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Original Article

SMARCA4 as a support for the differential diagnosis of poorly differentiated lung carcinomas

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Summary

Among non-small cell lung cancers (NSCLCs), sarcomatoid carcinomas account for 3%. They are rare tumours with a poor prognosis, classified into three subgroups, namely pleomorphic carcinoma, pulmonary blastoma and carcinosarcoma. In the 5th edition of WHO Classification of Thoracic Tumours more space is given to SMARC4-deficient lung cancers. Although studies on SMARCA4-deficient lung tumours are limited, a small percentage of SMARCA4 loss is present within NSCLCs. This finding is clinically relevant, as the loss of the SMARCA4 gene is associated with a worse prognosis. In our study, we analysed the presence of the main catalytic subunit of the SMARCA4 gene, the BRG1 protein, in 60 sarcomatoid lung tumours. The results of our study show that 5.3% of sarcomatoid carcinomas have BRG1-loss in tumour cells, proving that a non-negligible amount of lung sarcomatoid carcinomas are SMARCA4-deficient. These data open the debate on the necessity of including the detection of SMARCA4 within a standardised immunohistochemical panel.

Key words: SMARCA4, BRG1, NSCLC, sarcomatoid lung cancer, pleomorphic lung cancer

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Introduction

In the new "WHO Classification of Thoracic Tumours" [1], the tumour entity of lung sarcomatoid tumour has been replaced with pleomorphic carcinoma, carcinosarcoma, and pulmonary blastoma. Among the NSCLCs, the group of sarcomatoid carcinoma has the worst prognosis and a poor response to conventional chemotherapy treatment [2].

Pleomorphic carcinoma is a rare subtype of lung carcinoma that represents only 2-3% of all NSCLC cases in surgical series. It is a poorly differentiated NSCLC with that may include foci of adenocarcinoma, squamous cell carcinoma, and/or large cell carcinoma with at least 10% component of spindle and/or giant cells. Pleomorphic carcinoma may be composed only of frankly malignant spindle cells or giant cells [1].

Carcinosarcoma represents 0.1-0.2% of all lung cancers. Histologically, it is a mixture of carcinomatous and sarcomatous elements, usually a NSCLC, like adenocarcinoma or squamous cell carcinoma, and heterologous elements, such as osteosarcoma or chondrosarcoma, respectively [1].

Pulmonary blastoma accounts for a maximum of 0.1% of all lung cancers. It is composed of a mixture of fetal adenocarcinoma, a well-differentiated adenocarcinoma, and undifferentiated mesenchymal stroma [1].

In the latest "WHO Classification of Thoracic Tumours", a new entity, "Thoracic SMARCA4-deficient undifferentiated tumour" (SD-UTTs), was included. The SD-UTTs are adult-only carcinomas that have SMARCA4 loss at immunohistochemistry. Although they are classified among NSCLCs, these tumours show peculiar clinicopathological features. In particular, they have poor prognosis, diffuse thoracic involvement, absence of any epithelioid differentiation [1], show a complete epithelial-mesenchymal transition and sometimes exhibit stem-cell markers. The differential diagnosis between these tumours and the other poorly differentiated carcinomas can be very difficult: they share many histological similarities and require a panel of immunohistochemical stains as no one stain alone is diagnostic. In these settings, the use of SMARCA4 can be decisive for the correct diagnosis.

SMARCA4 (full name is "*SWI/SNF* related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4") is a gene that encodes a protein involved in chromatin remodelling. The encoded protein is part of the SNF/SWI (Switch/Sucrose-Non-Fermentable) complex that activates the transcription of specific genes by altering their chromatin structure. BRG1 (Brahma-related gene-1) is the central catalytic subunit of this chromatin-modifying complex. Moreover, BRG and other catalytic subunits of SNF/SWI such as BRM, may act as tumour suppressor proteins. For this reason, some studies claim that BRG/BRM may influence the clinical outcomes and survival rate of patients with NSCLCs [3].

