A Modified Vimentin Histological Score Helps Recognize Pulmonary Sarcomatoid Carcinoma in Small Biopsy Samples

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Abstract. *Background: As pulmonary sarcomatoid carcinomas (PSCs) are life-threatening tumors, an improvement in their recognition in small-sized tumor samples is clinically warranted. Materials and Methods: Preoperative biopsy samples and paired surgical specimens from 20 pleomorphic carcinomas, two pulmonary blastomas and one carcinosarcoma (training set) were studied for vimentin immunohistochemistry. A modified vimentin histologic score (M-VHS) was devised by multiplying three independently assessed parameters, i.e. the percentage of positive cells (from 0 to 5+, by quintiles), the intensity of immunostaining (low=1 vs. strong=2) and the distribution pattern within the cytoplasm (partial=1 vs. diffuse=2), so ranging from 0 to 20. Forty-eight consecutive and independent cases of non-small cell lung carcinoma (NSCLC), including two additional cases of PSC, were used as control groups (validation set). Results: No differences in M-VHS were found between biopsies and surgical specimens of PSC, thus confirming the occurrence of stable epithelial mesenchymal transition (EMT) and hence the specific diagnosis of PSC. All types of PSC shared the same M-VHS. The M-VHS of 46 conventional NSCLC was by far lower ($p<0.0001$), whereas two additional cases of PSC showed the same results as the training set. Poorly differentiated NSCLC with marked pleomorphism but not stable EMT did not exhibit significantly increased M-VHS values. Conclusion: M-VHS helped in morphological analysis to render more definite diagnoses on small biopsies of PSC.*

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Pulmonary sarcomatoid carcinomas (PSCs) make up a rare and deadly family of non-small cell lung cancer (NSCLC) encompassing pleomorphic carcinoma (PLC) (the most frequent), spindle cell carcinoma (SpCC), giant cell carcinoma (GCC), carcinosarcoma (CS) and pulmonary blastoma (PB). Albeit diverse genophenotypically, they share, however, similar clinical behavior and presentation of very aggressive tumors with dismal prognosis and poor life expectancy (1-8). PSCs are believed to derive from common protoepithelial stem cells undergoing stable epithelial mesenchymal transition (EMT) until complete mesenchymal phenotype "switch off" (2, 3, 6, 7, 9-11). Vimentin, a major component of intermediate filaments in mesenchymal cells, seems to play a role of central hub in inducing stable EMT during normal ontogenesis and tumor development (12). The sensitivity of PSC to current medical manipulation with platinum-based doublets, sarcoma-specific regimes, and radiotherapy has been completely disappointing (13-16), so it is clinically warranted to devise more effective and simple diagnostic tools for use upon small biopsy samples, as these tumors are presented most often at an advanced stage, where the limited availability of tumor tissue for diagnosis is almost the rule.

Although it has been widely established that assessing PSC is primarily a light microscopy exercise upon surgical specimens (1, 3), the identification of surrogates or adjuncts to morphology that are common to the diverse members of this heterogeneous tumor family could help identify them in demanding cases, especially when taking into account that striking pleomorphism with spindling or giant cell changes may also be shared by conventional poorly differentiated NSCLC (3, 17). In this regard, we have recently proposed, on a small series of PSC biopsies, that strong and diffuse immunoreactivity for vimentin, closely paralleled the eventual diagnoses of PSC on surgical specimens (18), but further investigation on a wider tumor series was thought to be necessary to solidify and generalize these preliminary data. The hope is that better identification of PSC may lead to substantial improvements in the treatment strategy for these life-threatening tumors and favor the performance of PSC-centered clinical trials, which have been thus far largely hampered by the rarity of these tumors and the trouble in their diagnostic recognition, too often assigned to the more generic NSCLC category especially when faced with small-sized diagnostic material (1, 3).

By means of a simple and reproducible immunohistochemical (IHC) approach, our study indicated that a modified-vimentin histological score (M-VHS) was a reasonable diagnostic adjunct to morphology for rendering more reliable diagnoses of PSC on small-sized biopsies. These findings may be relevant to the clinical practice and the design of specific clinical trials.

Materials and Methods

Patients and tumors. A series of 23 consecutive biopsies and corresponding surgical specimens of PSC from 20 males (range 38-87 years) and three females (range 30-62 years) were identified in the pathology archives of the participant Institutions. The need for pairing biopsy samples and surgical specimens for every patient accounted for the relatively small number of PSCs, which are rare tumors most often not amenable to surgery. The lack of a previous history of cancer elsewhere in the body and the availability of complete clinical information were also requirements for entering the study. Pertinent clinicopathological data regarding the 23 PSCs are presented in Table I. Small tissue fragments comprised 16 transcutaneous core biopsies, three bronchial/transbronchial biopsies, three mediastinoscopy biopsies and one video-assisted thoracic surgery-based biopsy, with the corresponding surgical specimens consisting of 13 (bi)lobectomies, nine pneumonectomies and one atypical resection. According to the 7th edition of the TNM staging system, there were one tumor staged IA, four IB, three IIA, eight IIB, six IIIA (four yIIIA), and one yIIIB.

