Gross Specimen Handling Procedures Do Not Impact the Occurrence of Spread through Air Spaces (STAS) in Lung Cancer

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Gross Specimen Handling Procedures Do Not Impact the Occurrence of Spread Through Air Spaces (STAS) in Lung Cancer

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Abstract

Spread Through Air Spaces (STAS) is a form of invasion characterized by neoplastic cell dissemination in the lung parenchyma surrounding the outer edge of the tumor. Its possible artifactual origin is widely debated in the literature. The aim of this study is to investigate the potential impact of gross sampling procedures in causing STAS. A prospective series of 51 surgical lung specimens was collected (35 adenocarcinomas, 68.6%; 13 squamous cell carcinomas, 25.5%; 2 large-cell neuroendocrine carcinomas, 3.9%; 1 atypical carcinoid, 2%). The fresh tissue was sectioned with a new and clean blade for each cut, to obtain a tissue slice comprising the upper lung parenchyma, the tumor, and the lower parenchyma. This slice was cut in half and separately processed. The same procedure was repeated in the residual (specular) specimen after formalin fixation. STAS was identified in 33/51 (64.7%) cases, the predominant pattern being cluster formation (29 cases, 87.9%), the remaining 4 cases having single-cell invasion. Comparing STAS detection in upper and lower lung parenchyma areas (ie, before and after the blade crossed the tumor), no significant preferential STAS distribution was observed, indeed being almost overlapping (60.6% and 63.6% for fresh and 61.3% and 65.6% for fixed tissues, respectively). There was no difference between STAS occurrence in freshly cut and fixed corresponding samples. These findings indicate that STAS is not a pathologist-related artifactual event because of knife transportation of tumor cells during gross specimen handling and support the notion that it is a phenomenon preexisting to surgical tissue processing.

The concept of Spread Through Air Spaces (STAS) was originally described in lung adenocarcinoma1 as a reinterpretation of older observations regarding free-floating tumor cells in alveolar spaces.2,3 It was incorporated in the current World Health Organization (WHO) classification of thoracic tumors as a form of invasion characterized by neoplastic cell dissemination in the lung parenchyma surrounding the outer edge of the tumor.4 It may occur as solid nests, micropapillary clusters, or single cells, possibly contributing to the significantly increased recurrence rate in STAS-positive patients.1,4 Subsequently, STAS was reported in a broad-spectrum of lung tumor subtypes5–8 showing its prognostic value.6,9,10 In a study by Warth et al,11 STAS was much more frequent in high-stage, node-positive lung adenocarcinomas with distant metastases. Moreover, in a recent work by Vaghjiani et al12 the presence of STAS was a predictor of occult lymph node metastasis in IA clinical stage lung adenocarcinomas.

Although, the presence of STAS is generally considered as one of the manifestations of lung cancer aggressiveness, the real nature of the STAS phenomenon is widely debated in the literature.13–15 The mechanism of STAS is yet to be understood, regarding the survival of free-floating tumor cells in the air spaces following detachment from the main tumor mass and their potential reattachment to alveolar walls to gain energy supply from blood vessels.16–18

The issue of the possible artifactual origin of STAS was raised, claiming that loose tumor fragments can be misplaced during tissue manipulation by the surgeon and/or in the pre-analytical phase of gross specimen handling, rather than being a biological event linked to intrinsic tumor invasion ability.15

The aim of the study is to investigate the potential impact of gross sampling procedures in the pathology laboratory in causing STAS in a series of resected lung cancers.

MATERIALS AND METHODS

Material Collection

We collected a prospective series of 51 surgically resected fresh lung specimens operated from May 2019 to April 2020 at the “Città della Salute e della Scienza” (Turin, Italy) and San Luigi (Orbassano, Turin, Italy) University Hospitals. Only cases that did not undergo neoadjuvant chemotherapy, harboring an easily palpable tumor mass, and with sufficient lung parenchyma surrounding the neoplasia (thus, as an example, not those with extensive visceral pleura infiltration/retraction) were eligible and entered into the study. In addition, centrally located carcinomas did not fit the grossing techniques described below and were excluded.

Clinicopathologic data such as age, sex, tumor location, largest diameter of the lesion, type of surgery (lobectomy, segmentectomy, bilobectomy, pneumonectomy), surgical technique (open vs. minimally invasive surgery), tumor grade, pTNM stage, visceral pleural invasion, angioinvasion, and presence of necrosis were collected from patients’ clinical reports in a dedicated database. Before the study started, all cases were deidentified and coded by a pathology staff member not involved in the study, and all data were accessed anonymously.

Grossing Technique and Staining

All samples were sent fresh to the pathology laboratory, either directly from the operating room in <1 hour or after under vacuum preservation at 4°C within 6 hours. After removing the tissue from the plastic container or bag, each specimen was handled on a clean surface under an anatomic dissection hood. After inking the pleural margin and using a new and clean blade, the first cut was performed perpendicular to the inked pleural surface through the peripheral parenchyma, the tumor mass, and the deep lung parenchyma on the other side of the tumor. After replacing the blade with a new one, a second cut was done along the

same direction obtaining a 4-mm-thick slice. This was divided into 2 parts cutting half the tumor mass, thus resulting in (i) a tumor area comprising the upper lung parenchyma (fresh upper sample) and approximately half tumor and (ii) the other half tumor area and the lower parenchyma portion (according to the blade cut direction) (fresh lower sample). The 2 tissues were placed into 2 separate biocassettes (labeled “fresh upper/lower blocks”). The residual surgical specimen was then fixed in neutral 10% formalin for 24 hours. The following day, 2 additional sections parallel and specular to the previous fresh-cut surface were obtained following the same cut direction and using a clean blade each time (fixed upper sample and fixed lower sample). This slice was also 4 mm thick and was cut half through the tumor mass and separately processed, labeling the tissues as “fixed upper/lower blocks.” The sampling procedure workflow for the fresh specimen step is presented in Figure 1.

