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THERAPEUTIC BIOMARKERS IN LUNG NEUROENDOCRINE NEOPLASIA

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THERAPEUTIC BIOMARKERS IN LUNG NEUROENDOCRINE NEOPLASIA

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ABSTRACT

1
2 The well known classification of neuroendocrine neoplasms of the lung into four major subtypes
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4 (including typical and atypical carcinoids and small and large cell neuroendocrine carcinomas) has
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6 a proven prognostic validity, but only partially helps to predict the response to specific therapies.
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9 Therapeutic biomarkers are incompletely known and include morphological, immunophenotypic
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11 and molecular markers. Morphology alone has no specific predictive role, nor has any
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13 immunophenotypic marker been proven to bear predictive implications. Ki67 is a relevant
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15 prognostic marker and can indirectly predict response to chemotherapy, when levels are extremely
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17 high in high-grade NE carcinomas. The expression of somatostatin receptors especially of the type
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19 2A, has been shown to predict response to somatostatin analog treatments, paralleling the
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21 information derived from octreotide scintigraphy. mTOR pathway is targeted by specific
22
23 inhibitors, but the exact cellular molecules predicting response are still to be defined. It seems that
24
25 high levels of phosphorylated forms of mTOR and of its downstream factor S6K are associated to
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27 a better response to rapalogs in experimental models. Data from gene expression profiling and
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29 mutational analyses are currently emerging, providing a more detailed map of different molecular
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31 activation pathways, potentially leading to a more accurate molecular classification of lung NE
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33 tumors as well as to the discovery of new therapeutic targets. The combination of mutational
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35 profiles with those of up- or down-regulated genes also by gene gains or losses may ultimately
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37 provide a better characterization of NE tumor histological types in terms of response to specific
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39 chemo- or bio-therapies.
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51 **KEY WORDS:** neuroendocrine tumor, lung, carcinoid, predictive marker, mTOR, somatostatin
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INTRODUCTION

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2 Therapeutic strategies for lung neuroendocrine neoplasms are not completely defined and greatly
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4 vary from carcinoids to high-grade carcinomas. In the former group, surgery is the mainstay for
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6 the therapy of resectable tumors, whereas adjuvant strategies are not well established yet. In
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8 recurrent or metastatic diseases, biotherapies (i.e. somatostatin analogs, mTOR inhibitors) have
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10 been proposed but no clinical evidence is currently available on their real impact to increase
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12 patient survival, and large prospective trials are still ongoing [1]. Chemotherapeutic strategies are
13
14 the only effective treatment in SCLC and LCNEC, although the high response rates (especially for
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16 SCLC) are paralleled by a very high likelihood of recurrence/progression and by a generally
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18 unfavorable outcome. Chemotherapeutic strategies are also considered in moderately proliferating
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20 carcinoids [2] generalizing to the lung the experience in gastrointestinal and pancreatic
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22 neuroendocrine neoplasms, but the evidence on the efficacy of chemotherapeutic agents derives
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24 from small retrospective series rather than from specifically designed prospective clinical trials.
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31 The present paper is aimed at summarizing the pathologist's role in determining predictive
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33 biomarkers of response to treatment in lung neuroendocrine neoplasms, with special reference to
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35 carcinoids (see **Table 1**).

MORPHOLOGICAL AND PHENOTYPIC MARKERS

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46 Morphology - Neuroendocrine (NE) neoplasms of the lung have been variably labeled for the
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48 purpose of better defining the different histotypes and differentiation grades [3]. The current WHO
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50 classification [4] recognizes four entities, defined by the term “carcinoid” for low/intermediate-
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52 grade tumors and “large or small cell carcinoma” for high-grade tumors [5,6].

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56 Typical carcinoids (TC) account for about 1-2% of lung tumors and exhibit a classical organoid
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58 (acinar, trabecular) pattern with polygonal, minimally atypical cells. Necrosis is absent and
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mitoses are $<2/2 \text{ mm}^2$. Similar tumors having a size $<5 \text{ mm}$ are labeled neuroendocrine tumorlets [7,8].

