PATHOLOGICA 2024;116:13-21;
DOI: 10.32074/1591-951X-957

Review

Biphenotypic lung carcinoma with coexpression of TTF-1 and ΔNP63/P40 within most of the same individual cells: a further case confirming poor prognosis and a review of literature

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Summary

The WHO Classification of Tumors, Thoracic Tumors, 5th edition, has outlined the use of TTF-1 and ΔNP63/P40 to discriminate between adenocarcinoma and squamous cell carcinoma. In 2015, the first description of a rare non-small cell lung carcinoma featuring co-expression of glandular and squamous differentiation within most of the same individual tumor cells was reported on, with ultrastructural and molecular demonstration of such a biphenotypic differentiation. We herein describe an additional case of this rare tumor entity, which is confirmed to be an aggressive neoplasm despite potential targets of therapy.

Key words: biphenotypic lung cancer, lung adenocarcinoma, lung squamous cell carcinoma, lung cancer immunohistochemistry, lung adenosquamous carcinoma

Received: December 5, 2023
Accepted: December 8, 2023

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How to cite this article: Marando A, Zagni M, Negrelli M, et al. Biphenotypic lung carcinoma with coexpression of TTF-1 and ΔNP63/P40 within most of the same individual cells: a further case confirming poor prognosis and a review of literature. Pathologica 2024;116:13-21. https://doi.org/10.32074/1591-951X-957

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Introduction

Lung cancer is the most prevalent cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) being the predominant histotype [1]. The WHO Classification of Tumors, Thoracic Tumors, 5th Edition, well establishes the management and the classification of NSCLCs and suggests the use of TTF-1 and p40 to discriminate between adenocarcinoma (ADC) and squamous cell carcinoma (SCC) [1].

In 2012, a novel two-hit, sparing-material approach based on TTF1 and ΔNp63/p40 (not p63) was proposed to best differentiate ADC and SCC in small-sized diagnostic material [2]. Subsequently, ΔNp63/p40 (henceforth, simply p40) was confirmed as being the most specific marker of pulmonary SCC [3], paving the way to an innovative driver biomarker approach to lung cancer characterization [4]. The current WHO Classification recommends the use of a glandular marker (e.g., TTF-1) and of a squamous differentiation marker (e.g., p40) as the basic immunohistochemistry panel to identify ADCs and SCCs.

The WHO first acknowledged the diagnosis of adenosquamous carcinoma (ASC) of the lung in its 2nd Edition dating back to 1981. According

to the current definition, established in 1999, ASC is a neoplasm characterized by a coexistent population of tumor cells with morphological and immunohistochemical glandular differentiation and a population of tumor cells with morphological and immunohistochemical squamous differentiation in geographically separate tumor areas, with each accounting for at least 10% of the tumor [1]. In this entity, the glandular and squamous components are required to be morphologically and immunohistochemically distinct.

The first description of a lung neoplasm showing co-expression of glandular and squamous differentiation markers (i.e., TTF-1 and p40) in the same individual tumor cell dates back to 2015 [5]. In the report, Pelosi et al. investigated the molecular profile of this newly described entity, demonstrating the concurrent presence of *KRAS* mutation, typically found in ADCs, and the amplification of *FGFR1*, and usually related to SCCs. Additional confirmation of the bi-phenotypic nature of this neoplasm came from electron microscopy, which highlighted the concomitant presence of secretion granules and desmosomes in the cytoplasm of tumor cells, features respectively associated with ADCs and SCCs [5]. Similar electron microscopy findings were reported in three subsequent reports published between 2015 and 2022 [5-7].

Subsequently, other studies on lung tumors with co-expression of TTF-1 and p40 have been published and, in 2023, Savari collected the largest series, with 14 cases reported [8-14].

We herein report an additional case of such a rare bi-phenotypic lung carcinoma with confirmatory morphological, immunohistochemical and molecular findings, which emphasizes once again the poor prognosis imparted by this tumor entity. A brief review of the current literature was also carried out.