The 4 samples were separately processed for paraffin embedding together with all the other main tumor and distant parenchyma samples taken according to the laboratory guidelines for diagnostic purposes). Three-micrometer-thick sections were cut from each tissue block and routinely stained with hematoxylin and eosin (Leica ASP 300 processor and automated Leica ST5020 Multistainer; Leica Microsystems, Wetzlar, Germany). Selected cases with controversial interpretation of STAS were submitted to immunohistochemical stains (TTF1, p40, or chromogranin A) to highlight suspected intra-alveolar tumor cells, using an automated platform (Ventana BenchMark AutoStainer; Ventana Medical Systems, Tucson, AZ).

Evaluation Criteria

Histologically, all glass slides were blindly screened by 3 of us (J.M., L.R., M.P.) and discrepant evaluations were discussed and solved using a multihead microscope. The following parameters were recorded: (i) tumor histotype; (ii) presence or absence of single and/or clusters of cells identical to those of the primary tumor in the alveolar spaces beyond the first alveolar space at the tumor edge (according to the WHO 2015 definition19); (iii) location of STAS cells, when present, either near the tumor edge (when found in the second to the fifth alveolar space) or distant (in case of their detection beyond fifth alveolar space, from the tumor edge); (iv) peritumoral lung tissue alterations (such as atelectasis or emphysema) with particular attention to conditions that caused intra-alveolar deposition of cells or fluids, including, edema, hemorrhage, hyaline membranes, granulation tissue, and histiocytic desquamation. According to the strict definition of STAS, the detection of neoplastic cells or clusters at the tissue section borders (or, obviously, within vascular and bronchial lumina, close or far away from the main tumor) was not considered STAS, but rather a possible artifact due to passive transportation of tumor fragments.

Quantification of STAS

STAS-positive cases were further analyzed, by performing STAS quantification, for a more detailed case evaluation. Two different methods were used. First, we used the method described by Blaauwgeers et al.13 In brief, the count of tumor fragments in each slide was estimated by counting the number of positive ×10 objective fields independent of the actual number of clusters. Second, to better quantify the presence of STAS, we also assessed the number of tumor cluster cells in each slide. Cases with the single-cell STAS pattern were not included in the quantification assessment.

Statistical Analyses

All analyses were performed using Stata/MP 15.0 Statistical Software (StataCorp, College Station, TX). Continuous variables were summarized as the mean and SD, whereas for categorical variables the frequency was provided. Patient’s characteristics were compared using the $\chi 2$ test for categorical variables according to Bonferroni corrections and the t test or for continuous variables. Agreement among different STAS quantification systems were performed using the Cohen κ test and Pearson r test. All statistical tests were 2 sided. P-values <0.05 were considered significant.

RESULTS

STAS Characterization and Distribution

The current series represents 28% (51/184) of the total number of lung cancers operated in the same period in the 2 academic hospitals and included all potentially adequate cases according to the selection criteria described in the material and method section. On the basis of the described sampling procedure (see the Materials and methods section) of the 51 cases, 201 glass slides were available for evaluation, having 3 slides been excluded because of insufficient peritumoral lung parenchyma on the histology section.

As shown in Table 1, STAS was identified in 33/51 (64.7%) cases (Figs. 2A–F), whereas 18/51 (35.3%) were STAS negative. The cases were diagnosed as adenocarcinoma (35, 68.6%), squamous cell carcinoma (13, 25.5%), large-cell neuroendocrine (NE) carcinoma (2, 3.9%), and an atypical carcinoid (1, 2%).

In particular, considering STAS-positive cases, its presence was found in 20/33 (60.6%) upper fresh samples and in 19/31 upper fixed samples (61.3%, 2 glass slides not evaluable). Regarding lower portions, it was observed in 21/33 lower fresh samples (63.6%) and 21/32 lower fixed samples (65.6%, 1 glass slide not evaluable) (Table 1). Mirroring each other, there was no significant difference in the presence of STAS between the corresponding fresh and fixed tissue areas (Table 1).

The STAS predominant pattern was cluster formation (29 cases, 87.9%), whereas single-cell invasion was observed in 4 cases only (12.1%). Moreover, in 5/29 cases with clusters as the predominant pattern, single-cell alveolar spread was noted in some areas, as well. Regarding the distance from the main tumor edge, STAS was observed in the nearby alveoli in 16 cases (48.5%) (ie, those from second to fifth alveolar space) and also in distant air spaces (ie, those beyond the fifth from tumor edge) in the other 17 cases (51.5%) (data not shown).

