Expression of p-AKT and p-mTOR in a large series of bronchopulmonary neuroendocrine tumors

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Abstract. Bronchopulmonary neuroendocrine tumors (BP-NETs) are separated into four subgroups: typical carcinoid tumor (TC), atypical carcinoid tumor (AC), large-cell neuroendocrine carcinoma (LCNEC) and small-cell lung carcinoma (SCLC). The signaling pathway involving AKT/mammalian target of rapamycin (mTOR) is crucial to the regulation of cell growth, proliferation and survival, and is frequently activated in human cancers. Consequently, mTOR is considered an attractive target for anticancer agents. The present study aimed to evaluate the expression of phosphorylated AKT and mTOR in a series of BP-NETs, and to analyze the correlations with clinicopathological parameters. p-AKT and p-mTOR levels were determined by immunohistochemistry in a series of 210 BP-NETs, including 85 SCLCs, 17 LCNECs, 26 ACs, 75 TCs and 7 tumorlets. Higher p-AKT and p-mTOR expression levels were identified in the majority of tumorlets and carcinoids in comparison to the LCNECs (P=0.0001) and SCLCs (P=0.0002). Furthermore, a significant association was observed between p-mTOR expression and tumor size (T) in SCLCs (P=0.04) and LCNECs (P=0.03): T3-T4 tumors exhibited significantly lower p-mTOR expression compared to T1-T2 tumors. In conclusion, most of the BP-NETs examined in this study expressed p-AKT and p-mTOR, suggesting that the AKT/mTOR pathway plays an important role in these tumors. Additionally, our results confirm that low- to intermediate-grade tumors are more closely associated to each other than to high-grade tumors, despite sharing common classification and a common origin from neuroendocrine cells. These findings improve our knowledge of the biological characterization of these tumors and indicate new therapeutic opportunities for the treatment of BP-NETs.

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Introduction

Bronchopulmonary neuroendocrine tumors (BP-NETs) comprise approximately 20% of all lung cancers and represent a distinct spectrum of tumors arising from the neuroendocrine cells of the BP-epithelium that share ultrastructural, morphologic and immunohistochemical characteristics (1). BP-NETs are separated into four subgroups of increasing aggressiveness: low-grade typical carcinoid (TC) with <2 mitoses/2 mm^2 and lacking necrosis; intermediate-grade atypical carcinoid (AC) with 2-10 mitoses/2 mm^2 and/or foci of necrosis; high-grade small-cell lung carcinoma (SCLC) and high-grade large-cell neuroendocrine carcinoma (LCNEC), characterized by abundant mitotic activity with >11 mitoses/2 mm^2 and prominent necrosis (1,2). Neuroendocrine tumorlets (NTs) display the same architectural and cytologic features as TCs; however, NTs are microscopic in size: an arbitrary size of 4 mm or less has been suggested as the classification of NTs, and they are often associated with chronic lung disease (3,4).

Despite common classification, these neuroendocrine tumors differ regarding the natural course of disease and treatment strategies. Although TCs are generally regarded as low-grade carcinomas, approximately 10-23% of cases metastasize to the regional lymph nodes at presentation, with 5-year overall survival rates ranging from 82 to 100% (5,6). By contrast, 40-50% of ACs metastasize to the regional lymph nodes at presentation, with 5-year overall survival rates ranging from 25 to 78% (2,7). The highly malignant LCNECs and SCLCs are generally widespread at diagnosis with a poor overall prognosis, despite aggressive treatment with extensive surgical resection, multi-agent chemotherapy and radiotherapy (8).