Case report

A 64-year-old woman, active smoker (pack-year 20), without any previous oncologic history, underwent thorough radiological assessment due to widespread body aches. Firstly, a pelvic MRI was carried out: a thin transverse fracture line of the ileopubic branch and a well-defined 14 mm lesion of the ischiopubic branch root were noted. Subsequently a total body CT scan showed a 5 cm solid formation in the right

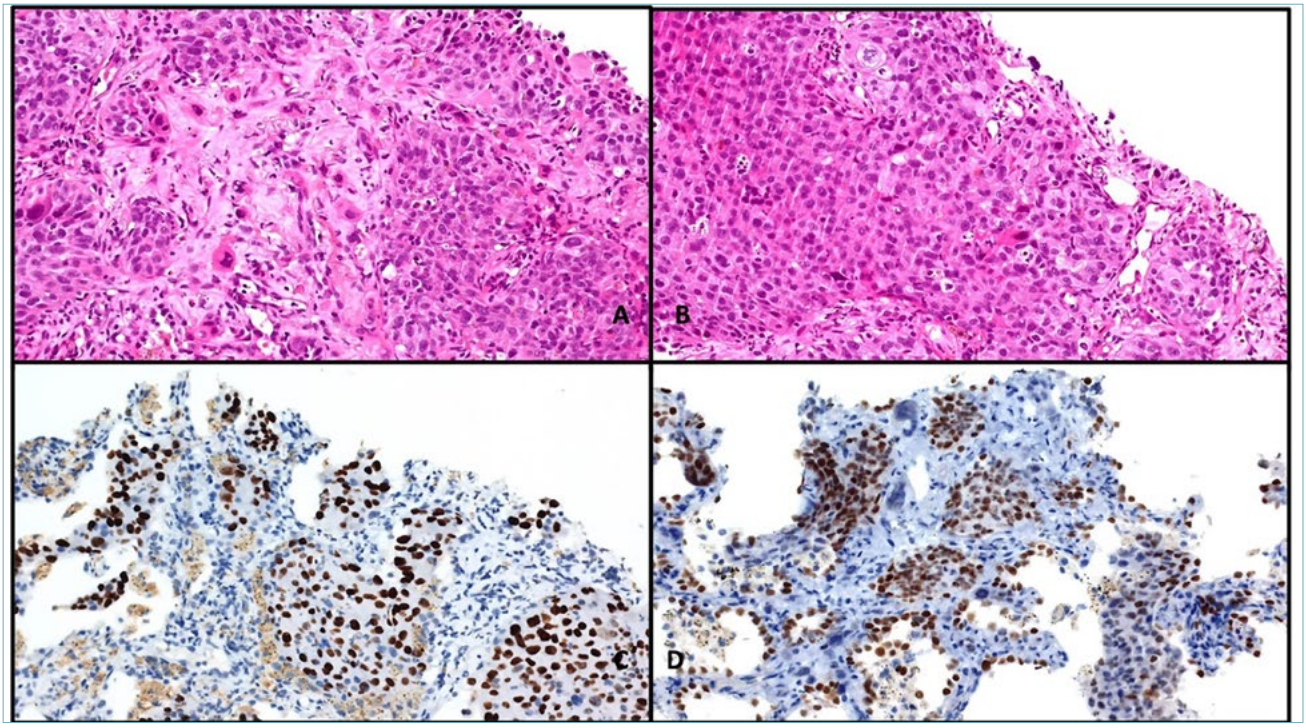

Figure 1. Histologic appearance and immunoreactivity of the reported case. The tumor showed a solid growth pattern with necrotic areas, moderate nuclear atypia and focal features suggestive of squamous differentiation, with no evidence of obvious keratinization (1A-B). Immunohistochemical analysis found the tumor cells to be diffusely reactive for p40 and, to a lesser extent, for TTF-1 (1C-D).