BRG1 and BRM are the most frequently down-regulated members of the SWI/SNF complex. This down-regulation of expression has been found in 3/40 cell lines (10%) [3]. SMARCA4/BRG1 loss has been identified in a subset of poorly differentiated/undifferentiated adult carcinomas appearing in many anatomical sites, including the lung [4]. Regarding lung tumours, Medina PP et al. [5] analysed the entire coding sequence of BRG1 in 59 lung cancer cell lines and mutations were detected in 24% of them, predominantly in NSCLCs. In the literature, NSCLCs with mutations in BRG1 that lead to loss of his immunohistochemical expression have been found in a range between 2.4% and 23.1% [1,4,6-12].

Moreover, in the setting of NSCLCs, BRG1-negative tumours are associated with a more aggressive clinical behaviour than NSCLCs: patients with high SMARC4 expression have a reduced risk of death compared with patients with low SMARCA4 expression, regardless of age, gender, stage, and degree of tumour differentiation [13]. Reisman et al. [9] also came to the same conclusion: BRG1-positive patients, even with a more advanced clinical stage, had a statistically higher survival rate than BRG1-negative patients, with the same therapies or treatment.

For many years, we have known about the existence of SMARCA4-deficient NSCLC, but there is a very limited number of studies about this type of tumours and, even less, of SMARCA4-deficient sarcomatoid lung carcinoma specifically. In this study, we reviewed and described the morphologic features and immunoprofiles of 3 cases of SMARCA4-deficient sarcomatoid carcinoma. The aim of the study is to assess the expression about SMARCA4 in sarcomatoid tumour and to see if this marker can help in the diagnosis of poorly differentiated lung cancers.

Materials and methods

Case selection

This study was approved by the Ethics Committee "Comitato Etico di Area Vasta Nord Ovest" (CEAVNO) for Clinical Experimentation (Prot. n° 9989). A total of 60 lung sarcomatoid carcinomas specimens were retrospectively collected from the archives of the Operative Unit of Pathological Anatomy III of the University Hospital of Pisa. In our research, we included pleomorphic carcinomas, carcinosarcomas, and pulmonary blastomas. The samples came from patients who had been submitted to surgical resection at the Unit of Thoracic Surgery of the University Hospital of Pisa from January 2012 to August 2022. Participation in this study required informed consent. Age, sex, histological tumour type, histological tumour components, immunohistochemistry, and clinical stage were revaluated for each patient. All cases were revised independently by two pathologists (GA and MP) on the criteria of the 2021 World Health Organization Classification of Thoracic Tumours.

Immunohistochemistry

For each case, we selected at least one tumour block, first fixed in formalin and then embedded in paraffin. The tumour block contained the best preserved and the most representative part of the neoplasm, including both the carcinoma and sarcoma components and as little necrosis as possible.

We performed immunohistochemical stains on freshly cut 3-µm paraffin sections, using the fully automated Benchmark XT System (Ventana Medical Systems Inc., Tucson, AZ, USA) and antibodies against SMARCA4 (anti-BRG1 antibody) (clone EPNCIR111A, dilution 1:100, Abcam, Cambridge, UK). SMARCA4 has a nuclear staining of immunohistochemical expression, so SMARCA4-deficient neoplasms have a complete loss of nuclear staining in tumour cells while the ac-

companying cells remain positive. Usually the so-called accompanying cells are lymphocytes, normal bronchial epithelial cells, and endothelial cells. The interpretation of SMARCA4 immunohistochemistry is based on comparison with the surrounding normal tissue [14]. In detail, loss of nuclear labelling was scored from the staining intensity of the benign internal control cells (inflammatory, stromal or benign epithelial cells). Loss was defined as diffuse absence of staining or severe overall reduction (minimal weak nuclear labelling) compared to internal controls [15]. We also performed immunohistochemical stains of NUT (Rabbit monoclonal antibody C53B1, dilution 1:100, Cell Signaling Technology, Danvers, MA, USA) in all cases with squamous differentiation in order to exclude any misdiagnosed NUT-carcinomas.