The molecular profile of BP-NETs has been extensively investigated with the aim of identifying features for diagnosis, prognosis and even therapy for this particular category of lung tumors. The gradual increase of certain molecular abnormalities along the spectrum of neuroendocrine lung tumors strongly supports the grading concept of typical carcinoid as

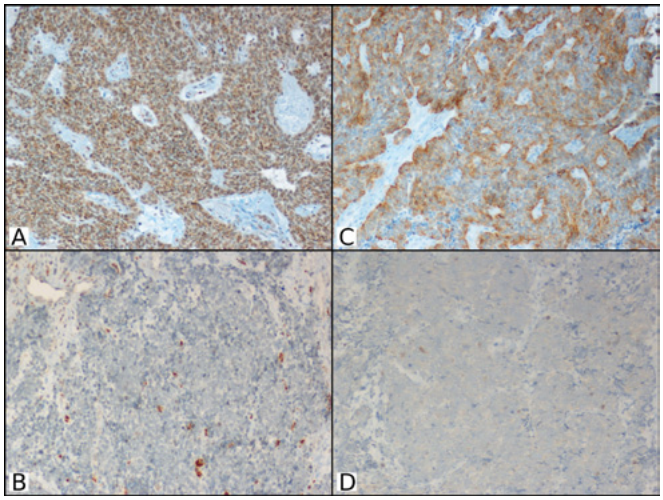

Figure 1. Immunohistochemical staining of p-AKT and p-mTOR in BP-NETs. (A and C) High p-AKT and p-mTOR expression levels in a case of TC. (B and D) Low p-AKT and p-mTOR expression levels in a case of SCLC. Original magnification, x100.

low grade, atypical carcinoid as intermediate grade and large cell neuroendocrine and SCLCs as high-grade neuroendocrine tumors. However, while BP-NETs share certain molecular abnormalities, several differences have been observed. For example, SCLCs and LCNECs display high rates of p53 mutations, while TCs and ACs are characterized by mutations in the menin gene (1,9,10).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is ubiquitously expressed in mammalian cells (11). mTOR is activated downstream of multiple distinct growth factor receptors that have been implicated in lung cancer biology. Additionally, it is of crucial importance in the regulation of cell growth, proliferation and survival (12-14). mTOR acts as a point of convergence of several different signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which responds to growth factors and nutritional status. Both AKT and mTOR are activated through phosphorylation of specific amino acid residues (15). The AKT/mTOR signaling pathway is aberrantly activated in many tumor types (13,14,16,17). In particular, this pathway seems to play a role in tumor cell growth and proliferation in human pulmonary carcinoid cells (18,19), as well in small-cell lung cancer cells (12,20). Because of these functions, mTOR has been considered an attractive target for anticancer agents. *In vitro* studies have established the potential of rapamycin to inhibit cellular transformation, and several types of tumors that exhibit the activation of the AKT/mTOR pathway, including SCLCs, are hypersensitive to rapamycin *in vitro* (12,21).

However, overexpression or activation of AKT and mTOR in the spectrum of BP-NETs and/or their associations with clinicopathological characteristics remain unclear. Therefore, the present study aimed to examine the expression of the phosphorylated forms of AKT and mTOR in a large series of neuroendocrine lung lesions, including tumorlets, TCs, ACs, LCNECs and surgically resected SCLCs. Additionally, the direct correlations between the expression of these genes and clinicopathological parameters were investigated.

Materials and methods

Patients and lung tissue specimens. Neuroendocrine lung tumor specimens (n=210) were obtained from patients (116 males and 94 females) who consecutively underwent surgical resection at the Department of Cardio-Thoracic Surgery of the University of Pisa from January 2000 to July 2009. No patient had received chemotherapy or radiotherapy prior to surgery. All material from the neuroendocrine lesions was obtained from the primary lung tumors. No cytological or bioptical material was included in the present study. Clinical information, including patient gender, age, tumor size and lymph node metastasis, was reviewed for each patient with SCLCs, LCNECs, TCs and ACs.

All tumor samples were formalin-fixed and paraffin-embedded for microscopic examination. The most representative paraffin block for each tumor was selected for analysis. Histological diagnosis and pathological features were reviewed by two pathologists (G. Alì and G. Fontanini) according to the WHO 2004 histologic criteria (1). Neuroendocrine differentiation was detected by positive immunohistochemical staining for chromogranin A, synaptophysin or CD56.

Disagreements concerning histologic diagnosis were discussed, and following which a mutual agreement was reached. Pathological staging was performed according to the TNM classification (22).

