful markers in distinguishing SCLC from poorly differentiated nonkeratinizing SCC, on surgical, biopsic and cytological sections. The results obtained in this study confirm that the p63 expression, alongside negative TTF-1 reaction, indicates a consistent poorly differentiated nonkeratinizing SCC, and the opposite staining pattern is frequently seen in SCLC.

Using the p63 and TTF-1 IHC panel allows the restatement of the diagnosis in the cases that initially were positioned as SCLC, the intermediate type. The TTF-1 and p63+ immunoreactivity confirm the diagnosis for poorly differentiated nonkeratinizing SCC.

Based on our results, we confirm that tumoral markers we investigated can be used as indicators of the tumoral proliferative potential and as prognostic factors in lung cancer. A correct approach of tumor diagnosis involves responsibility for a targeted therapy, according to distinct receptivity of each tumoral type.

Conflict of interests

The authors declare no conflict of interests.

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