Peritumoral lung tissue alterations were also assessed to investigate if some specific pathologic condition might have favored or hampered alveolar spread of neoplastic cells. Focal interstitial fibrosis was present in majority of the cases (36/51, 70.6%), whereas nearly half cases had atelectasis (23/51, 45%) and only 10 (19.6%) emphysematous changes. Regarding intra-alveolar deposition of fluids and/or cells, edema, hemorrhage, and histiocytic desquamation were noted in 4/51 (7.8%), 20/51 (39.2%), and 16/51 (31.4%) cases, respectively (Fig. 2G). No significant difference in peritumoral lung tissue alterations was noted between STAS-positive and STAS-negative cases (Table 1). Interestingly, 1 case showed lipoid pneumonia with alveoli packed by foamy macrophages intermingled with rare STAS clusters (Fig. 2H).

In 2 equivocal cases, hematoxylin and eosin coloration was insufficient to provide an accurate STAS assessment; hence, immunohistochemical stains were performed to better highlight intra-alveolar tumor cells. In detail, in 2 morphologically suspected cases, p40 and chromogranin A stains (in a case of squamous cell carcinoma and of atypical carcinoid, respectively) were able to highlight the single tumor cell spread pattern (Fig. 3).

In addition, we quantified the presence of STAS in 29 positive cases (cases with single-cell patterns were not included) according to the number of tumor clusters around the main tumor mass, in each of the 4 slides, and then we summed the 4 values to obtain the amount of STAS per single case. We observed an excellent agreement between ours and the method adopted by Blaauwgeers et al,13 either in terms of ability of detection (Supplemental Table 1, Supplemental Digital Content 1, https://links.lww.com/PAS/B50) and in terms of tumor clusters/tumor-positive field correlation (Supplemental Table 2, Supplemental Fig. 1, Supplemental Digital Content 1, https://links.lww.com/PAS/B50), regardless of upper/lower sample section of fresh/fixed status.

The median value of tumor clusters per case was 9 (range: 2 to 92) and the mean value was 21.5±23.3 clusters (Supplemental Table 2, Supplemental Digital Content 1, https://links.lww.com/PAS/B50). Moreover, we divided cases according to the median value of number of clusters (cutoff 10) into high and low STAS groups. Specifically, 14/29 (48.3%) of cases had a high number of STAS clusters (>10 median value), whereas 15/29 (51.7%) cases were pertinent to the low STAS group (≤10 median value). However, correlation with clinicopathologic parameters did not show any significant differences between the cases pertinent to the high and low STAS groups (Supplemental Table 3, Supplemental Digital Content 1, https://links.lww.com/PAS/B50).

Clinical and Pathologic Characteristics of Lung Tumors

Our case series consisted of 33 men and 18 women with a median age of 70 years (range: 55 to 83 y).

A larger part of cases (26, 51%) were assigned to stage I, whereas 13 (25.5%) and 12 (23.5%) cases were assigned to stages II and III, respectively. Regarding tumor grade, most tumors fell into G2 category (40, 78.4%), whereas 11 cases (21.6%) were assigned to G3 group. Penetration of visceral pleura by tumor cells was diagnosed in 21 cases (41.2%), whereas 30 (58.8%) were assigned to PL0 pleural stage. Angioinvasion was observed in 23/51 cases, whereas necrosis was present in 32/51 samples. Lymphocytic tumor infiltration was moderate to high in 26/51 tumors, low in 22/51, and absent in 3 cases (Table 1).

Lobectomy was performed in majority of cases (43, 84.3%), whereas the remaining patients were treated by segmentectomy (3, 5.9%), bilobectomy (2, 3.9%), and pneumonectomy (3, 5.9%). Technically, the patients were mainly treated with minimally invasive keyhole surgery (36, 70.6%), rather than with the traditional open surgery approach (7, 13.7%). This information was not available in the remaining 8 cases. Thirty-five specimens were received under vacuum (68.6%), whereas 16 (31.4%) were transported fresh from the operation theater.

As shown in Table 1 no significant association was observed between STAS status and above-described clinicopathologic characteristics, including sex, age, tumor diameter, histotype, clinical stage, tumor grade, pleural status, angioinvasion, necrosis and tumor-infiltrating lymphocytes, surgical procedure, shipping and preservation conditions.

DISCUSSION

In this study, we demonstrated that STAS is not an artifact due to pathologist's handling of the gross surgical specimen. Unfortunately, we could not exclude any other possible artifactual events occurred before the specimen reached the pathology laboratory, including manipulation during the surgical procedure.

Regarding tissue specimen handling in the pathology laboratory, several steps are crucial for the appropriate management of the sample and it is known that some actions by the pathologists and/or technicians may well determine tissue misplacements, including those along gross specimen processing procedures, paraffin-embedding phase, and microtome cutting, among others.20

The specific issue of STAS involves mainly the first of these steps at the gross specimen handling phase, as the others may induce abnormal tissue transportation and misplacement, but not within alveolar spaces only.