Immunohistochemistry. Immunohistochemical analyses were performed on 3-μm tissue sections using specific antibodies. Immunoreaction was displayed using the avidin-biotin-peroxidase complex (ABC) method. Peroxidase activity was visualized with diaminobenzidine. Counterstaining was performed with hematoxylin. Immunostaining was performed using a Benchmark immunostainer (Ventana, Tucson, AZ, USA). In all cases, the immunohistochemical evaluations were independently performed by two pathologists (G. Alì and G. Fontanini) who were blinded to the clinicopathological characteristics of the patients. In all discordant cases, mutual agreement was reached.

For immunohistochemical staining, sections were incubated with a rabbit anti-human phospho-AKT (Ser473) polyclonal antibody (Abcam, Cambridge, UK) at a dilution of 1:50 and with a rabbit anti-human p-mTOR (Ser2448) (clone 49F9; Cell Signaling Technology, Inc., Danvers, MA, USA) antibody used at a dilution of 1:100.

Normal bronchial epithelial cells were used as internal positive controls for p-AKT and p-mTOR staining. Negative controls were conducted by omitting the primary antibodies.

p-AKT expression was assessedd in both the cytoplasm and the nucleus of the neuroendocrine tumors. p-mTOR expression was assessed in both the cytoplasm and plasmatic membrane.

Immunohistochemical expression of both p-AKT and p-mTOR was evaluated as the percentage of tumor cells displaying immunoreactivity. At least 1,000 cancer cells (100 cells in 10 HPFs) were counted for each section (Fig. 1). The median value of p-AKT and p-mTOR for each type of BP-NET was used as a cutoff value to distinguish tumors with low p-AKT and p-mTOR expression levels from those with high expression levels. The staining intensity was analyzed by distinguishing four categories: negative (0), weak staining (+), intermediate staining (++) and strong staining (+++).

Table I. Patient characteristics.

Clinicopathological characteristics	Tumorlet n=7	TC n=75	AC n=26	LCNEC n=17	SCLC n=85
Age, years					
Median (range)	68 (53-77)	61 (24-82)	65 (23-82)	67.5 (45-84)	68 (45-83)
Gender					
Male	3 (42.9%)	32 (42.7%)	6 (23.1%)	13 (76.5%)	62 (72.9%)
Female	4 (57.1%)	43 (57.3%)	20 (76.9%)	4 (23.5%)	23 (27.1%)
Tumor size (T)		n=74	n=26	n=16	n=70
T1 (T1a-T1b)		50 (67.6%)	11 (42.3%)	4 (25.0%)	21 (30.0%)
T2 (T2a-T2b)		19 (25.7%)	12 (46.2%)	7 (43.8%)	33 (47.1%)
T3-T4		5 (6.7%)	3 (11.5%)	5 (31.2%)	16 (22.9%)
Lymph node metastasis (N)		n=65	n=22	n=13	n=58
Absent, N0		62 (95.4%)	15 (68.2%)	11 (84.6%)	34 (58.6%)
Present, N1+N2		3 (4.6%)	7 (31.8%)	2 (15.4%)	24 (41.4%)

TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma.

Statistical analysis. All statistical analyses were conducted using Statistica software. A Chi-square test was used to analyze the associations between the different variables. The *a priori* level of significance was set at a p-value of <0.05.

Results

Clinicopathological characteristics. BP-NETs were reevaluated and reclassified according to the WHO classification of tumors 2004 criteria (1). The most common histological type was SCLC (40.5%; 85 cases), followed by TC (35.7%; 75 cases), AC (12.4%, 26 cases), LCNEC (8.1%; 17 cases) and tumorlet (3.3%, 7 cases). The TNM classification was applied to carcinoids, SCLCs and LCNECs (22). Other histopathological and clinical characteristics of the patients are summarized in Table I.

p-AKT expression according to histology. p-AKT expression was analyzed as a dichotomous variable in all tumor samples, using the median value as the cutoff to distinguish tumors with low expression from tumors with high expression. High p-AKT expression was observed in 6 cases (85.7%) of tumorlets, whereas only 1 case (14.3%) displayed low expression (median 80; range 20-90). Regarding staining intensity, 3 tumorlets exhibited weak immunoreactivity, 2 exhibited intermediate staining and 2 displayed strong staining (Table II). p-AKT expression was high in 45 cases of TCs (60%) (median 60; range 0-90). The staining intensity was weak in 34 cases, intermediate in 23 cases and strong in 10 cases (Table II). p-AKT expression was also high in the majority of ACs. In fact, 73.1% (19/26) of the cases displayed high p-AKT expression (median 70; range 0-90). In terms of staining intensity, 9 cases exhibited weak staining, 11 exhibited intermediate staining and 3 exhibited strong staining (Table II).