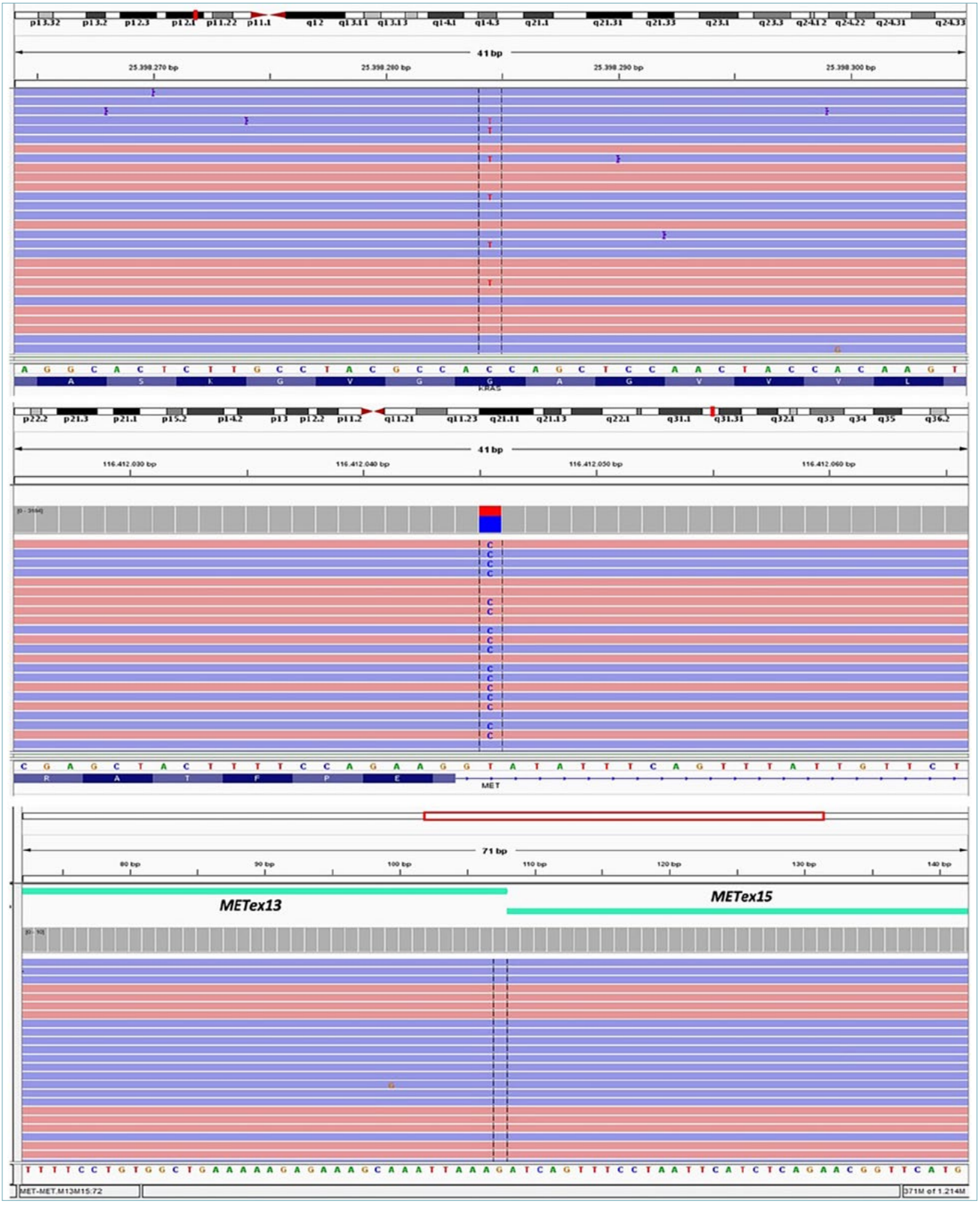

Figure 2. Next generation sequencing results. Integrative Genomics Viewer snapshots of base change c.35G > A (p.G12D) in the exon 2 of *KRAS* gene, Variant Allele Frequency (VAF) 14.6% (2A) and of exon 14-skipping nucleotide substitution c.3082+2T > C in the *MET* gene, Variant Allele Frequency (VAF) 61.2% (2B). Integrated Genomics Viewer of MET(13)-MET(15) variant, Read Counts 82159 (2C).