All biopsies and surgical specimens had been fixed in 4% to 10% buffered formaldehyde solution for 12-24 hours and embedded in paraffin according to standard histopathological methods. All the original hematoxylin and eosin (H&E)-stained sections of both biopsies and surgical specimens were blindly reviewed without knowledge of the patients' identities or original tumor categorization, according to the current lung cancer classification (1), assuming that revision of surgical specimens made-up the gold standard for any comparison. The study comprised of 20 PLC, two PB and one CS when addressing surgical specimens, whereas the diagnosis of PLC or CS was necessarily a descriptive one when reviewing biopsies according to the diverse tumor cell components, either conventional NSCLC or spindle and/or giant cells, independent of their relative amount. The diagnosis of PB was instead allowed on biopsy provided that a fetal type adenocarcinoma was mingled with mesenchymal stroma featuring primitive blastema (1, 7). Regarding grading, all tumors were considered poorly differentiated by definition. Details on conventional chemotherapy protocols or driver mutation analysis were beyond the scope of this study dealing with the discovery and validation of a novel diagnostic tool for PSC. Forty-six consecutive and independent surgical cases of NSCLC and two additional cases of PSC, either biopsies or surgical specimens, were used for validating results as negative and positive cancer control groups, respectively.

Immunohistochemical (IHC) evaluation. The list of the antibodies used in the current investigation and the basic technical specifications are summarized in Table II. All tumor samples, whether biopsy or surgical specimen, were assessed for vimentin, an indicator of EMT in the lung (4, 18); p40 and p63, two markers of squamous cell differentiation with the remarkable difference that only p40 is consistently negative in lung adenocarcinoma (19-21); thyroid transcription factor-1 (TTF1) and cytokeratin 7 (CK7), two markers of lung adenocarcinoma; and cytokeratins 5/6 (CK5/6), a further marker of squamous cell carcinoma (19, 22-24). In surgical specimens, the epithelial and mesenchymal components were kept separate while assessing the relevant antibodies, whereas in biopsies tumor cells were evaluated as a whole without trying to distinguish different components, if any.

Briefly, 3- to 4-μm thick sections were made to react with the relevant antibodies and then incubated with a commercially available detection kit (EnVision™ FLEX+; Dako, Glostrup, Denmark) following the manufacturer's instructions according to previously refined IHC methods (18, 19). For all the relevant antibodies, IHC results were rendered semiquantitatively on a scale from 0 to 5+, taking into account the entire tumor area on paraffin block samples and the cellular compartmentalization (nuclear area for TTF1, p40 and p63; cytoplasmic domain for CK5/6, CK7, and vimentin). Tumors were considered negative (0) if staining was completely absent from the relevant cells; 1+ cases exhibited immunoreactivity in up to 10% neoplastic cells, 2+ cases in 11-25% neoplastic cells, 3+ cases in 26-50% neoplastic cells, 4+ cases in 51-75% neoplastic cells and 5+ cases in 76-100% neoplastic cells. This choice was determined by the need for minimizing variability in the slide assessment when trying precise percentages.

In order to devise a modified vimentin histological score (M-VHS), additional characteristics were also recorded, *i.e.* the immunostaining intensity [dichotomized as low=1, if fainter than that seen in the internal control represented by normal mesenchymal cells (fibroblasts, lymphocytes or endothelium) *vs.* strong=2, if as intense as these normal mesenchymal cells] and the distribution pattern (dichotomized as partial=1, if limited to part of the cytoplasm with variably reticular to membranous quality *vs.* diffuse=2, if diffusely distributed to occupy the entire cytoplasmic area). Taking into account these three independently assessed parameters, an M-VHS was then obtained by multiplying all these,

Table I. *Clinicopathological data regarding the 23 pulmonary sarcomatoid carcinomas under evaluation.*