The main criticism to the concept of STAS is that a blade cutting a lung specimen can transfer cells and clusters from the tumor mass to the surrounding nonneoplastic pulmonary tissue, thus supporting the notion that any free-floating neoplastic cell in the alveoli (or even bronchi and vessel lumina) are passively

moved by the blade passage rather than being the result of a biologically active neoplastic spread process. Several papers supported this view, including some experiments on how a “dirty” or contaminated blade can move/misplace any type of tissue fragment or, by analogy, any piece of food cut with kitchen knives.15 A paper from the same group supported this notion even after performing an experiment similar to the one designed here, with quite a large number of cases that had tumor cell spread (or better “transportation”) mostly into the alveoli crossed by the blade after cutting the tumor mass, thus strongly supporting the possibility of a knife effect during specimen grossing.13 Here, a strict experimental procedure has been designed, based on the use of new clean blades for each cut, and the sequential analysis of fresh (within 6 h from surgery) and formalin-fixed (after 24 h) specular samples containing the lung tumor and the peritumoral parenchyma. Our results indicate that STAS occurred in 64.7% of cases, a figure largely in the range of published reports, and that both areas of the pulmonary parenchyma located before and after the blade passage into the tumor mass have a consistent presence of STAS, with no statistical differences between the 2 locations, nor between freshly cut or postfixation cut lung specimens. In particular, we found 48.3% (14/29) of cases with a higher number (>10 median value) of tumor clusters surrounding the main tumor mass. However, possibly because of the limited number of cases, no statistically significant differences were observed across the 2 groups.

As it is against physical laws that the possibility of neoplastic cell clusters to be misplaced in the reverse direction of an acting physical force, as that produced by a cutting blade, it seems virtually impossible to interpret STAS images in the upper parenchymal portion as a knife-induced artifact.13,15 This is particularly true for the rare cases having “distant” STAS clusters in the subpleural alveoli, far away from the tumor edge.

The use of a new, clean blade for each cut also excluded the possibility of transferring cells from previously contaminated blades within the same case. Care was also taken to produce a linear cut deep in the nodule avoiding horizontal movements (saw-like), to minimize artifactual cell spread in any direction. Even after these strict rules and procedures, STAS seemed to occur in several cases and locations with no specific preference of distribution in the lung parenchyma located before the tumor mass or after the tumor mass, according to the cutting blade direction.

A further support to the real (biological) nature of STAS is linked to its occurrence in any histologic type, in either near or distant alveolar spaces and in virtually all pulmonary pathology conditions affecting the peritumoral alveoli, including edema, hemorrhage, macrophage desquamation, and even in 1 case of an extensive lipoid pneumonia that massively filled the air spaces. In this latter, it would be very difficult to interpret as passive transportation the presence of neoplastic cells clusters “embedded” in the alveoli completely laden of foamy histiocytes, meaning that it would seem impossible to get through by a passive mechanism.

Even the pattern of STAS (single cells vs. clusters) did not seem to differ in the current series, with a predominance of the latter. Clusters generally occur as irregular aggregates rarely filling the alveolar space, but mostly floating in partially atelectatic spaces or, more rarely, attached to the pneumocyte lining or admixed with red blood cells and desquamated macrophages.

As reported, rare cases created difficulties in taking true STAS apart from desquamated macrophages or atypical (reactive) pneumocytes. This was especially true in the case of well-differentiated adenocarcinomas for which specific markers can be of help in correctly interpreting intra-alveolar neoplastic cells. Another controversial area regards NE neoplasms where STAS was equally found to be prognostic,5,6 thus recommending identifying STAS and including this information in the pathology report. In the current prospective series, 2 large-cell NE carcinoma and 1 atypical carcinoid occurred and, in the latter, STAS was confirmed by a chromogranin A staining as this case had a predominant single-cell pattern of NE tumor cells that were not readily evident in standard hematoxylin and eosin sections. In addition, in

the case of carcinoids, the possible coexistence of NE cell hyperplasia and/or tumorlets14 in the peritumoral parenchyma needs an accurate and cautious interpretation as STAS of NE cell clusters far away from the main NE neoplasm, although these latter have a more cohesive growth than a free-floating cell pattern.

Finally, having demonstrated that pathologists' gross handling of the specimen did not determine knife dissemination-related false STAS, the current findings cannot exclude artifactual events occurred before the specimen reached the pathology laboratory. Since a crucial step is the way the lung specimen is manipulated either during surgery or final extraction from the thorax, it can be noticed that in this series lung tissues are derived from both open surgery and thoracoscopy resection procedures. In the latter case, a higher incidence of (false) STAS could be expected because of the technical procedure, which requires the resected lobe (or lung) to be extracted through an ∼4 cm thoracic incision. In this series, only 5 of the STAS-positive cases underwent open thoracic surgery and no statistical significance could have been expected to ascertain an effect of the surgical procedures on STAS occurrence because of the small sample.

Dischoesiveness of individual tumor cells was claimed to be the real reason for tumor cells more easily spreading in alveolar spaces favored by tumor handling procedures. At the same time, it may well be supposed that such a spread can occur spontaneously as well because of such claimed higher propensity for neoplastic cell detachment and free floating in alveolar spaces, particularly in more aggressive cases. In this regard, our series could not respond to the clinical significance of our findings in reported cases because of the prospective design of our study in resected early-stage lung cancer. Consequently, even in the most former cases, the follow-up data would not have been enough reliable and complete to carry out analyses. In addition, the main goal of this study was to distinguish true STAS from artifactual transportation in the setting of pathologic laboratory, thus exploring the impact of grossing procedure on STAS presence and amount.

In conclusion, these findings indicate that STAS most probably is not a pathologist-related artifactual event due to knife transportation of tumor cells during gross specimen handling and support the notion that it is a phenomenon preexisting to surgical tissue processing.

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Fig. 1

Sampling procedure workflow (clockwise). A surgical specimen containing a palpable lung tumor is subjected to a cut with a clean blade perpendicular to the pleural surface (A). After changing the blade (B), a second parallel cut is performed obtaining a 4 mm thick slice (C), subsequently divided (dotted line) into upper and lower portions (D) and placed into dedicated biocassettes (E). The remaining specimen was fixed in 10% formalin for 24 hours for further processing the specular cut surface of the tumor, following the same procedure as above (F).