Atypical carcinoids (AC) are extremely rare, cigarette smoking-related tumors, though often associated with regional and distant metastases. Their morphology overlaps that of TC, except that necrosis is present and/or the mitotic count is $2-10/2 \text{ mm}^2$ [9,10].

Large cell NE carcinoma (LCNEC) partly resembles the organoid architecture of AC, but is made of larger cells, necrosis is extensive and the mitotic index exceeds by far 10 in 2 mm^2 . In the 2004 WHO scheme [4] LCNEC is classified among non-NE large cell carcinomas, from which it should be distinguished based on the recognition of a NE phenotype, by either morphology or immunohistochemistry for neuroendocrine markers [11]. A subgroup of undifferentiated large cell carcinomas with NE morphology but no NE marker expression has been also described [12].

Small cell lung carcinoma (SCLC) is the most common lung NE neoplasm and classically is characterized by small cells with scant cytoplasm and condensed chromatin, a diffuse growth pattern, extensive necrosis and a very high mitotic index (largely exceeding $10/2 \text{ mm}^2$).

“Combined NE carcinomas” are the result of a relatively uncommon association of SCLC or LCNEC with conventional squamous cell- or adeno-carcinoma component. Focal NE differentiation in conventional lung carcinomas is excluded from this definition, because its relevance to clinics and tumor behavior is unclear.

Immunophenotype - Chromogranin A or synaptophysin expression, in the absence of high molecular weight cytokeratins [13] or p40 [14] are the most reliable NE markers, whereas PGP9.5, NSE and CD56 are less specific. In high-grade NECs (but not in carcinoids), hASH-1 (a transcription factor driving NE differentiation during human development) expression has been reported [15]. NE markers are mandatory for recognizing the NE nature of a lesion, although any of them has been demonstrated to be superior to morphology in terms of prognostication or response-to-therapy prediction. Lung (and thyroid) specific marker TTF-1 is mostly expressed by

1 high-grade NE carcinomas of both pulmonary and extra-pulmonary origin, while carcinoid tumors
2 are either unreactive or may show variable positivity especially in peripheral lesions [4,16,17]. A
3 non NE marker has been recently proposed to help identifying mitotic figures, namely
4 phosphohistone 3 [18].
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10 11 **PROGNOSTIC AND PREDICTIVE MOLECULAR MARKERS**