upper lobe of the lung (RUL, encasing the adjacent vascular structures), multiple hilar and mediastinal lymphadenopathies along with concomitant mild pleural effusion, a nodular lesion measuring 6 mm in the right encephalic paramedian region, and several other nodular lesions in the liver, adrenal glands (bilaterally) and iliac bone.

A fine needle biopsy on the RUL lesion showed a solid growth pattern with necrotic areas, moderate nuclear atypia and focal features suggestive of squamous differentiation, with no evidence of obvious keratinization (Fig. 1A-1B).

Immunohistochemical analysis found the tumor cells to be diffusely reactive for p40 (Fig. 1C) and, to a lesser extent, for TTF-1 (Fig. 1D): remarkably, immunoreactivity for both antibodies was detected in most of the same individual cells. Additionally, neoplastic elements showed positivity for CK5/6 and negativity for synaptophysin, and chromogranin-A. Scattered cells were also intensely positive for p53.

The diagnosis of biphenotypic lung carcinoma with co-expression of TTF-1 and p40 was made, accordingly to Pelosi et al. 2015 [5].

Due to advanced disease stage, the patient was not eligible for surgical treatment and the tumoral molecular profile was investigated to assess the feasibility of chemotherapeutic or immunotherapeutic approaches. PDL-1 was expressed in about 40% of the neoplastic cells. Genomic DNA and RNA were extracted from 5-7 micro-dissected sections of FFPE samples (4 µm thickness) using Maxwell® CSC DNA FFPE Kit and Maxwell® CSC RNA FFPE Kit RNA (Promega), respectively, according to the manufacturers' protocols. Nucleic acid quantification was performed on a Qubit 3.0 Fluorometer using Qubit dsDNA HS and RNA HS Assay kits (Thermo Fisher Scientific), respectively. Genomic profiling was performed by NGS analysis on the Ion S5™ System using Oncomine™ Focus Assay (OFA) (ThermoFisher Scientific) according to the manufacturer's instructions. OFA is a targeted NGS assay for the simultaneous and rapid identification of single nucleotide variants (SNVs), short insertions and deletions (indels), copy number variations (CNV) and gene rearrangements across 52 cancer genes with therapeutic relevance, and can detect potential targets and current actionable genetic variants for personalized medicine. NGS analysis on DNA revealed a *KRAS* exon 2 c.35G > A and a *MET* exon 14 skipping c.3082+2T > C pathogenic variants with variant allele frequency (VAF) of 14.6 and 61.2%, respectively (Fig. 2A, 2B). The same *MET* exon 14 skipping was also detected by RNA sequencing with 82159 read counts (Fig. 2C).

Palliative radiotherapy was performed on the hip lesion (20 Gray dose) as well as stereotactic Gamma Knife radiosurgery on the brain lesion (21 Gray dose). According to the molecular profile, the patient underwent a combination of chemotherapy plus immunotherapy with carboplatin, paclitaxel and pembrolizumab. However, the general conditions and performance status rapidly worsened so that the systemic treatment was permanently discontinued after one cycle.

Two months later, the general conditions rapidly worsened (ECOG PS 2): no additional pharmacological treatment was pursued. The patient was referred to the Palliative Care Unit and died three months after the initial diagnosis.

Review of the literature

There are currently few cases reported in the literature. A total of 25 individual cases, including the present one, have been described, with Savari et al. reporting the largest series with 14 individual cases [14].

The mean age of the patients was 68.3 years (median age 69 years), with a wide range between 26 and 94 years. There is no clear gender prevalence, with 13 male (52%) and 12 female patients (48%), while a correlation with smoking history is strong, with 15 active or past smokers of 19 patients being investigated (79%) (Tab. I).