At the time of diagnosis, neuroendocrine markers had only been made in a limited number of cases (4 out of 60). We performed immunoistochemical stains of synaptophysin (Syn), chromogranin A (CgA), and CD56 in a selected group of cases, specifically in the ones with SMARCA4-loss.

Therefore, we revised the immunohistochemistry for the following antibodies: CK7, CK-PAN, CK-CAM, TTF-1, vimentin, CD10, P40, P63, desmin, and actin. The staining intensity was graded as negative, moderate or strong, and the extent was classified as negative (< 1%), focal (1-50%), and positive (> 50%).

Statistical analysis

The data in this study were analysed using descriptive statistics. Median and mean calculation was used for age, whereas percentage was used for sex, immunohistochemistry results, and pathological findings.

Results

The patients ranged in age from 43 to 87 years old, with a mean age of 70 years old and a median of 71.5. Sarcomatoid carcinomas were more common in males than females with a M:F ratio of more than 2:1. Clinical features and stage at the time of diagnosis are summarised in Table I.

Pathological findings

Of the 60 cases of sarcomatoid carcinomas, 54 were pleomorphic carcinomas, 1 was pulmonary blastoma, and 5 were carcinosarcomas. Twenty had a biopsy prior to resection and only 6 were diagnosed as pleomorphic carcinomas in the biopsy, while the other 14 were diagnosed as NSCLC not otherwise specified or as adenocarcinoma (ADC)/squamous cell carcinoma. The 54 pleomorphic carcinomas showed a significant

Table I. Clinical features and stage of sarcomatoid carcinomas.

Features	Pleomorphic carcinomas (n = 54)	Carcinosarcomas (n = 5)	Pulmonary blastoma (n = 1)
Age, median (range)	71 (43-87)	77 (67-78)	64
Sex N (%)			
Male	36 (66.7%)	3 (60%)	0
Female	18 (33.3%)	2 (40%)	1 (100%)
Pleural involvement, N (%)			
Absent	21 (38.9%)	4 (80%)	1 (100%)
Present	33 (61.1%)	1 (20%)	0
Vascular invasion, N (%)			
Absent	47 (87%)	5	1 (100%)
Present	7 (13%)	0	0
Thoracic chest invasion, N (%)	5 (9.3%)	0	0
Clinical T stage, N (%)			
T1	4 (7.4%)	2 (40%)	0
T2	19 (35.2%)	2 (40%)	0
T3	21 (38.9%)	1 (20%)	1 (100%)
T4	10 (18.5%)	0	0
Clinical N stage, N (%)			
N0	35 (64.8%)	5 (100%)	1 (100%)
N1	9 (16.7%)	0	0
N2	10 (18.5%)	0	0
Pathological AJCC stage, N (%)			
I	12 (22.2%)	3 (60%)	0
II	18 (33.3%)	2 (40%)	1 (100%)
III	23 (42.6%)	0	0
IV	1 (1.9%)	0	0

Table II. Data summary of SMARCA4-deficient sarcomatoid carcinomas.

Cases	Age/Sex	Diagnosis	Histological characteristics	Tumour size	IHC positive	IHC negative
1	66/W	Pleomorphic carcinoma	Spindle and giant cells + ADC	4 cm	CKPAN, CK7, vimentin	CD10, p40, CD56, Syn, CgA
2	77/M	Pleomorphic carcinoma	Giant cells	4.5 cm	CKPAN, CKCAM, CK7	TTF-1, p40, CD56, Syn, CgA
3	75/M	Pleomorphic carcinoma	Spindle cells + ADC	2.4 cm	CKPAN, CK7	TTF-1, p40, CD10, CD56, Syn, CgA

Abbreviations: IHC, immunohistochemical; ADC, adenocarcinoma; CKPAN, pankeratin; CK7, cytokeratin 7; CKCAM, cytokeratin CAM 5.2; Syn, synaptophysin; CgA, chromogranin A; TTF-1, thyroid transcription factor 1.

variety of histological patterns. The predominant one was ADC plus spindle cells (11 out of 54, 20.4%), followed by ADC plus giant cells (9 of 54, 16.7%) and giant cells plus spindle cells (9 of 54, 16.7%); giant cells alone (8 of 54, 14.8%), ADC plus both spindle and giant cells (8 of 54, 14.8%) and spindle cells only (1 of 54, 1.8%). The other 8 cases showed a commixture of giant and spindle cells with squamous differentiation (14.8%).