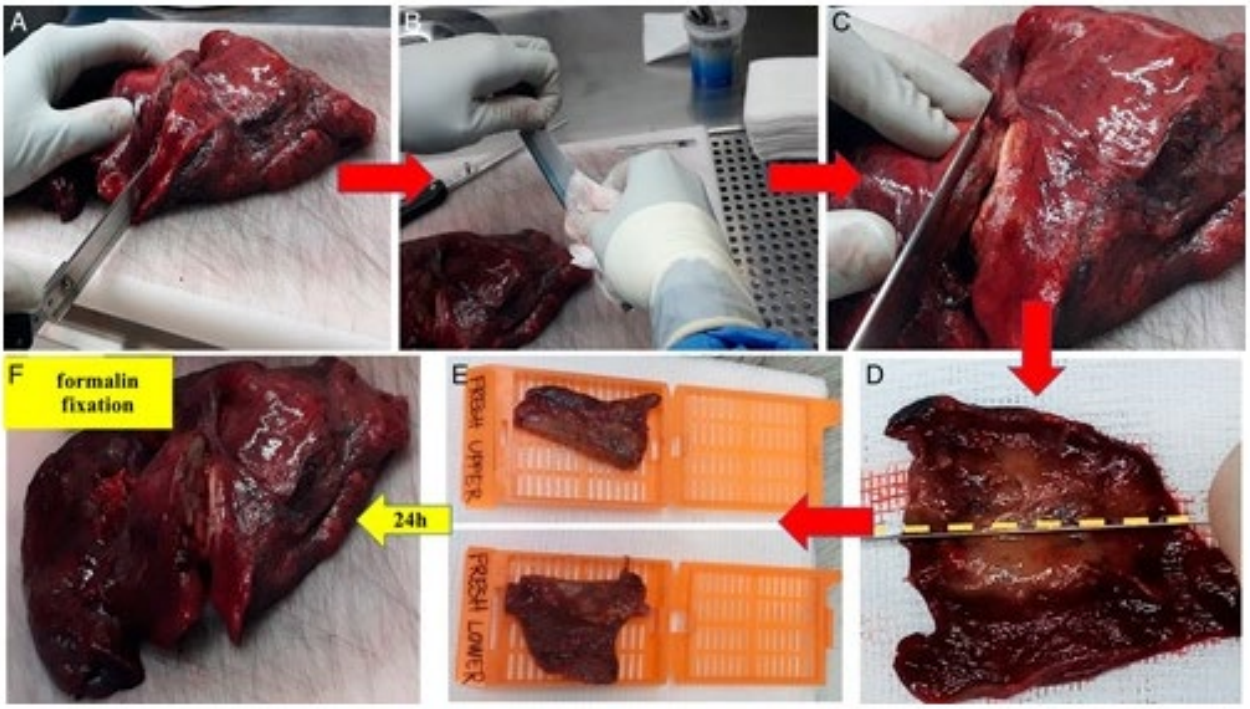


Fig. 2

On the basis of the sampling procedure and the cutting blade direction, upper parenchyma and part of the tumor (A, panoramic illustration) and the other tumor portion with the lower parenchyma (D, panoramic illustration) were separately obtained. Both samples contain STAS in the marked areas, as shown in the corresponding high-power fields (B, C, E, F). STAS in a case of adenocarcinoma showing a tumor cell aggregate associated with desquamated macrophages (G). A case of adenocarcinoma with lipoid pneumonia in the peritumoral alveoli, which appear filled with foamy macrophages also “embedding” a single STAS cluster (H). Arrows (G, H) indicate tumor cell clusters.