Neoplasm dimensions were reported in 23 cases. Mean and median diameter was 3.5 and 3.1 cm, ranging from 0.6 to 8.5 cm. Fifteen of 20 tumors were peripherally located, 5 were central and for the remaining 5 data were not available. Laterality was reported in just 11 cases: 7 tumors involved the left lung and 4 the right lung (Tab. I).

Clinical information about disease staging was known for 20 patients. Twelve presented at diagnosis with an advanced stage and presence of metastasis, while for the other 8 there was no evidence of distant spread. Lymph nodes, namely mediastinal and hilar ones, were the most common metastatic localization, with pathologic findings in 8 patients, followed by the brain (6 patients), ipsilateral or contralateral lung and pleura (4 patients), liver, adrenal gland, and bone (3 patients each) (Tab. I).

Histologically, every tumor reported presented with high grade NSCLC morphology: the majority (17 cases, 68%) showed focal features reflecting a squamous differentiation without evidence of frank keratinization, which was reported in only 4 cases (16%). Only 1 case (4%) was characterized by a glandular morphology and the remaining 3 (12%) did not show any glandular or squamous differentiation. All the tumor samples were, by definition, diffusely positive for TTF-1 and p40.

For the former, the antibody utilized was reported in 23 cases: only one author reported the use of SPT24 clone, the remaining 22 used the 8G7G3/1 clone. For the latter, 18 reports used the BC28 clone and two the ERB clone. DAK-p40, SP1/Mo and a polyclonal antibody were used respectively in one case. For the remaining two cases the clone was not known (Tab. II).
Lastly, electron microscopy analysis was conducted in four cases to study the cellular ultrastructure and to detect features of glandular and squamous co-differentiation. In all four cases the authors were able to detect secretion granules and in three of them also microvilli-like protrusions, features typically associated with glandular cell line. In the same four cases ultrastructure consistent with squamous differentiation was also observed (perinuclear tonofilaments and fascicles of keratin fibers). These characteristics were simultaneously present in the same individual neo-

Table I. Clinical and pathological characteristics of the reviewed cohort.

Reference	Age	Sex	Smoking history (Y/N)	Neoplasm diameter (cm)	Neoplasm localization	Metastasis localization	Therapy	Follow-up (months)
Pelosi, 2015 [5]	77	M	Y	8,5	Left (hilar)	Pleural effusion	None	DOD (1)
Hayashi, 2018 [8]	73	M	Y	1,9	LUL	No	Surgery	NA
Spinelli, 2019 [9]	51	M	Y	3,1	RUL	Brain, adrenal gland, mediastinal LN	RT+CT	DOD (3)
Pelosi, 2021 [15]	62	F	Y	4,5	Right	Brain, intralung, mediastinal LN	CT+gefitinb	DOD (48)
	62	M	Y	4,7	LLL	Mediastinal LN, liver	CT+pembrolizumab	DOD (3)
Li Hui, 2021 [10]	54	F	N	NA	LUL	Brain, bone, mediastinal LN	CT+pemetrexed	AWD (NA)
Chen Bing, 2022 [11]	58	F	NA	2,2	LUL	NA	NA	NA
Yang, 2022 [7]	58	M	Y	1,9	LUL	No	Surgery, CAR-NK cell therapy	AWD (9)
Cai, 2022 [12]	38	M	N	4,5	Right (mediastinum)	Bilateral intralung, bone, mediastinal LN	Carboplatin and pemetrexed	NA
Savari, 2022 [13]	26	F	N	5,2	LLL, central	No	Surgery	AWD (17)
Savari, 2023[14]	77	M	Y	4,4	Peripheral	Brain, liver	NA	DOD (10,5)
	68	F	Y	4,2	Peripheral	Brain	NA	AWD (55)
	84	F	NA	2,3	NA	No	Surgery	NA
	80	M	Y	6,4	Central	NA	NA	DOC (22,6)
	67	F	NA	0,6	Peripheral	No	Surgery	NA
	90	F	Y	3	Peripheral	Intralung, supraclavicular LN	Surgery	DOD (51,3)
	69	M	Y	2,2	Peripheral	Liver, adrenal gland, bone, pancreas, stomach	NA	DOD (11,8)
	65	M	Y	3,4	Peripheral	No	NA	AWD (34,5)
	79	F	NA	NA	NA	NA	NA	NA
	86	M	NA	2,2	NA	NA	NA	NA
	73	F	NA	5,2	NA	NA	NA	NA
	79	M	Y	1,3	Central	No	Surgery	NA
	74	M	Y	1,3	Peripheral	Mediastinal LN, pleura	NA	AWD (1,3)
	94	F	N	1,9	Peripheral	No	RT	AWD (12)
Present case	64	F	Y	5	RUL	Mediastinal LN, bone, adrenal gland, liver, brain	RT	DOD (3)