Concerning the 5 carcinosarcomas cases, 3 (60%) were composed of a part of squamous cell carcinoma plus a part of chondrosarcoma (3, 60%), while the other 2 cases (40%) were made of a part of squamous cell carcinoma plus a part of undifferentiated sarcomatous component.

The pulmonary blastoma was composed of a well-differentiated epithelial component, with tubulo-papillary structure, and mesenchymal stroma with spindle and oval cells. It was characterised by wide areas of necrosis and the absence of any differentiated sarcomatous tissues.

The histological diagnosis of the 3 BRG-negative sarcomatoid carcinomas is specified in Table II. Specifically, all 3 BRG-negative tumours were pleomorphic lung carcinomas with spindle cell and giant cell components equally represented. Whereas, as representative of the part of NSCLC, only the histological type of ADC was present: none of the BRG-negative tumours had squamous differentiation or large cell carcinoma areas. The glandular elements and the giant cells were well demarcated from one another, while the spindle cells were closely admixed with the other histological types. All 3 tumours showed large necrotic and haemorrhage areas and severe cell atypia in the ADC component with a high mitotic index. The spindle tumour cells showed bland atypia with storiform arrangement admixed with little collagenous stroma. The giant cell component had diffuse pleomorphism, wide clear cytoplasm, and irregular nuclear membrane. None of the above tumours had areas of heterologous differentiation or rhabdoid areas.

Immunohistochemical findings

All tumours were subjected to immunohistochemistry for BRG1 antibody and 54 cases showed retained nuclear staining for SMARCA4 both in the neoplastic cells and in normal bronchial epithelial and infiltrating lymphocytes (Fig. 1). Of the 6 cases that did not test positive, 3 had complete and diffuse loss of BRG1 antibody in tumour cells and each sample showed internal positive control staining (Fig. 2). The last 3 cases

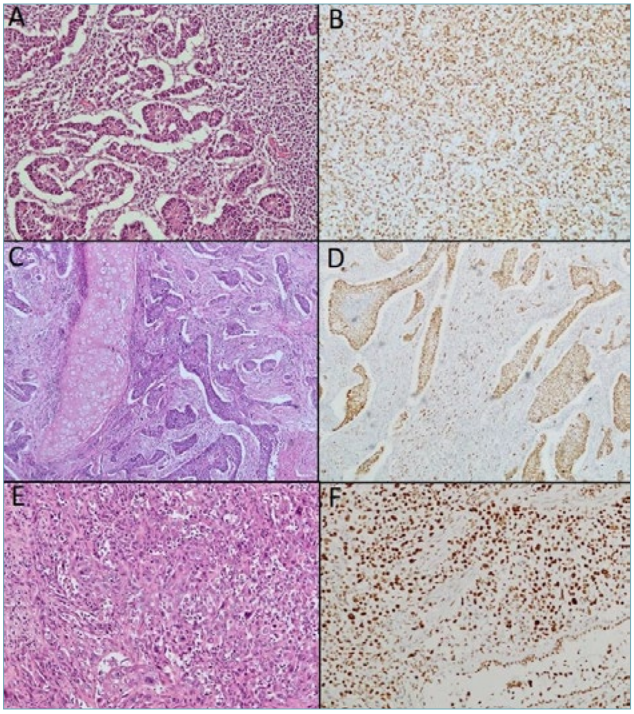

Figure 1. Histomorphologic features of BRG1-positive sarcomatoid carcinoma. A-B, Pulmonary blastoma (hematoxylin and eosin stain) (A), immunostained sections showing retained BRG1 nuclear expression both in tumour cells and in the background cells (B). C-D, Carcinosarcoma (hematoxylin and eosin stain) (C), BRG1 stain both in tumour cells and in the background cells (D). E-F, Pleomorphic carcinoma (haematoxylin and eosin stain) (E), BRG1 stain both in tumour cells and in the background cells (F).