Patient	Gender	Age	Adjuvant CT	Surgery	pTNM	Smoking status	Original tumor diagnosis on BS	Revised tumor diagnosis		Sarcomatous component on SS		Epithelial component on SS		M-VHS on BS	M-VHS on SS
								On BS	On SS	Type	%	Type	%		
INT-1	M	56	Yes	Pneumonectomy	ypT4N0	Never	SpCC	SpCC	PLC	Spindle cells	40	AD	60	20	20
INT-2	M	73	No	Lobectomy	pT2aN0	Current	SpCC	PLC (SpCC+GCC)	PLC	Spindle and giant cells	30	AD	70	20	20
INT-3	M	73	No	Segmentectomy	pT3N0	Current	AD	PLC (AD+GCC)	PLC	Giant cells	30	AD	70	20	20
INT-4	M	56	No	Lobectomy	pT3N0	Current	SpCC	SpCC	PLC	Spindle and giant cells	70	AD	30	20	20
INT-5	M	62	Yes	Pneumonectomy	ypT3N2	Current	AD	SpCC	PLC	Spindle cells	80	AD	20	20	20
INT-6	M	67	No	Bilobectomy	pT2bN0	Current	PLC (SpCC+GCC)	PLC (SpCC+GCC)	PLC	Spindle and giant cells	100	-	-	20	20
IEO-1	F	52	Yes	Pneumonectomy	ypT3N1	Current	NSCLC, NOS	PLC (AD+GCC)	PLC	Giant cells	65	AD	35	16	20
IEO-2	M	61	No	Pneumonectomy	pT2bN1	Former	SQC	SQC	PLC	Spindle and giant cells	65	SQC	35	1	20
IEO-3	F	62	Yes	Pneumonectomy	ypT4N1	Current	NSCLC, NOS	AD	PLC	Giant cells	30	AD	70	0	20
IEO-4	M	85	No	Lobectomy	pT3N0	Current	NSCLC, NOS	PLC (AD+SpCC/GCC)	PLC	Spindle and giant cells	90	AD	10	20	20
IEO-5	M	58	No	Pneumonectomy	pT3N1	Current	SQC	SQC	PLC	Spindle cells	60	SQC	40	0	20
IEO-6	M	56	Yes	Bilobectomy	ypT3N2	Current	AD	AD	PLC	Spindle and giant cells	90	AD	10	0	20
IEO-7	M	39	No	Lobectomy	pT3N0	Current	NSCLC, NOS	PLC (AD+GCC)	PLC	Giant cells	60	AD	40	20	20
RE-2	M	59	No	Pneumonectomy	pT3N1	Current	SQC	SQC	PLC	Spindle and giant cells	95	SQC	5	0	20
RE-3	M	87	No	Lobectomy	pT3N0	Current	NSCLC, NOS	SpCC	PLC	Spindle cells	60	AD	40	20	20
RE-4	M	67	No	Pneumonectomy	pT1bN0	Former	SQC	SQC	PLC	Spindle cells	60	SQC	40	2	20
RE-5	M	55	No	Lobectomy	pT2aN0	Current	PLC (SQC+SpCC)	PLC (SpCC+GCC)	PLC	Spindle and giant cells	100	-	-	20	20
RE-6	M	69	No	Bilobectomy	pT3N0	Former	NSCLC, NOS	SpCC	PLC	Spindle and giant cells	100	-	-	20	20
MO-1	M	74	No	Lobectomy	pT2bN0	Current	SpCC	SpCC	PLC	Spindle cells	30	SQC	70	10	10
MO-2	M	83	No	Lobectomy	pT2aN0	Current	PLC (SpCC+GCC)	PLC (SpCC+GCC)	PLC	Spindle and giant cells	70	AD	30	20	20
JPN-1	F	30	No	Lobectomy	pT2bN0	Never	Favor PB	PB (fetal AD + fetal stroma)	PB	Blastema and rhabdomyosarcoma	60	Fetal AD	40	20	20
JPN-2	M	38	No	Lobectomy	pT2bN0	Unknown	Teratoma	PB (fetal AD + fetal stroma)	PB	Blastema	70	Fetal AD	30	20	20
JPN-3	M	79	No	Lobectomy	pT3N0	Current	PLC (ADSQC+SpCC)	PLC (ADQSC+SpCC)	CS	Focal osteosarcoma	70	ADSQC	30	20	20

M: Male; F: female; BS: biopsy samples; SS: surgical specimens; PLC: pleomorphic carcinoma; SpCC: spindle cell carcinoma; GCC: giant cell carcinoma; PB: pulmonary blastoma; FISH: fluorescence *in situ* hybridization; CS: carcinosarcoma; AD: adenocarcinoma; SQC: squamous cell carcinoma; M-VHS: modified-vimentin histological score.

Table II. *Antibody panel used in the current study.*

Antibody	Mono (m) polyclonal (p)	Clone	Source	Incubation time	Dilution	Pretreatment
Vimentin	m	V9	DAKO, Glostrup, Denmark	30'	1:50	PTLink-EDTA for 15'
p40	p	-	Calbiochem, Darmstadt, Germany	30'	1:3000	PTLink-EDTA for 30'
p63	m	4A4	DAKO, Glostrup, Denmark	30'	1:200	PTLink-EDTA for 15'
Cytokeratins 5/6	m	D5/16 B4	Invitrogen, Camarillo, CA	30'	1:25	PTLink-EDTA for 30'
Cytokeratin 7	m	K 72.7	NeoMarkers, Fremont, CA	30'	1:400	PTLink-EDTA for 15'
Thyroid transcription factor-1	m	8G7G3/1	DAKO, Glostrup, Denmark	30'	1:2000	PTLink-EDTA for 30'

Pretreatment: Pt Link (Target Retrieval Solution, High pH-EDTA pH-8) from DAKO.

thus resulting in a final score ranging from 0 to 20 (*i.e.*, maximum value resulting from 5×2×2).

Statistical analysis. Qualitative data were compared by the Fisher's exact probability test and the chi-square test as appropriate. Different M-VHS values were contrasted by the nonparametric Mann-Whitney test. For all tests, two-sided *p*-values were taken into account, with a threshold of <0.05 being statistically significant.

Results

The 23 PSCs had originally been diagnosed as sarcomatoid carcinoma in eight cases, NSCLC-NOS in six, squamous cell carcinoma in four, adenocarcinoma in three, suspected PB in one and teratoma in another one (Table I). Upon revision, a diagnosis of sarcomatoid carcinoma was reasonable in 17/23 (74%) biopsies by morphology alone, whereas in the remaining six tumors, a definitive diagnosis of squamous cell carcinoma and adenocarcinoma was rendered in four and two cases, respectively. However, the small size of the diagnostic material, the presence of variable necrosis with regressive changes, tissue fragmentation and crush artifacts made easy diagnostic recognition challenging and subjective for most tumors. Moreover, among the 46 cases of conventional NSCLC used as a negative control group, there were poorly differentiated tumors exhibiting marked pleomorphism with spindling and/or giant cell changes as seen in biopsy samples of PSC.