Conversely, in high-grade tumors (SCLCs and LCNECs), lower p-AKT expression was observed compared to low-grade lesions, such as ACs, TCs and tumorlets (p=0.0001) (Table III). Low p-AKT expression was observed in 54 of the 85 cases (63.5%) of SCLCs (median 40; range 0-90) and in 13 out of 17 cases (76.5%) of LCNECs (median 10; range 0-90). The staining intensity in SCLCs was weak in 42 cases, intermediate in 19 cases and strong in 11 cases, whereas the intensity in LCNECs was weak in 11 cases and intermediate in 3 cases (Table II).

p-mTOR expression according to histology. p-mTOR expression was also analyzed as a dichotomous variable, using the median value as a cutoff to distinguish two categories: tumors with high m-TOR expression and those with low or no m-TOR expression. High p-mTOR expression was observed in the majority of well-differentiated tumors: 71.4% (5/7) of tumorlets (median 50; range 0-90), 64% (48/75) of TCs (median 25; range 0-90) and 61.5% (16/26) of ACs (median 30; range 0-80) (Table II). Regarding staining intensity, weak intensity was observed in 1 case of tumorlets, 27 cases of TC and 12 cases of AC, whereas intermediate intensity was observed in 4 cases of tumorlets, 26 cases of TC and 5 cases of AC. Strong intensity was noted in 3 cases of TC and 3 of AC (Table II).

By contrast, p-mTOR expression was significantly lower in high-grade BP-NETs, such as LCNECs and SCLCs (p=0.0002) (Table III). High p-mTOR expression was observed in only 35.3% (6/17) of the LCNECs (median 10; range 0-70) and in 30.6% (26/85) of the SCLCs (median 5; range 0-90) (Table II). Immunoreactivity in LCNECs was weak in 9 cases, intermediate in 1 case and strong in 1 case. In SCLCs, staining was weak in 35 cases, intermediate in 13 cases and strong in only 1 case (Table II).

Associations between p-AKT and p-mTOR expression and clinicopathological characteristics in BP-NETs. No significant association was found between p-AKT expression and other clinicopathological parameters, including age, gender, tumor size and lymph node status, in the BP-NETs (data not shown).

A significant association was observed between p-mTOR expression and tumor size (T) in high-grade tumors, SCLCs

Table II. p-AKT and p-mTOR expression in tumor tissue.

	Tumorlet n=7	TC n=75	AC n=26	LCNEC n=17	SCLC n=85
p-AKT expression (p-AKT %)					
Low (< median)	1 (<80)	30 (<60)	7 (<70)	13 (<10)	54 (<40)
High (≥ median)	6 (≥80)	45 (≥60)	19 (≥70)	4 (≥10)	31 (≥40)
p-AKT (p-AKT +)					
0	0	8	3	3	13
1	3	34	9	11	42
2	2	23	11	3	19
3	2	10	3	0	11
p-mTOR expression (p-mTOR %)					
Low (< median)	2 (<50)	27 (<25)	10 (<30)	11 (<10)	59 (<5)
High (≥ median)	5 (≥50)	48 (≥25)	16 (≥30)	6 (≥10)	26 (≥5)
p-mTOR (p-mTOR +)					
0	1	19	6	6	36
1	2	27	12	9	35
2	4	26	5	1	13
3	0	3	3	1	1

TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma.

Table III. Correlations between p-AKT and p-mTOR expression and histology.

Lesion type	p-AKT expression (no. of patients)			p-mTOR expression (no. of patients)		
	Low	High	p-value	Low	High	p-value
Tumorlet	1	6	0.0001[a]	2	5	0.0002[b]
TC	30	45		27	48	
AC	7	19		10	16	
LCNEC	13	4		11	6	
SCLC	54	31		59	26	

TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma. p-values comparing [a]p-AKT and [b]p-mTOR expression between low-grade and high-grade BP-NETs.