AWD alive with disease, *CT* chemotherapy, *DOC* dead of other causes, *DOD* dead of disease, *LLL* left lower lobe, *LN* lymph nodes, *LUL* left upper lobe, RUL right upper lobe, *NA* not available.

Table II. Histological, immunohistochemical, molecular and electron microscopy features of the reviewed cohort.

Reference	Histology	p40	TTF-1	Molecular alterations	PDL-1	Electron microscopy
Pelosi, 2015 [5]	HG, focal squamous differentiation	+ (clone BC28)	+ (clone 8G7G3/1)	*KRAS* K117N, *TP53* V272G, *FGFR1* amplification	NA	Extracellular lumen formation with microvilli-like protrusion, mucous granules and perinuclear tonofilaments
Hayashi, 2018 [8]	Solid, focal glandular-like	+ (clone BC28)	+ (clone 8G7G3/1)	*ATK* phosphorylation, *PTEN* H123D, *TP53* V272L	NA	NA
Spinelli, 2019 [9]	Solid	+ (polyclonal)	+ (clone SPT24)	WT	70%	NA
Pelosi, 2021 [15]	HG, resembling squamous differentiation	+ (clone BC28)	+ (clone 8G7G3/1)	*EGFR* E746_A750del, *TP53* E224D, *RAD51B* P365R[1], *CCND3* S259A	Negative	Intracellular luminal bordered by microvilli and fascicles of keratin fibers
	HG, resembling squamous differentiation	+ (clone BC28)	+ (clone 8G7G3/1)	*NF1* R1769[1]	2%	Intracytoplasmatic lumina and fascicles of keratin fibers
Li Hui, 2021 [10]	HG, hints of squamous differentiation	+ (clone ERB)	+ (clone 8G7G3/1)	*EGFR* L747_S752del, *TP53* C135R	NA	NA
Chen Bing,2022 [11]	HG	+ (NA)	+ (NA)	NA	NA	NA
Yang, 2022 [7]	HG, resembling squamous differentiation	+ (clone ERB)	+ (clone 8G7G3/1)	*STK11* A205T, *NF1* R1132C, *ARID1A* Q1334del, *BRCA2* D104N, *FGFR1* D125del, *MSH2* V3M, *RET* N975I, *TSC1* H120D	NA	Microvillous and extended cytoplasm, with intracellular mucus granules and perinuclear tension filaments
Cai, 2022 [12]	HG	+ (NA)	+ (NA)	*EML4::ALK*, *PIK3CA* S553T	NA	NA
Savari, 2022 [13]	HG, resembling SCC with focal keratinization	+ (clone SP1/Mo)	+ (clone 8G7G3/1)	*DEK*::*AFF2*, *PATCH1* H1316Y, *FGFR3* K403[1], *BLM* T1015I, *SHH* R244S, *OLIG2* A227_A223del, *WNK1* I726[1]	Negative	NA
Savari, 2023 [14]	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* and *CDKN2A* missense mutation, FGFR*1* amplification	NA	NA
	HG resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* missense mutation, *KRAS* G12C, *FGFR1* and *MYC* amplification	NA	NA
	HG, resembling SCC with focal keratinization	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* splice site mutation, *FGFR1*, *MYC*, *NKX2-1* and *AKT1* amplification	NA	NA
	HG, resembling SCC with focal keratinization	+ (clone BC28)	+ (clone 8G7G3/1)	NA	NA	NA