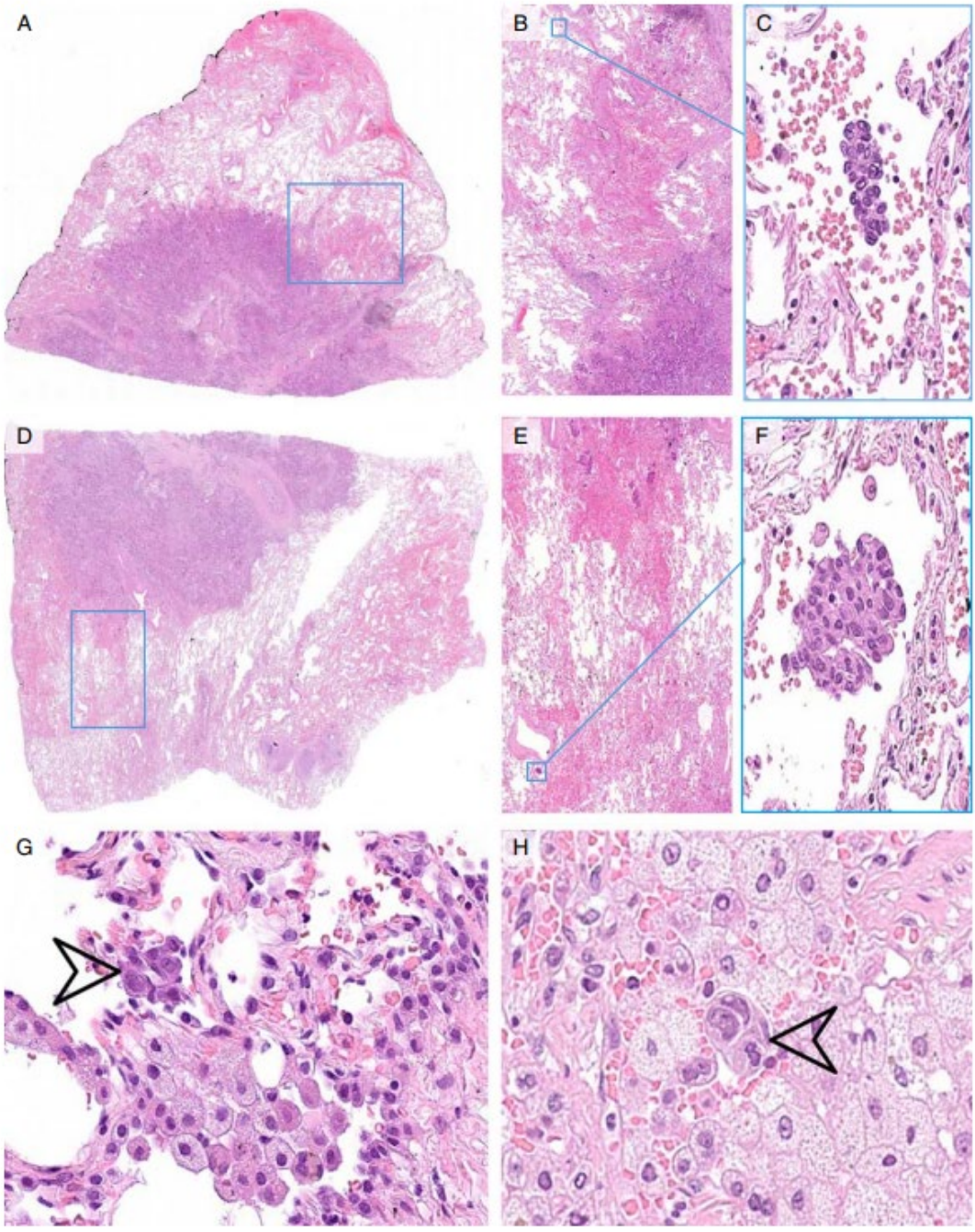


Fig. 3

Immunohistochemical stains supporting STAS identification. Clusters of tumor cells reactive for chromogranin A are present in the peritumor alveoli in a case of atypical carcinoid (A, B), STAS-positive area that is shown at greater magnification in the background (blue rectangles). Single-cell pattern of STAS (blue rectangle) (C) is highlighted by p40 immunoreactivity in a case of squamous carcinoma (D, inset).