In PSC samples, the mean M-VHS appeared to be marginally lower in biopsies (mean value=14.7, range=0-20) than in the sarcoma/sarcoma-like component (mean value=19.6, range 10-20) of the relevant surgical specimens (p=0.118). This marginal difference, however, disappeared completely once the six tumors containing only conventional NSCLC elements in biopsy were eliminated [mean value=19.7 (range=16-20) *vs*. 19.4 (range 10-20), respectively, p=0.984]. Differences were striking when comparing all biopsies with the epithelial elements of the relevant surgical specimens [mean value=14.7, (range=0-20) *vs*. 2.0 (range=0-8), respectively, p=0.0001).

Table III. *Differences in vimentin immunoreactivity.*

Vimentin immunoreactivity	PSC	NSCLC	*p*-Value
Semiquantitative scale			2.10E-17
0	0	30	
1+	0	5	
2+	0	2	
3+	0	5	
4+	0	3	
5+	23	1	
Pattern of staining within the cytoplasm			1.99E-17
Absent	0	30	
Partial	0	15	
Diffuse	23	1	
Intensity of staining within the cytoplasm			1.51E-11
Absent	0	30	
Low	1	9	
Strong	22	7	

PSC: Pulmonary sarcomatoid carcinoma; NSCLC: non-small cell lung cancer.

Representative features of vimentin IHC in PSC and NSCLC according to the criteria of M-VHS are depicted in Figure 1. The 46 surgical specimens of NSCLC used as independent negative controls for validating M-VHS (validation set) showed that 16 (35%) cases were actually positive for vimentin, including 7/24 squamous cell carcinomas, 8/20 adenocarcinomas and 1/2 adenosquamous carcinomas. However, the mean M-VHS was 4.1 (range 1-8) in these tumors in comparison with 19.6 (range 10-20) for PSC (p<0.0001). It emerged that PSC and conventional NSCLC differed significantly in all the parameters under evaluation, *i.e.* the percentage of vimentin-immunoreactive cells (exclusively 5+ in PSC *vs*. 1+ to 5+ in NSCLC), the intensity of immunostaining (almost always strong in PSC *vs*. low in NSCLC), and the staining pattern of the cytoplasm (always diffuse in the cytoplasm in PSC *vs*. reticular to membranous in NSCLC) (Table III). Interestingly, M-VHS of conventional NSCLC was double the corresponding value of the epithelial component in PSC [4.1 (range=1-8) *vs*. 2.05

Table IV. *Distribution of the relevant immunohistochemical markers in biopsy and surgical specimen.*

Marker under evaluation	Pleomorphic carcinoma (n=20)*						
	IHC score	Positivity score	Biopsy	Sarc-SS	Epith-SS**	*p*-Value[1]	*p*-Value[2]
Cytokeratin 7	Negative	0	10	9	4	0.136	0.038
	≤10%	1+	1	0	1		
	11-25%	2+	1	4	1		
	26-50%	3+	3	2	1		
	51-75%	4+	1	2	0		
	76-100%	5+	4	3	10		
Tyroid transcription factor-1	Negative	0	17	16	11	0.999	0.307
	≤10%	1+	0	1	2		
	11-25%	2+	1	2	0		
	26-50%	3+	1	1	2		
	51-75%	4+	1	0	0		
	76-100%	5+	0	0	2		
Cytokeratins 5/6	Negative	0	15	19	8	0.047	0.0002
	≤10%	1+	0	0	3		
	11-25%	2+	0	0	0		
	26-50%	3+	2	0	3		
	51-75%	4+	0	1	0		
	76-100%	5+	3	0	3		
p63	Negative	0	11	14	9	0.439	0.063
	≤ 10%	1+	2	0	1		
	11-25%	2+	2	4	0		
	26-50%	3+	0	0	1		
	51-75%	4+	2	1	2		
	76-100%	5+	3	1	4		
p40	Negative	0	14	16	12	0.606	0.068
	≤10%	1+	1	0	0		
	11-25%	2+	1	2	0		
	26-50%	3+	0	0	0		
	51-75%	4+	2	2	1		
	76-100%	5+	2	0	4		
Vimentin#	Negative	0	0	0	9	0.412	6.30E-11
	≤10%	1+	0	0	1		
	11-25%	2+	0	0	4		
	26-50%	3+	0	0	2		
	51-75%	4+	1	0	1		
	76-100%	5+	13	20	0		

IHC: Immunohistochemistry; *carcinosarcoma and pulmonary blastoma were excluded because of their small number; **three pleomorphic carcinomas were composed exclusively of spindle and giant cells with no overt epithelial component; Epith-SS: epithelial components in surgical specimens; Sarc-SS: sarcoma-like components in surgical specimens; # the six biopsies containing only epithelial components (four squamous cell carcinomas and two adenocarcinomas), which were negative (4 cases), 1+ (1 case) or 2+ (1 case) for vimentin, were excluded from computation. [1]Comparing biopsy with sarc-SS; [2]Comparing sarc-SS with epith-SS.