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16 *Ki67 index* - The proliferation index detected by Ki-67 is also a useful tool to better classify a lung
17 NET, although not included in the WHO classification criteria [4], at variance with the digestive
18 tract NETs [19]. The reason of this difference may be related to the lack of validation of Ki-67
19 index in lung NETs, yet [20]. Indeed, the proliferative activity of pulmonary NE tumors has been
20 extensively investigated (reviewed by Pelosi et al 2014 [21]). The diagnostic role of Ki67 is so far
21 well established in small biopsy or cytology samples, in which artifacts may hamper the differential
22 diagnosis between small cell lung cancer and carcinoid tumor in individual cases [22]. Analyzing
23 the Ki67 indexes in over 1800 reported cases, it appears that the mean proliferation values for TC,
24 AC, LCNEC and SCLC are 1.6%, 7.3%, 48.5% and 58.9%, respectively, and these figures are
25 paralleled by the different survival rates reported for the single histotypes [23]. There may be,
26 however, significant overlap of Ki-67 indexes between biologically adjacent tumor categories (TC
27 vs. AC; AC vs. LCNEC; LCNEC vs. SCLC), this preventing its reliable diagnostic use in individual
28 cases (**Figure 1**). Some authors found a Ki-67 performance equal or higher than that of mitotic
29 count for both diagnostic (cutoff between TC and AC proposed at 4%) and prognostic purposes [24-
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52 In a recent study on 399 NE tumors of the lung [27], Ki67 index was incorporated into a newly
53 proposed grading system, which also considered two conventional morphological parameters
54 (mitotic count and necrosis). Adapting cut-off values for mitoses and Ki67 at 4 and 25%, it was
55 found that a three grade system can be reliably obtained when at least two of the three parameters
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1 were identified which allows to stratify NE tumors in three subgroups with significantly different
2 survivals. In terms of prediction, Ki-67 has not been associated with the response to any specific
3 treatment in carcinoids. By contrast, in high-grade carcinomas the higher Ki-67 values detected in
4 SCLC (90%) as compared to LCNEC (50-60%) have been postulated to be associated to different
5 chemotherapy responses [28].
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14 *Molecular profile* – The molecular profile of lung NE tumors has been extensively investigated to
15 identify diagnostic, prognostic and predictive factors, and to possibly lead to a “molecular
16 classification” of NETs [29]. Specific chromosomal alterations (e.g., 11q22.3-q25 losses) [30],
17 oncogene mutations and cell cycle deregulation [31] were documented in lung NETs [32]. The
18 mutational profile of lung NETs is still largely uncovered. MEN1 gene is the most largely
19 investigated, and found to be mutated in approximately 13% of carcinoids but very rarely in high-
20 grade NE carcinomas [33]. In term of prognosis, MEN1 gene mutation or loss were significantly
21 related to shorter survival in carcinoid patients, together with tumor stage [34], in keeping with its
22 function of tumor-suppressor gene. Novel data are coming from next generation sequencing
23 analysis, showing a genetic similarity between pulmonary and pancreatic well differentiated
24 neuroendocrine tumors, but surprisingly not between TC and AC in the lung [35].
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41 Gene and protein expression profiles were also extensively investigated. The latter were analyzed
42 by proteomics and immunohistochemistry in carcinoid and SCLC, ultimately leading to the
43 identification of over 300 differentially expressed proteins in each tumor subtype [36]. Gene
44 expression profiling studies identified also novel prognostic markers in the group of lung
45 carcinoids, independent of the histological type. Among others, three genes were found to bear
46 prognostic implications, namely orthopedia homeobox (OTP), CD44 and RET. In particular,
47 significant associations with reduced 20-year survival were observed in the case of low mRNA
48 levels of CD44 (p=0.000018) and OTP (p=0.00054), and high RET levels (p=0.025) [37]. The
49 same Authors also found a different gene expression profile in a small series of 10 bronchial
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1 carcinoids having a favorable or a poor outcome (five cases each). The latter had a significantly
2 higher number of down-regulated genes at chromosome 11q, a region frequently lost in carcinoids
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4 (p=0.00017). Up-regulated genes involved in the mitotic spindle checkpoint, the chromosomal
5 passenger complex (CPC), mitotic kinase CDC2 activity and BRCA-Fanconi anemia pathway.
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7 The above mentioned CD44 and OTP genes, as well as others, including BIRC5 (survivin),
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9 BUB1, IL20RA and KLK12 were found to be independent predictors of patient outcome [38].