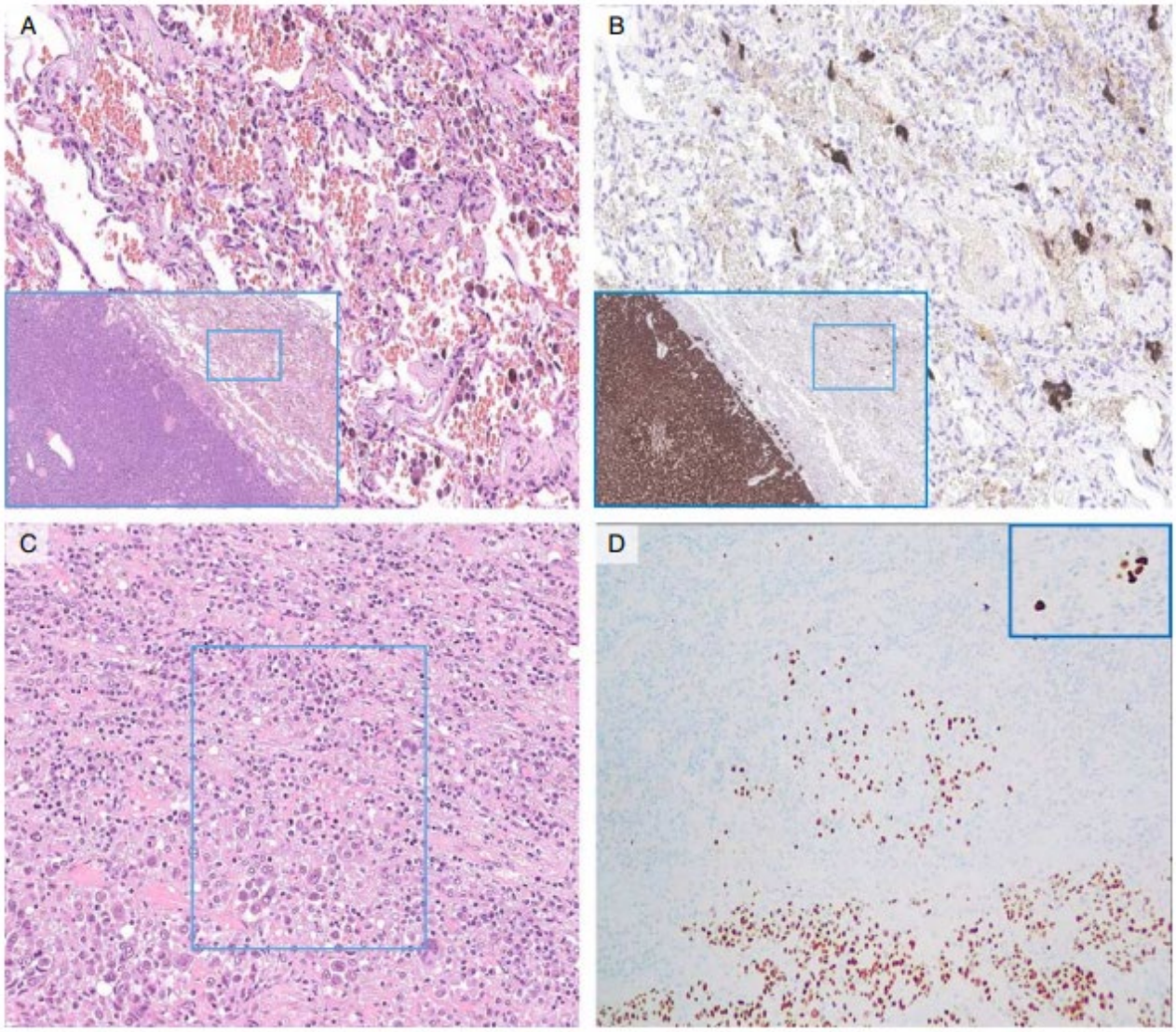


TABLE 1 - Clinicopathologic Characteristics and Distribution of STAS in Different Samples

Parameters	STAS⁻ #18	STAS⁺ #33	*P*
Histotype			
Adenocarcinoma	11	24	0.651
Squamous cell carcinoma	6	7	
Large-cell NE carcinoma	1	1	
Atypical carcinoid	0	1	
Tumor diameter			
Mean±SD	3.6±1.43	3.5±2.27	0.827
STAS distribution, n/N (%)			
Fresh upper lung samples	—	20/33 (60.6)	0.975
Fixed upper lung samples*	—	19/31 (61.3)	
Fresh lower lung samples	—	21/33 (63.6)	
Fixed lower lung samples†	—	21/32 (65.6)	
STAS distribution grouped			
Fresh+fixed upper lung samples*	—	27/33 (82)	—
Fresh+fixed lower lung samples†	—	28/33 (85)	
Background parenchyma			
Atelectasis	7	16	0.922
Fibrosis	15	21	
Hemorrhage	6	14	
Emphysema	4	6	
Histiocytic desquamation	6	10	
Edema	1	3	
Clinical stage			
I	10	16	0.271
II	6	7	
III	2	10	
Tumor grade			
2	14	26	0.933
3	4	7	
Viscera pleural invasion			
PL0	11	19	0.806
PL⁺	7	14	
Angioinvasion			
Absent	12	16	0.212
Present	6	17	
Necrosis			
Absent	8	11	0.433
Present	10	22	
Tumor-infiltrating lymphocytes			

Parameters	STAS- #18	STAS+ #33	*P*
Absent	2	1	0.493
Low	7	15	
Moderate/high	9	17	
Surgical procedure			
Lobectomy	16	27	0.593
Segmentectomy	1	2	
Bilobectomy	1	1	
Pneumonectomy	0	3	
Surgical technique			
Open surgery	2	5	0.620
Keyhole surgery	12	24	
Missing information	4	4	
Specimen transportation			
Under vacuum	11	24	0.393
Fresh	7	9	

*Two glass slides not evaluable.

†One glass slide not evaluable.

Supplemental

Content

Supplemental Table 1. Number of cases with and without STAS identification according to Metovic and Blaauwgeers[13] quantification methods (cases with single cells pattern were not included).

		Cases with NO cluster identification (Blaauwgeers[13])	Cases with cluster identification (Blaauwgeers[13])	K*	Agreement	Expected agreement	*P* value
Fresh upper sample	Cases with NO cluster identification (Metovic)	12	0	1.000	100%	51,5%	<0.001
	Cases with cluster identification (Metovic)	0	17				
Fresh lower sample	Cases with NO cluster identification (Metovic)	11	0	1.000	100%	52.9%	<0.001
	Cases with cluster identification (Metovic)	0	18				
Fixed upper sample	Cases with NO cluster identification (Metovic)	11	0	1.000	100%	52.3%	<0.001
	Cases with cluster identification (Metovic)	0	17				
Fixed lower sample	Cases with NO cluster identification (Metovic)	9	0	1.000	100%	56.3%	<0.001
	Cases with cluster identification (Metovic)	0	19				