(p=0.04) and LCNECs (p=0.03); patients with T3-T4 tumors exhibited significantly lower levels of p-mTOR expression compared to those with T1 (T1a-T1b) or T2 (T2a-T2b) tumors (Table IV). Conversely, no significant association between p-mTOR expression and tumor size was observed in low- and intermediate-grade tumors, such as TCs and ACs. Additionally, no association was identified between m-TOR expression and any other clinicopathological characteristic (age, gender, lymph node status) in the spectrum of BP-NETs (Table IV).

Discussion

SCLCs, LCNECs and pulmonary carcinoids are classic neuroendocrine tumors that reflect all of the characteristics of neuroendocrine cells. WHO classification divides BP-NETs into four categories: low-grade TCs, intermediate-grade ACs, high-grade SCLCs and high-grade LCNECs (1). While these tumors share certain clinical, molecular and genetic abnormalities, they also exhibit differences (1,9,10).

mTOR is a serine/threonine kinase that is ubiquitously expressed in mammalian cells (11), and which regulates cell growth and proliferation. mTOR is also one of the main downstream effectors in the PI3K/AKT pathway that is critically involved in the mediation of cell survival (12-14). This pathway is aberrantly activated in several different tumor models (13,14,16,17). Due to these functions, mTOR has been regarded as an attractive target of anticancer agents; the functions of mTOR are blocked by rapamycin, as well as by other mTOR inhibitors, such as everolimus and temsirolimus (23).

Various studies have evaluated the effect of the inhibition of the PI3K/AKT/mTOR pathway in experimental models of human pulmonary carcinoid and SCLC cells. In particular, the treatment of lung carcinoid cells with inhibitors of the PI3K/AKT/mTOR pathway significantly reduced cellular growth and neuroendocrine marker expression *in vitro* (18,19). In

Table IV. Association of p-mTOR expression with clinicopathological parameters.

Clinicopathological characteristics	Tumorlet (p-value)	TC (p-value)	AC (p-value)	LCNEC (p-value)	SCLC (p-value)
Age	0.32	0.61	0.33	0.31	0.41
Gender	0.14	0.45	0.76	0.66	0.98
Tumor size (T)	NE	0.94	0.34	0.03[a]	0.04[a]
Lymph node metastasis (N)	NE	0.98	0.66	0.22	0.19

[a]Significant difference; TC, typical carcinoid; AC, atypical carcinoid; LCNEC, large-cell neuroendocrine carcinoma; SCLC, small-cell lung carcinoma; NE, not evaluable.

SCLC cells, the inhibition of PI3K/AKT signaling resulted in the inhibition of cellular growth, promotion of apoptosis and enhanced sensitivity of cancer cells to chemotherapy (20,24). Moreover, Marinov *et al* (12) reported that targeting mTOR with the clinically approved inhibitor RAD001 (everolimus) significantly reduced the cell growth of SCLCs and increased their sensitivity to the antitumor effects of commonly used chemotherapeutic agents. However, recent preliminary data from a phase II clinical trial evaluating the effects of the rapamycin derivate temsirolimus in SCLC patients following chemotherapy failed to exhibit any beneficial effect (25).

Nevertheless, the efficacy of mTOR inhibitors in the treatment of neuroendocrine lung tumors remains unclear, and more appropriate molecular classification criteria and therapeutic strategies against BP-NETs have yet to be clearly established. These facts prompted us to investigate whether mTOR and its upstream effector p-AKT were expressed across the whole spectrum of BP-NETs, including tumorlets.

In the present study, the expression of p-AKT and p-mTOR was analyzed in a large retrospective series of 210 patients whose tumors were classified according to low and high p-AKT and p-mTOR expression.

Other studies have also investigated the p-AKT/p-mTOR pathway in BP-NETs, but their analysis of the percentage of immunoreactive tumor cells was combined with the staining intensity of the cells (26-29). We decided to analyze our cases using only the percentage of positive cells, as our experience has indicated that the scoring system is highly subjective.