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17 Predictive markers – Predictors of response to chemo- or bio-therapies are increasingly being
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19 evaluated [39-41], but investigational studies specifically designed in lung neuroendocrine
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21 neoplasms are meagre.
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24 Thymidylate synthase (TS) is the target of antifolate drugs and intratumoral expression levels may
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26 predict response to an antifolate-based regimen. A differential expression of TS mRNA and
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28 protein in the spectrum of pulmonary NE neoplasms was observed. TS levels were higher in
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30 poorly differentiated NE carcinomas, thus supporting the extremely poor activity of these drugs in
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32 small cell lung cancer [42].
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36 The expression of specific receptors or enzymes implicated in the response to biotherapies has
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38 been demonstrated [16]. Somatostatin receptors (SSTR) have been identified in NE tumors by
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40 different techniques [43]. The immunohistochemical expression of SSTR types 2 and 3 was
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42 investigated in 218 aggressive lung NE tumors (metastatic TC, AC, LCNEC and resected SCLC).
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44 SSTRs expression was progressively reduced in poorly differentiated forms, and correlated with
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46 octreotide scintigraphy in 70% of cases [44] (**Figure 2**). The mTOR pathway has been explored in
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48 lung NET [45] and a lower expression of active forms of mTOR and S6K was detected in high-
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50 grade carcinomas (of either large or small cell types) [46] (**Figure 3**). Indeed, the mTOR pathway
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52 is a complex network of factors and the potential role of its players in predicting response to
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54 mTOR inhibitors (rapalogs) is unknown. Several proteins belonging to the mTOR complexes, or
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56 downstream and upstream to mTOR interplay in regulating such central intracellular signaling
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1 pathway, ideally all being candidate biomarkers of prediction of response to mTOR inhibition.
2 Moreover, additional players interact with mTOR, such as nutrient transporters or somatostatin
3 receptors themselves. Preliminary data from our laboratory (Rapa & Volante, unpublished results)
4 indicate, among others, a significant inverse correlation between the expression of glucose
5 transporter GLUT-1 and mTOR signaling. In addition, the expression of some of the above
6 molecules (in particular p-mTOR and GLUT-1, as well as of the aminoacid transporter LAT-1,
7 were strongly associated with SSTR2A expression, suggesting that somatostatin receptor inhibitor
8 effects may result from mTOR pathway control, and that synergies can be obtained by combined
9 treatments, as suggested in intestinal NETs [47]. Indeed, in *in vitro* models, bronchial carcinoid
10 cells of patients responding to mTOR inhibitors were shown to have higher levels of
11 phosphorylated mTOR [48].
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26 Other synergistic combination effects (ie apoptosis induction) were recently reported, involving
27 erlotinib (targeting EGFR) combined with everolimus (targeting mTOR) in AC and LCNEC [49].
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29 In another study, significant differences in c-KIT and HER2 expression were seen between
30 LCNEC and AC, while EGFR mutations were more common in AC than LCNEC. A potential role
31 for VEGF, c-KIT (and possibly HER2) targeting agents in the treatment of LCNEC was therefore
32 suggested [50,51].
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41 Finally, c-MET oncogene has been investigated in pulmonary NE neoplasms. PAX5 was shown to
42 up-regulate c-MET in small cell lung carcinoma and PAX5 and c-MET co-inhibition produced a
43 synergistic effect in killing tumor cells, probably related to paxillin inactivation (a downstream
44 target of activated c-Met involved in cell motility and tumor spread) [52]. However, regardless of
45 TKI treatment, c-Met activation (phosphorylation) was not influenced by the mutational status,
46 which was detected in 25% of SCLC cell lines, in 8.3% of NETs and in 6.5% of SCLC cases [53].
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FIGURE LEGENDS