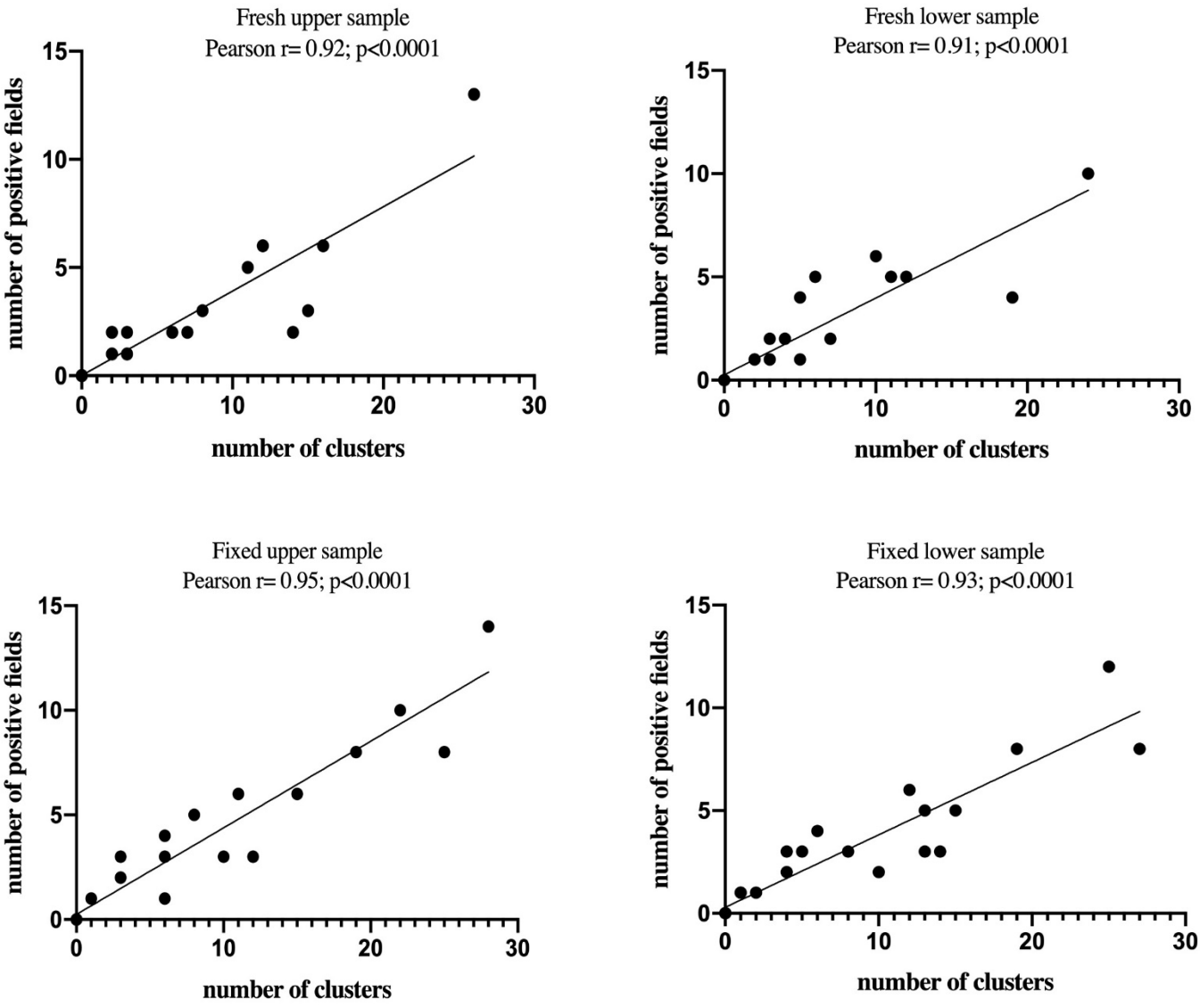
*Cohen K coefficent.

Supplemental Table 2. Median and mean±SD values of STAS quantification according to the two different methods (Metovic et al. *versus* Blaauwgeers et al.) (cases with single cells pattern were not included).

STAS quantification	Metovic et al. method median (interval)	Metovic et al. method mean±SD	Blaauwgeers et al.[13] method median (interval)	Blaauwgeers et al.[13] method mean±SD
Fresh upper sample	6 (2-26)	7.9±6.8	2 (1-13)	3.1±3.04
Fresh lower sample	5 (2-24)	6.9±6.2	2 (1-10)	3.0±2.5
Fixed upper sample	8 (1-28)	10.8±8.3	4 (1-14)	4.9±3.5
Fixed lower samples	8 (1-27)	9.5±8.0	3 (1-12)	3.8±3.00
Total (all four samples)	9 (2-92)	21.5±23.3	5 (1-35)	28.76±30.35

Supplemental Table 3. Clinicopathological characteristics and distribution of high and low STAS cases (*cases with single cells pattern were not included).

Parameter		**HIGH STAS cluster median >10 (#14 cases)**	**LOW STAS cluster median ≤10 (#15 cases)**	*P* value
Histotype	Adenocarcinoma*	12	10	0.282
	Squamous cell carcinoma*	1	5	
	Large cell neuroendocrine carcinoma	1	0	
	Atypical carcinoid*	0	0	
Tumor diameter	Mean	2.8	4	//
STAS distribution	Fresh upper lung samples	11	6	0.634
	Fixed upper lung samples*	13	4	
	Fresh lower lung samples	10	8	
	Fixed lower lung samples§	12	7	
Number of clusters mean (interval)	Fresh + fixed upper and lower samples	40	5	//
Background parenchyma	Atelectasis	5	10	0.376
	Fibrosis	11	7	
	Hemorrhage	8	6	
	Emphysema	1	2	
	Histiocytic desquamation	6	4	
	Edema	2	0	
Clinical stage	I	7	7	0.962
	II	3	3	
	III	4	5	
Tumor grade	2	12	10	0.231
	3	2	5	
Viscera pleural invasion	PL0	7	10	0.363
	PL+	7	5	
Angioinvasion	Absent	7	8	0.704
	Present	7	6	
Necrosis	Absent	4	6	0.517
	Present	10	9	
Tumor infiltrating lymphocytes	Absent	0	0	0.984
	Low	7	7	
	Moderate/High	7	8	



Supplemental Figure 1. Number of tumor clusters/number of tumor positive fields correlation according to the method presented in this study and described by Blaauwgeers et al[13].