(range 0-8), respectively, p=0.008]. There were no statistically significant differences in M-VHS among different histologies of NSCLC (squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma, p=0.960). Poorly differentiated NSCLC simulating PSC in regard to spindling and/or giant cell changes did not present with significant vimentin immunoreactivity (Figure 2). Of note, the two additional cases of PSC used as the positive control group in the validation set, exhibited the same high M-VHS value in both biopsy and surgical specimens as seen in the training set.

No differences in the distribution of semiquantitative scores were found between biopsies and sarcoma-like

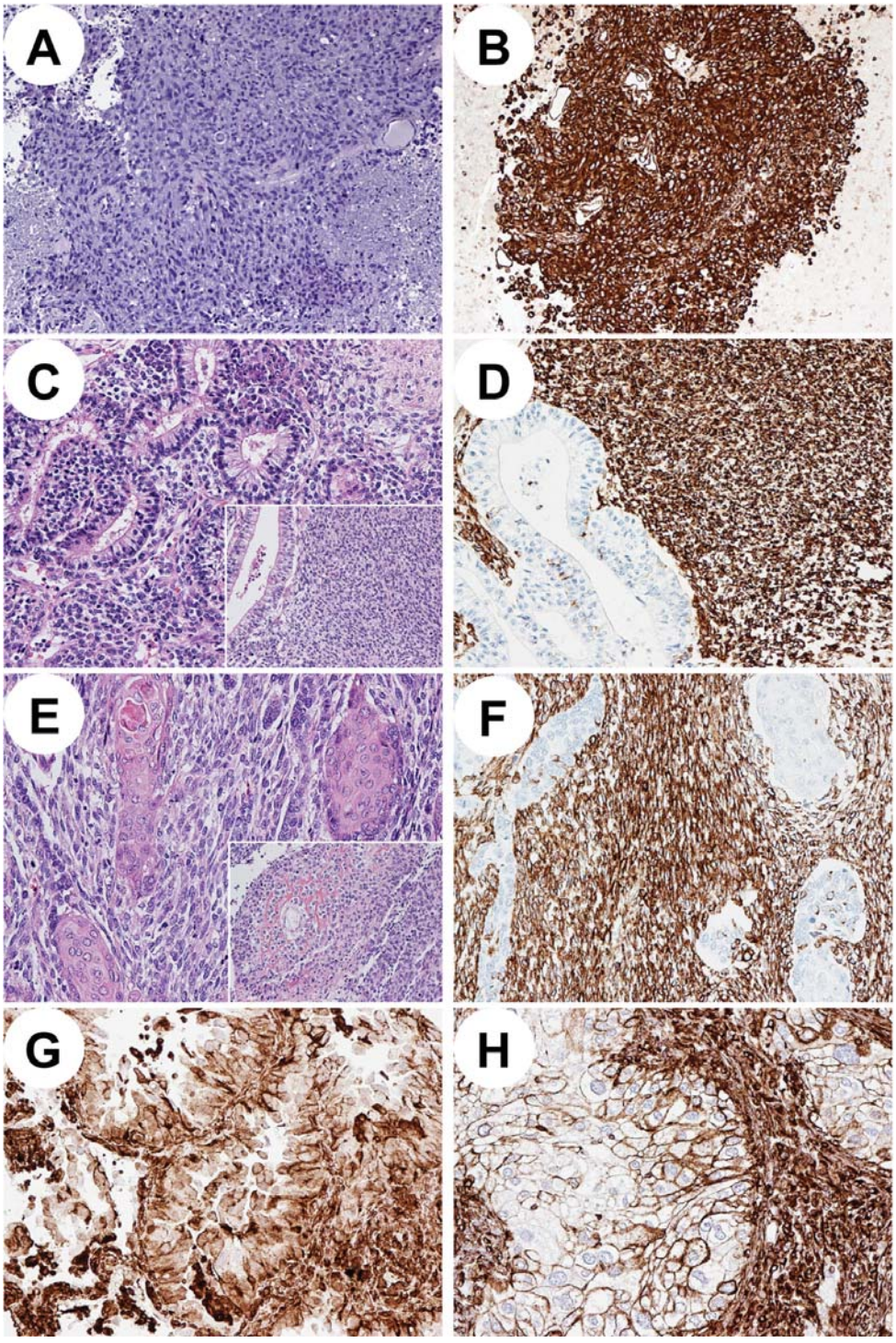

Figure 1. *A pleomorphic carcinoma composed of spindle and giant cell carcinoma, as seen in a hematoxylin and eosin-stained section (A), exhibited an impressively high modified-vimentin histological score (M-VHS) (B). Pulmonary blastoma with fetal type adenocarcinoma and primitive looking-appearing stroma (inset) (C) reacted strongly and diffusely for vimentin in the mesenchymal component whereas the glandular structures were consistently negative (D). A case of carcinosarcoma composed of adenosquamous carcinoma and sarcoma component with focal osteosarcomatous differentiation (E, inset) exhibited a high M-VHS in the latter component, whose pattern decidedly contrasted with the almost complete negativity for vimentin of its epithelial component (F). Exemplification of vimentin-expressing conventional adenocarcinoma (G) and squamous cell carcinoma (H): many tumor cells actually reacted for vimentin, but their resulting M-VHS values remained negligible because of the faint immunostaining level and the partial quality of the cytoplasmis expression (G and H) (all microphotographs are taken at ×200 magnification).*