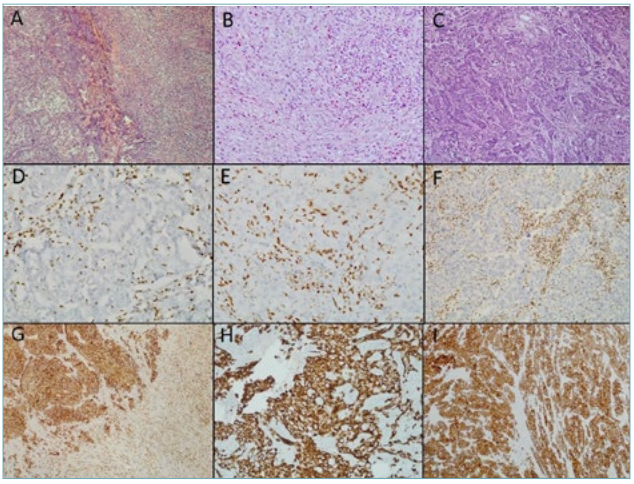

Figure 2. Histomorphologic features of SMARCA4-deficient pleomorphic carcinoma. A-C, Pleomorphic carcinoma (haematoxylin and eosin stain): spindle cells and poorly differentiated areas of adenocarcinoma (A); spindle cells, epithelioid cells with glandular formations and eosinophilic, vacuolated and clear cytoplasm giant cells (B); tumour composed only by giant cells (C). D-F, Immunostained sections showing loss of BRG1 expression in tumour cells and retained nuclear staining in the background cells in the pleomorphic carcinoma in A (D), B (E) and C (F). G-I, positive nuclear staining for CK7 in the pleomorphic carcinoma in A (G), B (H) and C (I).

were non-assessable due to the absence of positive internal control. This may result from the degeneration of the material and the consequent loss of its antigenicity.

Of all 60 cases, 17 (28.3%) had squamous differentiation or had focal p40/p63 positivity at immunohistochemistry. These 17 cases underwent NUT staining in order to exclude the NUT-carcinomas. They were all negative, thus excluding misdiagnosed NUT-carcinomas.

Of all the reviewed immunohistochemical stains, cytokeratins (CK-PAN, CK-CAM, CK7) were the most represented: 39 of 60 cases (65%) had diffuse immunohistochemical staining to at least one of the cytokeratins, while the remaining 21 cases (35%) had focal or mild positivity for one of them. The staining for vimentin was also mildly represented with positivity in 23 out of 60 cases (38.3%). TTF-1 stain was positive in neoplastic cell nuclei of the adenocarcinoma component; in particular, it was diffusely positive in 3 out of 60 cases (5%), focal in 18 of 60 cases (30%), and negative in 20 of 60 cases (33.3%). The p40/p63 stains were positive in neoplastic cell nuclei of the squamous elements; in particular, they were diffusely positive in 2 of 60 cases (3.3%), focal in 12 of 60 cases (20%), and negative in 27 of 60 cases (45%).

Regarding the SMARCA4-deficient sarcomatoid carcinomas, CK-PAN and CK7 were consistently positive in all tumours (3 of 3) (Fig. 2), while p40 was consistently negative in all tumours (0 of 3). Moreover, TTF-1 showed focal staining for tumour cells with glandular differentiation in 1 of the 3 tumours and in the same tumour vimentin was moderately expressed. CD10 was performed in 2 of 3 tumours and was negative. Lastly, negative staining of Syn, CgA and CD56 were displayed in all of 3 tumours. The immunohistochemical characteristics of the SMARCA4-deficient sarcomatoid carcinomas are summed in Table II.