continue

Table II. *Follows.*

Reference	Histology	p40	TTF-1	Molecular alterations	PDL-1	Electron microscopy
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	NA	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* missense mutation, *FGFR1*, *MYC*, *NKX2-1* and *AKT1* amplification	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	NA	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* splice site mutation, *CDKN2A* missense mutation, *KRAS* G12C	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	NA	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	NA	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	NA	NA	NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* missense mutation	NA NA	NA NA
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*TP53* and *CDKN2A* truncating mutation, *FGFR1* amplification		
	HG, resembling NK-SCC	+ (clone BC28)	+ (clone 8G7G3/1)	*EGFR* N771_H773dup	NA	NA
Present case	HG, resembling squamous differentiation	+ (clone DAK-p40)	+ (clone 8G7G3/1)	*KRAS* G12D, *MET* 14 exon skipping	40%	NA

[1] Variant of Unknown Significance (VUS)

HG high grade, *NA* not available, *NK-SCC* non-keatinizing squamous cell carcinoma, *SCC* squamous cell carcinoma, *VUS* variant of unknown significance, *WT* wild type

plastic cells, supporting the notion of an intrinsic differentiation in both glandular and squamous direction (Tab. II).

PD-L1 immunohistochemistry was carried out in only five cases: two cases were negative, in the other three instances the expression was 2%, 40% and 70% (Tab. II).

Although there is limited data regarding biphenotypic tumors, almost all cases in the literature are characterized by dysregulation of the cell cycle. No specific pattern in gene mutations was observed in this neoplasm. Gene driver alterations of *TP53*, *KRAS*, *PTEN* alterations, *FGFR1* and *MYC* amplifications with PD- L1 expression and cyclin-dependent kinases *CDKN2A* alteration are highly recurrent and drive metastatic potential, and poor overall survival. Some alterations may be concomitant. Among them, *TP53* mutations and *FGFR1* amplification are recurrently aberrant (11 and 6 out of 18 cases, respectively). In our case, *TP53* gene was not tested. *EGFR, NF1, PIK3CA, FGFR3, ARID1A, STK11, BRCA2, TSC1, RET, MSH2, AKT1, NIXX2-1, PATCH1, BLM, SHH* mutations, *EML4::ALK*, *DEK::AFF2* fusion and *MYC* amplification were also detected in other cases (Tab. II). *KRAS* G12D and *MET* exon 14 skipping variants were concurrently observed in the present case. Regarding the pathogenesis of these tumors, it has recently been suggested that the double expression of p40/TTF1 in the same individual tumor cells has a normal counterpart in rare bronchiolar basal cells and tumors would derive according to a model of de novo-basal-like carcinomas with unbalanced glandular and squamous codifferentiation, upon functional recruitment of p63 and TTF1 master genes in double-positive progenitor cells [13-15].

Data regarding treatment were available for 15 cases. Seven (28%) patients underwent surgery, six of whom were diagnosed at an early stage. Survival data was available only for three of these patients: one was alive 9 months after surgery, one was alive 17 months after surgery, and one died of the disease after 51.3 months. Two patients received only chemotherapy,

with no available survival data. In one case, the presence of *EGFR* mutation allowed the patient to undergo chemotherapy plus gefitinib, with a 48-months survival. One patient received chemotherapy plus pembrolizumab, and one patient chemotherapy plus radiotherapy, but both died after three months. Two patients received only radiotherapy: one patient died after 12 months, and the patient in this case report died after two months. One patient was not treated and died after one month. No treatment data were reported for the remaining 10 patients: three were alive respectively after 1.3, 34.5 and 55 months, two died of the disease respectively after 10.5 and 11.8 months, and one died of other unknown causes after 22.6 months (Tab. I).