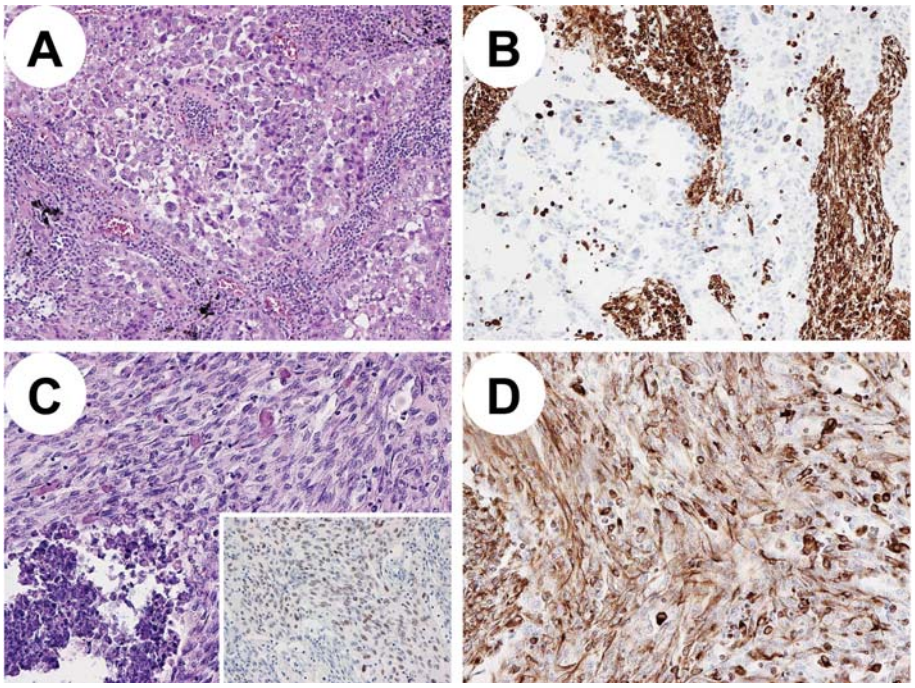

Figure 2. *Two examples of adenocarcinoma showing marked pleomorphism in the form of giant cell changes (A) and spindle cells (C), but not vimentin accumulation (B), and with low vimentin content (D) to denote the lack of stable epithelial mesenchymal transition. TTF1 was positive in the spindled tumor cells, confirming adenocarcinoma diagnosis (panel C, inset). All microphotographs are taken at ×200 magnification.*

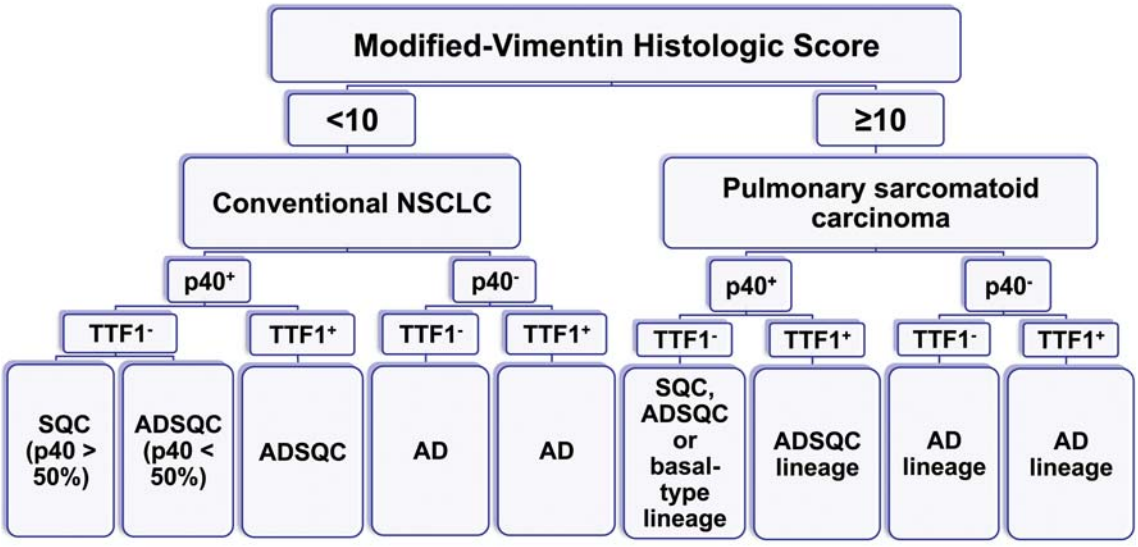

Figure 3. *Diagnostic algorithm constructed according to the different values of modified-vimentin histological score (M-VHSI for splitting lung cancer into PSC (M-VHS≥10) and conventional NSCLC (M-VHS<10), in turn further classified according to the addition of p40 and the thyroid transcription factor-1 (TTF1) immunostaining. In particular, adenocarcinoma (AD) in conventional NSCLC and AD lineage in PSC were identified by p40 negativity regardless of TTF1, squamous cell carcinoma (SQC) in conventional NSCLC and SQC lineage in PSC by p40+/TTF1- profile, with p40>50% tumor cells and adenosquamous cell carcinoma (ADSQC) in conventional NSCLC and ADSQC lineage in PSC by $p40^{+}/TTF^{+}$ or $TTF1^{-}/p40^{+}$, the latter with p40<50% tumor cells). PSC with $p40^{+}/TTF1^{-}$ profile could comprise tumor cells with basal-type properties heralded by progenitor/stem cells. Other diagnostic possibilities regarding sarcoma, melanoma, mesothelioma or anaplastic/large cell lymphoma for tumors featuring M-VHS values>10 have not been included in the diagnostic algorithm but could be reliably ruled out by means of accurate clinicopathological work-up.*