In our study, AKT and mTOR were widely expressed in the entire series of BP-NETs, including tumorlets. Significantly higher expression of p-AKT was observed in tumorlets and carcinoids, both typical and atypical, than in high-grade neuroendocrine carcinomas, LCNECs and SCLCs (p=0.0001).

Additionally, significantly higher immunohistochemical expression of p-mTOR was revealed in tumorlets, TCs and ACs compared to LCNECs and SCLCs (p=0.0002).

Furthermore, a correlation was identified between p-mTOR expression and tumor size (T) in SCLCs and LCNECs. Patients with high tumor sizes exhibited significantly lower levels of p-mTOR expression, compared to patients with smaller tumors.

Dobashi *et al* reported immunohistochemical expression of activated AKT and mTOR in 14/30 (46.7%) and in 3/30 (10%) SCLC specimens, respectively (28). They failed to reveal correlations between immunohistochemical expression of the two markers and the clinicopathological characteristics of patients. In our study, p-AKT and p-mTOR expression levels were observed in a larger number of SCLC cases (84.7 and 57.6%, respectively), probably due to the larger number of patients analyzed.

Our results agreed with the conclusions of the study conducted on 218 clinically malignant BP-NETs by Righi *et al*, who revealed a statistically significant higher expression level of p-mTOR in well-differentiated neuroendocrine tumors compared to high-grade carcinomas (29).

Since the mTOR pathway controls protein synthesis, cell growth and proliferation, lower expression of p-AKT in high-grade tumors was an unexpected result. One possible explanation could be that in tumors with low or no mTOR expression, another signaling pathway, such as Erk, could be activated (30). Another potential explanation is that the activity of mTOR may be controlled at the post-translational level (31). Moreover, experimental studies have shown that the mTOR pathway regulates cell growth at the expense of proliferation (32). However, the functions of mTOR are more complex than translational control alone, and cross-talk between pathways could alter its oncogenic potential.

However, the differences in the expression of p-AKT and p-mTOR among the various subsets of neuroendocrine tumors agrees with the molecular and genetic data indicating that TCs and ACs are more closely associated to each other than they are to LCNECs and SCLCs, which are themselves closely related. In addition to differences in clinical characteristics between the two groups, abnormalities in several genetic markers, such as p53, bcl2/bax, cyclin D1, RB loss and LOH at 3p, are observed in a high percentage of both SCLCs and LCNECs with minimal and intermediate percentages of TCs and ACs, respectively, exhibiting these abnormalities (9,33).

Currently, the only potentially curative treatment option for patients with pulmonary carcinoids is surgical resection (34). Unfortunately, effective therapeutic options for patients with unresectable disease are lacking, since radiotherapy, chemotherapy and biotherapy have exhibited only limited success (8). Promising results have been obtained in experimental models of carcinoid tumors using PI3K/AKT inhibitors (18). These findings, in addition to the results of the present study revealing high p-AKT and p-mTOR in lung carcinoids, indicate that innovative therapies that block the PI3K/AKT/mTOR

signaling pathway could represent new treatment options for patients with unresectable pulmonary carcinoid disease.

Regarding inhibition of the PI3K/AKT/mTOR pathway in SCLCs, results achieved in pre-clinical models (12,28,29) were not confirmed in a clinical trial (25). However, this disappointing result could be the result of different activation status of the AKT/mTOR pathway in distinct SCLC patients (12). Indeed, in our study, high p-AKT and p-mTOR expression levels were observed in only 36.5 and 30.6% of SCLCs, respectively. In this sense, the putative use of AKT/mTOR inhibitors in clinical treatments should be preceded by analysis of the status of the AKT/mTOR signaling pathway.

In conclusion, the expression of activated AKT and mTOR was examined in a large series of BP-NETs. The immunohistochemical expression levels observed indicate that this pathway plays an important role in this group of lung tumors. Moreover, the differences in the expression of these markers in the various types of neuroendocrine tumors confirm that low to intermediate tumors are more closely associated with each other than with high-grade tumors, despite sharing common classification and a common origin from neuroendocrine cells. Our results provide new knowledge of the biological characterization of these tumors and offer new potential therapeutic opportunities for the treatment of BP-NETs.

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