Integrated CT imaging and tissue immune features disclose a radio-immune signature with high prognostic impact on surgically resected NSCLC

Integrated CT Imaging and Tissue Immune Features Disclose a Radio-Immune Signature with High Prognostic Impact on Surgically Resected NSCLC

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List of abbreviations

CT: Computed Tomography
US: ultrasound
TIME: Tumor Immune MicroEnvironment
PD-L1: Programmed Death Ligand-1
TILs: Tumor Infiltrating Lymphocytes
PD-1: Programmed cell Death protein-1
CT-SFs: CT-derived Semantic Features
TXT: texture
EFC: effect
MRG: margins
CT-RFs: CT-derived Radiomic Features
ROC: Receiver Operating Characteristic
AUC: Area Under the Curve
ICI: Immune Checkpoint Inhibitor
IHC: ImmunoHistoChemistry
ROI: Region of Interest
VOI: Volume of Interest
ADC: Adenocarcinoma
SCC: Squamous Cell Carcinoma
PCA: Principal Component Analysis
GLCM: Gray Level Co-occurrence Matrix
GLDM: Gray Level Dependence Matrix
GLRLM: Gray Level Run Length Matrix
GLSZM: Gray Level Size Zone Matrix
DAPI: Diamidino-2-phenylindole

Abstract

Objectives: Qualitative and quantitative CT imaging features might intercept the multifaceted tumor immune microenvironment (TIME), providing a non-invasive approach to design new prognostic models in NSCLC patients.

Materials and Methods: Our study population consisted of 100 surgically resected NSCLC patients among which 31 served as a validation cohort for quantitative image analysis. TIME was classified according to PD-L1 expression and the magnitude of Tumor Infiltrating Lymphocytes (TILs) and further defined as hot or cold by the tissue analysis of effector (CD8-to-$CD3^{high}$/PD-1-to-$CD8^{low}$) or inert (CD8-to-$CD3^{low}$/PD-1-to-$CD8^{high}$) phenotypes. CT datasets acted as source for qualitative (semantic, CT-SFs) and quantitative (radiomic, CT-RFs) features which were correlated with clinico-pathological and TIME profiles to determine their impact on survival outcome.

Results: Specific CT-SFs (texture [TXT], effect [EFC] and margins [MRG]) strongly correlated to PD-L1 and TILs status and showed significant impact on survival outcome (TXT, HR:3.39, 95% CI 1.12-10-27, P<0.05; EFC, HR:0.41, 95% CI 0.18-0.93, P<0.05; MRG, HR:1.93, 95% CI 0.88-4.25, P=0.09). Seven CT derived radiomic features were able to sharply discriminate cases with hot (inflamed) vs cold (desert) TIME, which also exhibited opposite OS (long vs short, HR:0.09, 95% CI 0.04-0.23, P<0.001) and DFS (long vs short, HR:0.31, 95% CI 0.16-0.58, P<0.001). Moreover, we identified 6 prognostic radiomic features among which ClusterProminence displayed the highest statistical significance (HR:0.13, 95% CI 0.06-0.31, P<0.001). These findings were independently validated in an additional cohort of NSCLC (HR:0.11, 95% CI 0.03-0.40, P=0.001). Finally, in our training cohort we developed a multiparametric prognostic model, interlacing TIME and clinico-pathological characteristics with CT-SFs (ROC curve AUC:0.83, 95% CI 0.71-0.92, P<0.001) or CT-RFs (AUC: 0.91, 95% CI 0.83-0.99, P<0.001), which appeared to

outperform pTNM staging (AUC: 0.66, 95% CI 0.51-0.80, *P*<0.05) in the risk assessment of NSCLC.

Conclusion: Higher order CT extracted features associated with specific TIME profiles may reveal a radio-immune signature with prognostic impact on resected NSCLC.

Keywords: lung cancer, CT imaging, immune contexture, radiomics, prognostic signature

1. Introduction

In the past ten years several non-invasive strategies emerged as alternative ways to prevent or treat cancer patients, including NSCLC [1]. Advanced surgical procedures [2,3] coupled with the genomic characterization of the disease on circulating tumor DNA from exosomes or tumor cells (liquid biopsy),–improved the path to a rapid diagnosis, finally avoiding patient discomfort [4].