Figure 1: A) Pulmonary atypical carcinoid with vascular invasion and B) lymph node metastasis, both having a high Ki67 proliferation index (C, D) estimated at 16% per 10HPF (C, insert) (A,B: H&E, 100x; C,D: immunoperoxidase, 100x; insert: 400x)

Figure 2: Somatostatin Receptor Type 2 expression in a pulmonary atypical carcinoid (A, Score 2 [44]) with peritumoral vascular invasion (B) and lymph node metastasis (C, Score 3 [44]) (immunoperoxidase, 200x)

Figure 3: mTOR pathway activation in a typical carcinoid with lymph node metastases. A) organoid and trabecular growth pattern (E&E, 200x); B) phospho-mTOR protein expression, Hscore=110 [46] and C) phospho-p70S6K protein expression, Hscore=240 [45] (C, D: Immunoperoxidase, 200x)

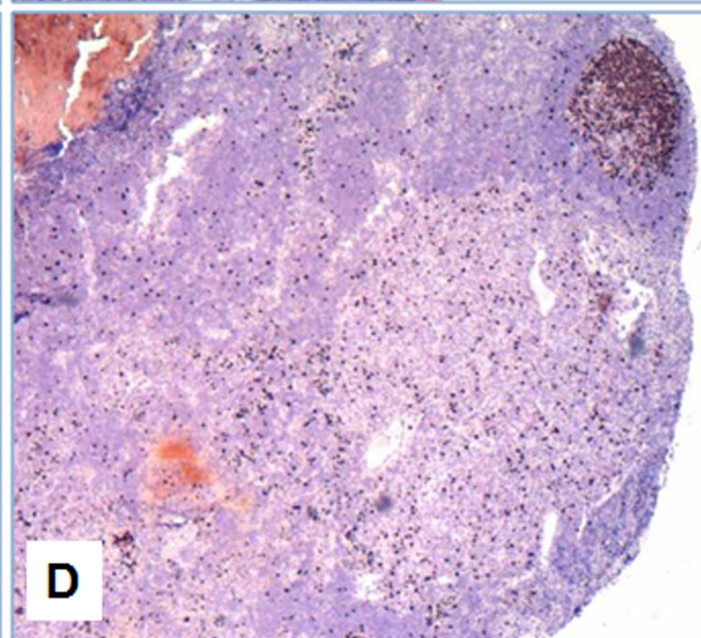
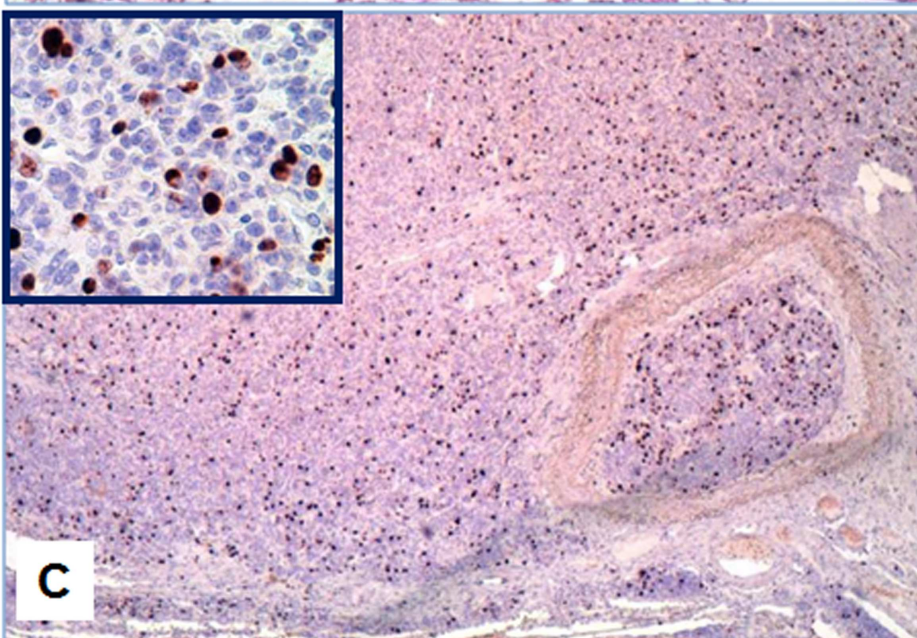
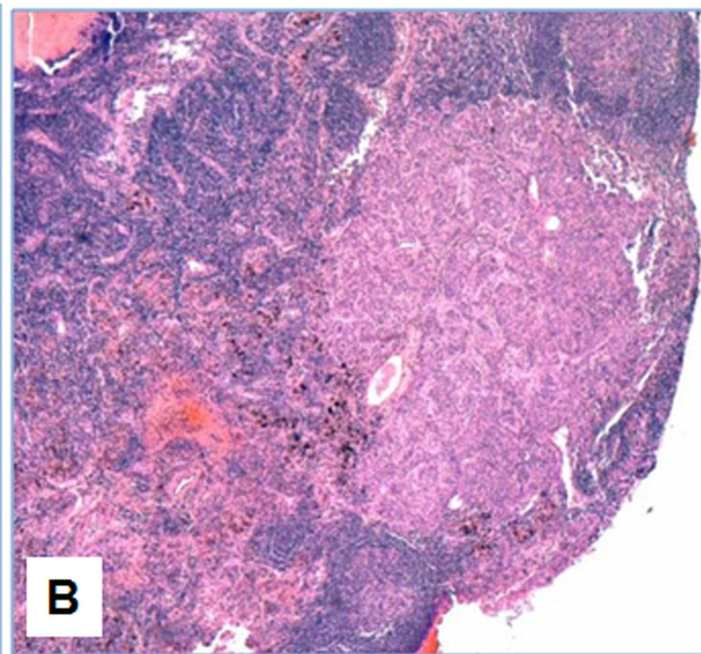
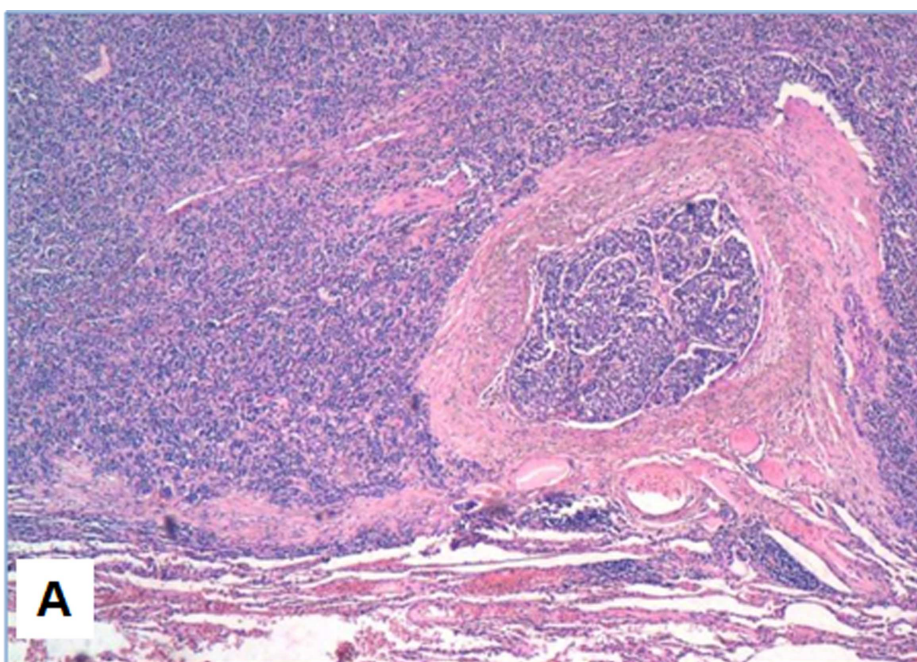
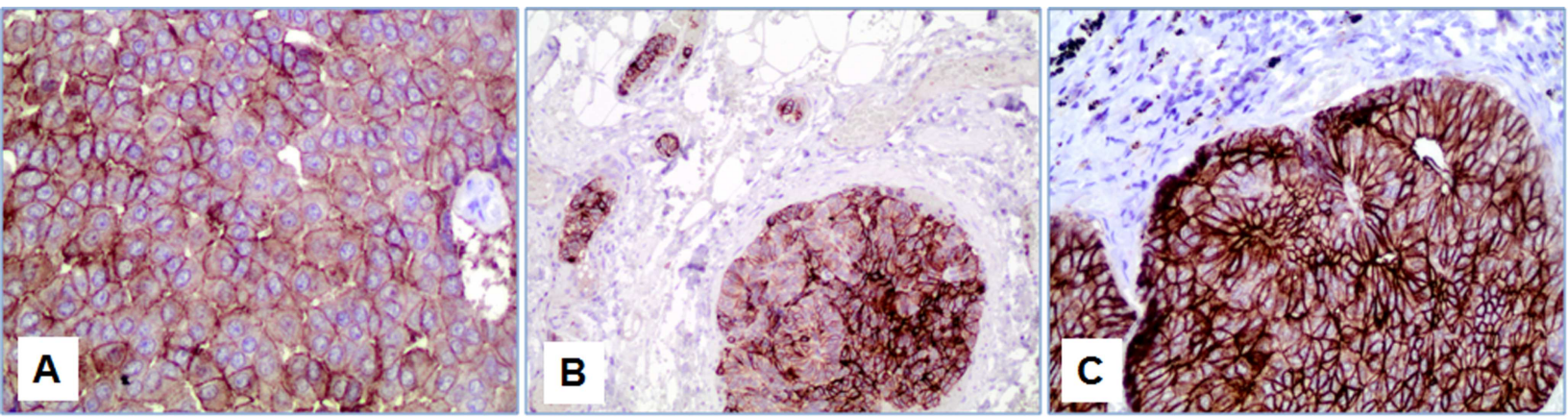