Discussion

For a few years, NSCLCs presenting loss of SMARCA4 has been an entity recognised by the international pathology community. Over the last 20 years, more studies have been done on SMARC4-loss NSCLC and almost all agreed on the percentage of NSCLC with loss of SMARCA4 expression. Naito et al. [6] found SMARCA4-loss in 2.4% of NSCLS in their study. Most studies found a rate of approximately 5%, in line with that reported in the WHO [1]. In particular, Herpel et al. [7] found the SMARCA4-loss in 5.1% of NSCLC and Nambirajan et al. [4] found a complete loss of SMARCA4 in 4% of all NSCLC tested. Schoenfeld et al. [8] tested a large number of NSCLCs for SMARCA4 alterations by next-generation sequencing (NGS) and found alterations in 8% of case. A slightly higher percentage was found in the study of Reisman et al. [9], who observed loss of BRG1/BRM expression in 10% of NSCLC samples. However, the higher percentage reported in this study can be explained by the fact that they also included the loss of BRM subunit along with the loss of BRG1. Very few studies focused on the research of SMARCA4-loss exclusively in sarcomatoid carcinomas. Among them, Zhou et al. [10] and Liang et al. [11] found mutations of SMARCA4 in 14% and 19%, respectively, of all pulmonary sarcomatoid carcinomas examined. To better understand these last two data, we must emphasise that SMARCA4-mutated tumours are not synonymous with SMARCA4-deficient tumours. Indeed, although many types of mutations exist in this gene, those that cause loss of SMARCA4 are mainly truncating mutations. Of all the SMARCA4 mutations, about one-third are truncating ones, so only a subset of SMARCA4 mutations results in BRG1 loss [16].

Our study focused on a specific and rare type of NSCLCs, the sarcomatoid tumour of the lung: an entity divided by the latest *WHO* (5th ed. 2021) into three subgroups, pleomorphic carcinoma, pulmunary blas-

toma, and carcinosarcoma, which have the characteristic of being very aggressive and have a poor prognosis. We searched the archives for all cases diagnosed as "sarcomatoid" in the last 10 years and found 60 cases. All cases underwent histological review and, for those with squamous differentiation (28.3%), immunohistochemical confirmation with NUT1 antibody. We excluded misdiagnosis of NUT-carcinoma and all cases were confirmed to be sarcomatoid carcinomas. We did immunohistochemistry for BRG1 and 6 cases came up negative: 3 of these were excluded from the study because they had no positive internal control, while the other 3 had loss of BRG1 specifically in the tumour cells, with strong and diffuse staining in the accompanying cells. Thus, our study showed that 5.3% of all pulmonary sarcomatoid carcinomas diagnosed from specimens are SMARCA4-deficient. Based on the above data, our study is in line with most studies in the literature. In particular, our result in accordance with the latest version of WHO [1] and with the studies by Herpel et al. [7] and Nambirajan et al. [4] regarding the reported loss of BRG1 in NSCLC. In contrast, it appears noticeably different from the finding of Zhou et al. [10] and Liang et al. [11] probably due to the different mutations taken into account: their studies consider all mutations present in the gene, whereas in our study only the subgroup causing immunohistochemical loss of BRG1 staining is considered, which in the majority of cases means to be truncating mutations only.

On the other hand, one of the most recent studies on SMARCA4-deficient lung sarcomatoid carcinomas found a loss of SMARCA4 in 23.1% of all the examined cases [12]. This percentage is one of the highest found so far in the literature and differs considerably from the data collected in our study. Unfortunately, there are still very few studies on SMARCA4-deficient lung sarcomatoid carcinoma and this fact does not allow more precise comparisons.

In the recent study by Rekhtman et al. [17] the features of SD-UTTs were compared with those of conventional SMARCA4-deficient NSCLCs. The authors showed undifferentiated round cells and rhabdoid morphology in SD-UTTs. Immunohistochemical markers expression in SD-UTTs was also peculiar and included negative or low expression of keratin, lack of the claudin-4 and positivity to some stem-cell markers such as SALL4, CD34 and SOX2. Therefore, our three SMARCA4-deficient cases could be classified as sarcomatoid NSCLCs and not SD-UTTs according to their histopathological and immunohistochemical features.