elements on surgical specimens for all the remaining markers, except CK5/6 (four PLCs had only squamous cell carcinoma component in the relevant biopsies) (Table IV). Significant down-regulation of epithelial markers (CK7 and CK5/6), marginal decrease of p40/p63 and marked increase of the number of vimentin-immunoreactive cells (a component of M-VHS) were seen when moving from epithelial to sarcoma-like elements (Table IV). In particular, immunoreactivity for p40 closely correlated with the squamous cell carcinoma component (cases IEO-2, IEO-5, RE-02, RE-04) if 50% or more (4+ to 5+) tumor cells were positive, and with squamous morulae in PB (case JPN-1) or adenosquamous carcinoma in CS (case JPN-3) if up to 25% (2+) tumor cells were positive. Three PLCs, however, devoid of overt squamous differentiation (cases INT-04, RE-03 and RE-05) and lacking CK5/6 surprisingly exhibited scores of 2+ to 5+ for p40 or p63 in the sarcoma-like cells only. Unlike p40, p63 expression was retained (1+ to 4+ immunostaining) in the adenocarcinoma component of three PLC (cases INT-2, IEO-4 and MO-02).

A practical diagnostic algorithm was then constructed in order to differentiate the diverse subsets of conventional NSCLC and to highlight the diverse differentiation lineages of PSC according to the values of M-VHS and IHC for p40 and TTF1 (Figure 3).

Discussion

The main result of our investigation is that the M-VHS helped to diagnose PSC in biopsy samples. As novel therapy options for PSC, a life-threatening tumor subset for which the current treatment is disappointing, could also stem from targeting EMT mechanisms, an ultimate recognition of PSC is of utmost clinical relevance especially when dealing with limited diagnostic material; moreover, distinguishing between the diverse histological variants could be clinically unwarranted due to the comparable clinical behavior underlying the diverse subtypes of PSC (3). Diagnostic criteria for PSC have been based on surgical specimens, with a 10% cut-off being required for classification in the event of PLC (1, 3, 25), hence the diagnosis of PSC using small biopsy specimens has been argued against (13, 26), albeit it may be suggested or at least reasonably suspected on biopsy (27, 28) and cytology or analysis of cell blocks (1, 29-31), even by morphology alone.

In this article, not only did we confirm our previous observations on the role of vimentin as a central hub (12) in the induction of stable EMT that is a hallmark of PSC (3, 4, 9), but we also re-appraised an old and apparently useless marker according to a novel and more objective method of assessment, *i.e.* M-VHS, to assist pathologists in diagnosing PSC more reliably, even on small biopsy samples. On this regard, sampling error rather than an insufficient biomarker robustness accounted for hampering of the final recognition of PSC in six biopsies of our tumor series, inasmuch as vimentin accumulation is deemed to be closely related to full-blown EMT in NSCLC in general and in this tumor type in particular (18, 19).

Our proposal of M-VHS relied on three independently assessed and easily evaluable parameters that were multiplied by each other to obtain a final score, namely the percentage of positive tumor cells, the immunostaining intensity and the labeling pattern inside the cytoplasm, hence the suffix modified in comparison with more traditional scoring systems. In fact, the main novelty was the introduction of an additional parameter, namely the labeling pattern inside the cytoplasm, which is easily appreciable even at low magnification and actually reflects the striking accumulation of vimentin in tumor cells due to stable EMT-related cytoskeleton remodeling.

These three parameters were evenly distributed at the highest level in PSC, while only few NSCLC exhibited high values of intensity for vimentin in a lower percentage of cells or a diffuse pattern and a high intensity in a more limited number of tumor cells with a resulting low M-VHS (Table III). This study represents, to the best of our knowledge, the first attempt of using vimentin for clinical purposes in order to obtain a substantial improvement in the diagnostic recognition of PSC in small biopsy specimens, especially when considering that several other markers thus far proposed for subtyping NSCLC (18, 24, 32) may prove to be completely negative or misleading in these tumors probably due to the irregular distribution of epithelial traits owed to EMT changes (18, 19). On this regard, it is worthwhile noting that cytological appraisal of poorly differentiated NSCLC (33), a cornerstone in lung cancer classification (1), may also be insufficient and inconclusive alone, when considering that spindling and/or giant cell changes not related to vimentin-driven cytoskeleton rearrangement, the biopsy fragmentation or crush and shrinkage tissue artifacts may be responsible for inconsistencies between morphology and function. Although conventional high-grade adenocarcinomas and squamous cell carcinomas of the lung are actually known to variably undergo EMT with vimentin accumulation (34-36) (as also suggested in our investigation by the double value of M-VHS in conventional NSCLC considered as a whole compared to that of the epithelial component of PSC), we noted that M-VHS based on diverse aspects of vimentin IHC in tumor cells (namely, percentage, intensity of staining and intracellular distribution) served as a powerful adjunct to morphology to substantiate the ultimate diagnosis of PSC in small-sized biopsies (Figure 1). The same held true for sarcoma/sarcoma-like elements of PSC, which could easily be distinguished from the corresponding epithelial cell component in bi-phasic tumors on the basis of the M-VHS value, indicating that a stable EMT was occurring in these tumors. Our results support the notion that a strong and diffuse vimentin expression by IHC by sarcoma-like tumor