Parallel to next generation sequencing (NGS) applications, several imaged-based systems have been exploited to implement cancer management from diagnosis to outcome [5,6]. For example, the high throughput data mining approach of radiologic images (radiomics) demonstrated the ability to define tumor masses by inferring volumetric, densitometric and morphological features [7–9].

The basic idea of radiomics is to overcome the “eye limited resolution” diagnosis intrinsic to computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) techniques by extrapolating high-computed data to resolute qualitatively and quantitatively complex images. If radiomics can resolve images in numbers, numbers can be used to define statistically testable algorithms, generate machine-learning approaches to define patterns, predict outcome or unveil hidden insights [10–12]. The relevance of this strategy in cancer has been established by the National Cancer Institute (NCI) imaging program, that promoted the Quantitative Imaging Network (QIN) initiative to provide clinical grade biomarkers for cancer detection, prognosis and prediction of response to therapy [13,14].

Several reports showed the prognostic/predictive power of CT, MRI, PET and ultrasound (US) based radiomics in different neoplasms [15], also revealing associations with pathological Tumor-Node-Metastasis (pTNM) staging, histotype, mutational status,

genotypic characteristics (radiogenomic), tumor metabolism and immune-related processes [6,16–21].

In spite of the striking success of immunotherapy, the potential correlation of imaging data with cancer immune profile has been only partially investigated [22]. Recent studies have identified radiomic based biomarkers of response to immune checkpoint inhibitors (ICI) in melanoma and NSCLC [23–25]. Accordingly, a radiomic signature for CD8+ cells has been proposed as predictor of ICI efficacy in advanced solid tumors [24].

Based on the assessment of tumor infiltrating lymphocytes (TILs) and PD-L1 score, new classifications of the tumor immune microenvironment (TIME) exhibited prognostic and predictive significance in ICI treated NSCLC patients [26–29].

The question whether radiologic features may reflect specific TIME characteristics remains unanswered, although under intense scrutiny. For example, TILs signatures have been correlated with defined imaging texture [22] or changes in tissue inflammatory cells composition may sustain a pseudoprogressive radiological patterns in NSCLC [30,31]. These observations indicate that the longstanding knowledge on cancer immunity cycle may be reprised by high-computed radiomics ultimately decoding TIME profiles.

The aim of the present study was to develop and validate a prognostic model intersecting radiologic and immunophenotypic features in surgically resected NSCLC patients. By this approach, we defined a radio-immune signature with significant impact on clinical outcome. Our findings suggest that a strategy involving TIME and CT imaging analysis might be endowed with potential predictive power in advanced lung cancer settings.

2. Materials and Methods

2.1 Study Population

Our study was approved by the Institutional research Review Board for human studies (Ethical Committee) of the University-Hospital of Parma (278/2018/OSS*/AOUPR) and in accord with principles listed in the Helsinki declaration. Informed consent was waived.

From January 2011 to December 2015 we collected information from patients with a diagnosis of primary NSCLC stage I to IIIa, undergoing lung resections with curative intent at the Unit of Thoracic Surgery at the University-Hospital of Parma. All cases were scored according to the staging system from the 7th American Joint Committee on Cancer (AJCC, [32]). One hundred patients (69 belonging to training and 31 to validation cohorts) fulfilled the following inclusion criteria: a) stage I to stage III disease; b) diagnosis of NSCLC with complete pathologic report; c) available preoperative thin-section CT images (Picture Archiving and Communication System, PACS) within three months before surgery; d) adequacy of tissue samples for immunohistochemical (IHC) analysis; e) available complete clinical records. Main exclusion criteria were represented by: a) history of drug abuse or medications with potential impact on lung diseases; b) active pneumonitis at the time of CT scan. A schematic representation of patient selection according to the above described criteria is shown in Supplementary Figure S1.

2.2 Morphometric and Immunohistochemical Analysis

Tissue sections (5 μm thick) were cut from formalin-fixed, paraffin-embedded blocks containing surgically resected tumor samples. Sections were stained with hematoxylin eosin (H&E), Masson's Trichrome and scored for morphometric and IHC analyses (see Data in Brief for details).