Discussion

The WHO 5th edition of "Thoracic tumors" does not include neoplasms in which the same neoplastic cells have simultaneous glandular and squamous differentiation, while admitting the category of adenosquamous carcinoma, meant as a tumor with two distinct and separate components of adenocarcinoma and squamous cell carcinoma [1].

However, careful analysis of the literature highlights the actual existence of lung tumors with biphenotypic profile (TTF-1 + and p40+): despite being rare, 25 cases have been described, with the first one being described by Pelosi et al. in 2015 [5]. Every case was reported to have a high-grade, non-small cell lung cancer morphology with a solid growth pattern and, although the invariable presence of the distinctive co-expression of TTF-1 and p40, the vast majority did show equivocal histological differentiation towards glandular and/or squamous lineage. Only four cases were reported to exhibit focal keratinization and one presented featured glandular differentiation.

Ultrastructural studies conducted on four cases confirmed the immunohistochemical findings of combined phenotype, showing the coexistence within the same neoplastic cell of features consistent with both squamous (perinuclear tonofilaments and keratin fibers) and glandular (secretion granules and microvilli-like protrusions) differentiation.

Further confirmation of the nature of this neoplasm can be found in the available molecular data: gene mutations associated to both adenocarcinomas and squamous cell carcinomas were found: six cases harbored *FGFR1* amplification (typical of squamous cell carcinoma). Moreover, mutations of *TP53, KRAS* and of other genes involved in cellular cycle regulation were frequently found (*PTEN, MYC* and *CDKN2A*).

The pathogenesis of these tumors is unknown, but it has been proposed an origin from double-positive distal airway stem-like basal cells through either de novo-basal-like or differentiating cell mechanisms according to a model of epithelial renewal [13-15].

From a clinical standpoint, these neoplasms appear to show a correlation with smoking habit: 79% of patients with available anamnesis were active or past smokers. Tumors are typically characterized by an aggressive behavior, as demonstrated by the advanced stage diagnosis made in 60% of cases, with lymph node localization or distance metastasis. Therapeutic approach showed a considerable variability in the reviewed cohort: patients at initial stages (small neoplasms and absence of distant localizations) were treated with surgery and, according to the scarce data, achieved a reasonable survival time. On the other hand, in patients at an advanced stage survival time was, almost invariably, considerably shorter, with a single patient with an *EGFR*-mutated tumor reported to obtain a long survival. Although scarce and, in some cases, incomplete, data on survival suggest that surgery may be the best therapeutic approach in early stages, while in advanced stages and in absence of molecular targeted treatment is not effective to prolong survival.

Our further report and the review of the literature indicate that, although exceedingly rare in clinical practice, these neoplasms, defined as "biphenotypic lung carcinoma with co-expression of TTF-1 an p40", can be nonetheless encountered and that they are consistently associated with an aggressive clinical behavior with no effective therapy options. The recognition of this peculiar entity is, however, crucial to get insights into the molecular landscape of these strange biphenotypic tumors, which could lead to the identification of potential therapeutic targets and an improvement in prognosis.

Conflicts of interest statement

CL reports honoraria from Roche. DS reports personal fees from AstraZeneca, Roche, BMS, Sanofi, MSD, Boehringer Ingelheim and Novartis, and non-personal fees from Lilly and Pfizer.

EGP reports personal fees from Roche.

All the remaining authors declare no conflict of interests.

Funding

The authors declare no financial or personal interest or belief that could affect their objectivity and haven't received any fund for this manuscript.

Authors' contributions

All the authors have seen and approved the final ver-

sion of the manuscript being submitted. The authors warrant that this article is their original work, that it hasn't receive any prior publication and it isn't under consideration for publication elsewhere.

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