cells featuring spindle and/or giant cell changes would authorize diagnosis of PSC in biopsy samples, whereas the only morphological changes of spindle and/or giant cells without overwhelming vimentin accumulation would be a necessary but insufficient criterion to corroborate such an ultimate diagnosis (Figure 2). On this regard, M-VHS was able to identify two additional cases of PSC used as the positive control group in the validation set, which exhibited the same high M-VHS value in both biopsy and surgical specimens as seen in the training set. The issue of PB was different; this could also be reliably rendered on biopsies by morphology alone upon concurrence of fetal adenocarcinoma and blastema-appearing primitive stroma (7). In addition, in these cases however, M-VHS confirmed its central role as a marker of stable EMT, helping to diagnose these tumors more confidently (3) (Figure 1). Interestingly, while vimentin expression closely parallels EMT in NSCLC (34-36), poorly differentiated neuroendocrine lung tumors, practically, never undergo significant vimentin expression according to our criteria of M-VHS (Pelosi G, unpublished observations), while they activate other mechanisms of cytoskeleton rearrangement, for example through the accumulation of actin filament-bundling proteins such as fascin (37).

Although vimentin has low diagnostic specificity because of its wide distribution in a variety of epithelial and non-epithelial tumors (38), it is worth stressing that once metastases to the lung are excluded by means of an integrated clinicopathological work-up, most of the remaining vimentin-positive malignancies occurring in this organ actually correspond to PSC, inasmuch as diagnostic alternatives of primary sarcomas, melanoma or anaplastic/large cell lymphoma are exceedingly rare occurrences, which may be assessed by additional IHC markers (see also Table IV), selective molecular investigations and clinical and case history evaluation. In turn, malignant mesothelioma, whether epithelioid or sarcomatoid, may be a formidable mimicker of pleura-based PSC in small biopsy samples as far as vimentin IHC is concerned, but highly specific markers, such as claudin-4 (39), may help distinguish them from PSC. Practically speaking, a high M-VHS (see below) in a lung-localized tumor should alert to the possibility of PSC until proven otherwise by using appropriate and accurate clinicopathological work-up.

For lung tumors, it was possible to construct a diagnostic algorithm for differentiating PSC from conventional NSCLC on the basis of the different values of M-VHS (median cut-off=10) and a recently released, two-hit IHC approach relying on p40 and TTF1 (19) (Figure 3). In PSC (M-VHS≥10), p40 negativity heralded adenocarcinoma lineage regardless of TTF1, whereas p40 positivity suggested squamous, adenosquamous or basal-type lineage. Conventional NSCLC (M-VHS<10) were in turn split into adenocarcinoma ($p40^-/TTF1^+$ or $p40^-/TTF1^-$), squamous cell carcinoma ($p40^+/TTF1^-$, with p40>50% tumor cells) or adenosquamous cell carcinoma ($p40^+/TTF1^+$ or $p40^+/TTF1^-$, the latter with p40<50% tumor cells). Negativity for vimentin in association with the lack of p40 and TTF1 confidently excluded PSC (but the same profile would exclude also melanoma, sarcoma, lymphoma and mesothelioma), paving the way to the possibility of being faced with uncommon pulmonary adenocarcinoma, non-conventional lung tumor or metastasis for which careful clinical integration is mandatory (18, 19).

Although not specifically addressed as the main endpoint of this study but inclusive to the diagnostic algorithm depicted in Figure 3, some considerations about the prevalence of p40 in PSC are worthwhile. In our experience, p40 strictly correlated with squamous cell differentiation in PLC (squamous cell carcinoma), PB (squamous morulae) and CS (adenosquamous carcinoma), should IHC be positive, and with adenocarcinoma differentiation, should it be negative according to recently proposed criteria (19). In contrast, p63 was overtly positive (1+ to 4+) also in the adenocarcinoma component of some PSC as previously shown (20, 21). However, PLC with no overt squamous cell differentiation by morphology and lack of other squamous cell carcinoma-related markers, such as CK5/6 (18) or desmocollin-3 (40) (data not shown), showed p40-positive tumor cells to be confined to the sarcoma-like cell component, suggesting a possible basal-type phenotype (41) heralded by progenitor/stem cells also in light of the distribution of the p40-positive elements in normal lung tissue (19). Further investigation on a wider tumor series is needed to confirm these preliminary findings, which could yet have some clinical relevance to personalized treatments.

In conclusion, we proposed M-VHS here as an effective tool to reliably distinguish PSC from other NSCLC in small biopsy specimens and indicated the possibility for p40 to reflect a basal-like phenotype of progenitor/stem cells potentially suitable for targeted therapy in a subset of PSC.

Conflict of Interest

The Authors declare that they have no conflicts of interest.

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