2.3 Definition of TIME

To integrate TIME-derived features in our prognostic algorithm we used two different approaches. The first was based on PD-L1 levels and CD3+ TILs incidence, as per general reported criteria that define type I to IV categories of immune contexture [33]. A second approach was adopted to implement this rather simplistic evaluation of TIME. Specifically, we computed the fraction of CD8+ cytotoxic cells over the total CD3+ T cell population together with the extent of the expression of PD-1 inhibitory receptor on CD8+ TILs. Both represents individual estimated TILs parameters whose relative contribution depicts an active or quiescent TIME functional state, as suggested by other reports [34,35]. Thus, we defined TIME as "hot" when displaying PD-L1^{high}, CD8-to-CD3^{high} and PD-1-to-CD8^{low} and "cold" when PD-L1^{low}, CD8-to-CD3^{low} and PD-1-to-CD8^{high}. High and low subsets were established according to cut-offs based on CART tree analysis for CD8-to-CD3 and PD-1-to-CD8 ratios, while for all other investigated parameters median values were employed. In addition to CD8 and PD-1, the population of effector phenotypes was investigated by the IHC detection of CD57 (NK) and Granzyme B while suppressor subpopulations were defined by the expression of CD25 and FOXP3 (Treg) CD4+ lymphocytes.

A detailed description of our TIME classifications is available in the associated manuscript (Data in Brief).

2.4 CT Examination

CT scans were performed by two CT scanners, either a 6-slice (Emotion 6; Siemens Healthineers, Forchheim, Germany) or a second-generation dual-source 128-slice (Somatom Definition Flash; Siemens Healthineers) scanner. All scans were acquired at end-inspiration in craniocaudal direction, capturing the entire lung volume from the apices to the pleural recesses and reconstructed with a slice thickness ranging 1–2.5 mm. For the purpose of this study, the Digital Imaging and Communications in Medicine (DICOM) datasets were retrieved from PACS.

2.4.1 Qualitative analysis

CT datasets reconstructed on lung and mediastinal window settings were qualitatively analyzed by a dedicated reader (PP, 3 years-experience in chest imaging), who reviewed all CT images and, following reported criteria [36,37], defined five categories of semantic features (SFs): shape, margins, texture, structure and effect on parenchyma.

A senior thoracic radiologist (NS, 13 years-experience in chest imaging) reviewed the scoring system and final decision was made in consensus. A detailed description of this analysis is reported in the associated manuscript (Data in Brief).

2.4.2 Quantitative analysis

All CT datasets were uploaded into a dedicated open-source software for quantitative analyses (3dSlicer 4.9.0, www.slicer.org [38]), to extract all radiomic features (RFs).

Tumors were semi-automatically delineated every three images by means of manually drawn regions of interest (ROIs) and a dedicated interpolation algorithm tool was launched to calculate a volume of interest (VOI), encompassing the whole lesions. The reader was allowed to modify VOI boundaries in case of inadequate segmentation. RF extraction was performed by a dedicated function embedded into the segmentation software (SlicerRadiomics). Overall, 841 RFs were computed, originating from eight main classes: 1. shape based; 2. first order statistics; 3. gray level co-occurrence matrix (GLCM); 4. gray level dependence matrix (GLDM); 5. gray level run length matrix (GLRLM); 6. gray level size zone matrix (GLSZM); 7. neighboring gray tone difference matrix (NGTDM); 8. wavelet transform. To test consistency of RFs extraction, a subset of the study population composed of twenty patients (33.3 %) was independently evaluated by a second radiologist (GM, with 6 years-experience in chest imaging).

To reinforce the specificity and reliability of our radiomic approach, CT-RFs from 13 control tissues (10 lymph nodes and 3 skeletal muscles) of patients not affected by neoplastic

diseases and 60 NSCLC lesions belonging to our training cohort were ~~simultaneously~~ extracted and subjected to cluster analysis (see Data in Brief for detail).

2.4.3 Validation Cohort

To assess the validity of our RF-signature and diminish inter-observer variability due to operators with different experience, we repeated our radiomic analysis in additional 31 NSCLC cases. Semi-automatic segmentations were performed by the same reader who was involved in the segmentation of a subset of the test population (GM) and by a junior reader (with 1 year-experience in chest imaging), trained for the purposes of lesions' segmentations.