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Figure

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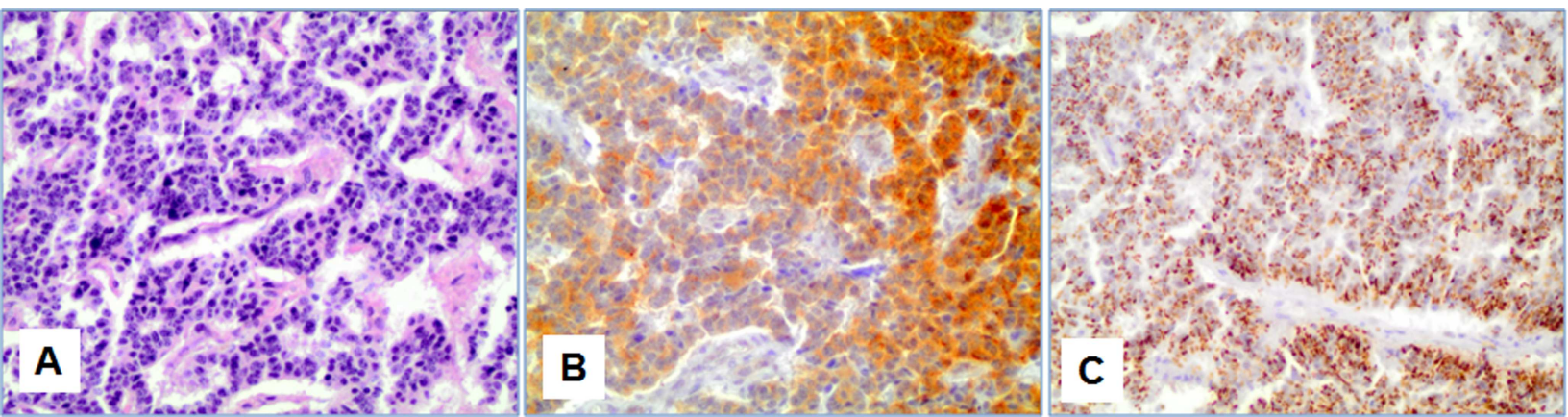


Table 1. Candidate biomarkers with predictive value in lung neuroendocrine neoplasms.

<i>Type of neoplasm</i>	Available therapeutic options	Candidate predictive biomarkers	Literature-based evidence [refs]
<i>Carcinoid</i>	surgery	None	-
<i>Carcinoid</i>	Somatostatin analogs	SSTR expression	SSTR2A positive immunohistochemistry is associated with response to somatostatin analog treatment [54]
<i>Carcinoid</i>	mTOR inhibitors	mTOR pathway molecules expression	mTOR pathway activation is associated with better response to everolimus <i>in vitro</i> [48]
<i>Carcinoid</i>	Interferon alpha	None	-
<i>Carcinoid</i>	PPRT	SSTR expression	SSTR2A positive immunohistochemistry is associated with intense ⁶⁸ Ga-SS analogue-based PET/CT uptake [55]
<i>Carcinoid</i>	streptozotocin	None	-
<i>Carcinoid</i>	antifolate drugs (5-fluorouracil, capecitabine)	thymidylate synthase expression	high thymidylate synthase mRNA expression associated with a lower response to 5-FU [42]
<i>Carcinoid</i>	temozolomide	MGMT expression	MGMT protein deficiency is associated with response to temozolomide in pancreatic neuroendocrine tumors [56]
<i>high grade NE carcinoma (small and large cell types)</i>	surgery (LCNEC, only)	None	-
<i>high grade NE carcinoma (small and large cell types)</i>	external beam radiation	None	-
<i>high grade NE carcinoma (small and large cell types)</i>	platinum derivatives	ERCC1 expression	low ERCC1 mRNA expression associated with better response in small cell carcinoma patients with limited disease [42]; not confirmed for ERCC1 protein [57].
<i>high grade NE carcinoma (small and large cell types)</i>	etoposide	topoisomerase II expression	low topoisomerase II alpha mRNA expression associated with better response in small cell carcinoma patients with limited disease [58].

Abbreviations: NE: neuroendocrine; LCNEC: large cell neuroendocrine carcinoma; SSTR: somatostatin receptor(s); MGMT: O6-methylguanine DNA methyltransferase; ERCC1: excision repair cross-complementing 1