Two of the three cases defined as SMARCA4-deficient sarcomatoid carcinomas were composed of spindle cells and neoplastic glandular formations, while the other one was made exclusively of giant cells. A characteristic feature of all these 3 cases was the widespread positivity for cytokeratins (CK7 and CKPAN). According to the WHO [1], pleomorphic carcinomas should have a diffuse cytokeratin positivity in the NSCLC component and a variable one in the spindle and giant cell component. Also related to the diffuse cytokeratins positivity and even after careful morphologic re-evaluation, these cases were still classified as sarcomatoid tumours.

Lastly, all 3 cases shown negativity for neuroendocrine markers (chromogranin A, synaptophysin and CD56). This finding is in disagreement with what expressed in the study of Mao et al. [18] where they analysed poorly differentiated/undifferentiated NSCLC. In their 5 SMARCA4-deficient cases they performed neuroendocrine markers: 3 of them showed moderate and diffuse staining for Syn, while 2 of 5 and 3 of 5 exhibited focal staining for CgA and CD56, respectively. Their study demonstrated how at least one neuroendocrine marker was expressed in poorly differentiated NSCLC with loss of SMARCA4. Positivity for neuroendocrine markers was also observed in SD-UTTs, with synaptophysin being the predominant staining [1,17]. Our study disagreed with their results probably because we only considered sarcomatoid carcinomas and not all the other poorly differentiated NSCLC. Given the absence of further scientific data on neuroendocrine markers in SMARCA4-deficient sarcomatoid tumours, we cannot say with certainty whether these tumours were indeed all negative for such markers.

Due to BRG1's apparent role as a tumour suppressor in lung cancer, loss of BRG1 has been proved to be associated with a worse prognosis. The overall survival (OS) of patients with NSCLC changes significantly if the tumour has low or intermediate/high SMARCA4 expression. NSCLCs with SMARCA4 expression of less than 30% are associated with poor OS compared to those with expression higher than 30% [13]. This difference in OS is maintained regardless of tumour stage and clinical characteristics. Additional, Bell et al. [13] have demonstrated cisplatin-based chemotherapy has greater benefit in resectable NSCLC tumours with low SMARCA4/BRG1 expression than in BRG-positive ones. Thus, it is evident how SMARCA4/BRG1 expression could be used as a predictive marker of diagnostic response in clinical practice, as well as a parameter for survival rate and prognosis. However, a limitation of the present study was the lack of clinical and outcome data that will be necessary to evaluate SMARCA4/BRG1 as prognostic and predictive factor in resectable NSCLC.

The inclusion of the detection of SMARCA4 in a standardised immunohistochemical panel has been proposed in previous studies. In particular, the detection

of BRG1 for the diagnosis of poorly differentiated/undifferentiated NSCLC has been recommended to better characterise these tumours and help the development of more targeted therapeutic options [18]. It has also been suggested to add SMARCA4 detection to the panel used to assess the pulmonary origin of a rare group of ADC characterised by the absence of TTF-1 stain [19]. Lastly, Ogunbona et al. [20] proposed the use of SMARCA4 as an aid in the differential diagnosis of poorly differentiated epithelial carcinomas exhibiting neuroendocrine and/or sarcomatoid features.

Conclusions

The present study is one of the largest on SMARCA4-deficient sarcomatoid lung carcinoma. Although SMARCA4-deficient NSCLCs are increasingly being recognised as a clinically important subset of NSCLCs, the available studies are few, and even less about the group of sarcomatoid tumours.
Since the percentage of SMARC4-deficient sarcomatoid carcinomas is not negligible, it would be worth considering the inclusion of BRG1 stain in a diagnostic immunonhistochemical panel when facing with poorly differentiated lung and/or thoracic tumours.

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Conflict of interest

The authors declare no conflict of interest in the writing and publication of this paper.

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Authors' contribution

MP, GA and AP conceived and designed the study; FM, CZ and ML provide specimens and clinical data; MP and GA participated in data collection, contributed to the interpretation of the results and in the writing the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethical consideration

This study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee "Comitato Etico di Area Vasta Nord Ovest" (CEAVNO) for Clinical Experimentation (Prot. n° 9989). Informed consent was obtained from each patient for study participation and data publication.

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