2.5 Statistical Analysis

The Fisher's exact test was used to examine the differences in categorical variables, and the Mann-Whitney *U* test to detect differences in continuous variables between groups of patients, given that the distribution of data was not normal (Kolmogorov-Smirnov test). Overall survival (OS) and disease-free survival (DFS) were estimated by means of the Kaplan Meier method. OS was defined as the interval from surgery or start of treatment to death from any cause, or the last date the patient was known to be alive; DFS was defined as the interval from surgery to the evidence of recurrence disease, or death, or the last date the patient was known to be recurrence-free or alive. Both OS and DFS data were censored at 5 years. Receiver operating characteristic (ROC) curves were used to test the sensitivity and specificity of a marker, with the area under the curve (AUC) being given with its 95% confidence interval (CI).

Log-rank test was performed to determine the difference in survival between groups. OS and DFS data were then analysed through Cox regression multivariate models. Classification and regression tree (CART) analysis identified specific cut-off values that segregated patients by clinical outcomes. *P* value of 0.05 was set as a threshold of statistical

significance. IBM SPSS Statistics v 25.0 (IBM) and Stata 13 with Cart module (Statacorp) were used to perform all computational analyses. Heatmaps and matrix analyses were performed by freely available modules in “Morpheus” software (Broad, Institute, Cambridge, MA, USA).

2.5.1 Radiomic signature and combined predictive models

To intersect TIME with radiomic images, we validated an internal pipeline where the tumor volume of interest (VOI) (Figure 3A) was used to extract scalable features, CT-RFs (n=841), collectively ascribable to one original- and eight wavelet-related major classes (Figure 3B). The first step toward our supervised analysis in search of a radio-immune signature was to exclude redundant radiomic features by a matrix correlation, setting a threshold at 0.7.

Next, we conducted univariate correlation test by Mann-Whitney U test evaluating the clinical impact of CT-RFs; the features showing significant level of correlation ($P < 0.05$) were selected. The designated CT-RFs, considered as continuous variables, were then challenged in a Cox proportional-hazard model. Finally, we developed multiparametric prediction models of overall survival, defined from a linear combination and regression coefficients of significant clinico-pathological, TIME and CT-imaging features. Model validation was performed with repeated 10-fold cross-validation, and the discrimination ability of the generated models was evaluated with ROC curves and their area under the curve (AUC) values.

3. Results

3.1 Patient Population

The training set involved 39 squamous cell carcinomas (SCC) and 30 adenocarcinomas (ADC) among which 42%, were in stage I (A and B), 33.3% in stage II (A and B) and 24.7% in stage IIIA. NSCLC samples were obtained by lobectomy (72%), pneumonectomy (18%) and atypical segmental resection (10%).

EGFR and *KRAS* mutation involved, respectively, 13% and 10% of tested ADC cases. Clinico-pathological characteristics of our patient population are reported in Figure 1A and Supplementary Table S1.

~~To partly overcome the limitation of sample size, we supported our results including a validation cohort of 31 clinico-pathologically matched NSCLC (Supplementary Table S1)~~. Noticeably, the entire population of 100 NSCLC patients reached a follow-up of 60 months.

The impact of clinico-pathological characteristics on survival outcome is reported in Table 1 and Supplementary Figure S2. Importantly, as documented by Cox Regression hazard models, the prognostic significance of N status in terms of OS was apparent on both uni- and multi-variate analysis, while its impact on DFS resulted significant only on univariate model.

3.2 Distinctive tumor immune contextures predict survival outcome

~~To define a tumor immune signature, we immunohistochemically assessed PD-L1 levels and the incidence of effector and suppressor subpopulations of TILs as major determinants of TIME characteristics (Figure 1B).~~

As predicted ~~by our previous experience [29]~~, PD-L1 levels, TILs incidence and the distribution of the four defined immune classes ~~[32]~~, significantly varied across patients and histotype (Figure 1A, B).

~~The distribution of the four defined immune classes [33] showed that more than 1/3 of our NSCLC cohort belonged to class II implying a rather desert immune contexture (Figure 1A). The quantitative assessment and the relative contribution of PD-L1 and TILs phenotypes to the composition of these classes of TIME are reported in Supplementary Table S2.~~

Collectively, immune categories (Figure 1C*i* and Supplementary Figure S3) or individual descriptors (PD-L1 and TILs) did not correlate with survival (Table 1). On the opposite, distinct immunophenotypic features predicted the clinical outcome on both univariate and multivariate analysis (Table 1). High CD8-to-CD3 ratio resulted in increased OS (HR: 0.426, *P*=0.05) and DFS (HR: 0.330, *P*=0.001), while low PD-1-to-CD8 ratio positively affected OS (HR: 2.955, *P*=0.007) (Figure 1C*ii*, *iii* and Supplementary Figure S3). When challenged on multivariate analysis (Table 1), PD-1-to-CD8 ratio maintained a statistically significant impact on OS (*P*=0.005), while CD8-to-CD3 ratio conditioned only DFS (*P*=0.027).

Thus, we integrated these two phenotypic descriptors with PD-L1 status to distinguish "hot" and "cold" TIME (Figure 1D) and test their prognostic significance. These two defined subsets were equally distributed in our study population, similarly involving 17% of patients. ~~displaying hot (PD-L1^{high}, CD8-to-CD3^{high} and PD-1-to-CD8^{low}) and cold (PD-L1^{low}, CD8-to-CD3^{low} and PD-1-to-CD8^{high}) TIME.~~ Importantly, the comparative analysis of NSCLC patients carrying these distinctive immune categories depicted different prognostic profiles (Figure 1E). Prolonged OS (Figure 1E*i*) and DFS (Figure 1E*ii*) were documented in patients carrying hot vs cold TIME (median OS: not reached [NR] vs 11 months, HR: 0.124, 95% CI 0.068-0.356, *P*<0.001; median DFS: NR vs 5 months; HR: 0.348, 95% CI 0.232-0.795, *P*<0.001), while intermediate survival duration was observed in the remaining cases.

3.3 Semantic imaging features impact on prognosis and encompass specific TIME

~~To initially assess the impact of qualitative CT descriptors on patients' outcome we analyzed shape, margins, texture, structure and effect on parenchyma, whose clinical relevance in NSCLC had been previously demonstrated [39].~~

~~The distribution of semantic CT features in our cohort of patients is reported in Supplementary Table S3. We observed that association of clinical parameters with CT-SFs could identify specific patterns of NSCLC. For example, active smoking correlates with CT non-homogeneous structure (P<0.05), while higher incidence of well-defined margins (P<0.05) was present in ex- and never smokers (data not shown).~~

NSCLC patients with lesions displaying evidence of parenchyma reaction, classified as effect, had significantly higher OS (HR: 0.411, P=0.033) compared to their counterpart (Table 1, Figure 2A), although without a parallel impact on DFS (Supplementary Figure S4). In addition, solid texture was associated with sustained OS (HR: 3.397, P=0.021) and DFS (HR: 2.615, P=0.031), while spiculated margins appeared to shorten OS (P=0.099) (Figure 2A, Supplementary Figure S4). Interestingly, CT effect and margins significantly correlated to OS (P<0.01) at multivariate analysis (Table 1).

As first step toward the integration of different risk scoring approaches, we correlated CT-SFs with tumor immune profiles. High PD-L1 levels were detected in radiologic lesions displaying a solid texture and any effects on the surrounding parenchyma (P<0.05), while well-defined CT margins were typically observed in TILs-rich cases (P<0.05) (Figure 2B). A prominent lymphocyte infiltrate was also associated with CT evidence of tumor effect (Figure 2C).

~~A prominent lymphocyte infiltrate was also associated with CT evidence of tumor effect (Figure 2B). The evaluation of CT-SFs occurrence according to the 4 main TIME categories indicated that nearly 80% of NSCLC samples displaying an intrinsic inductive TIME (Type III) exhibited CT effect on lung parenchyma (not shown).~~

These data collectively indicate that qualitative image analysis may decipher distinctive NSCLC immune contextures, potentially providing non-invasive prognostic tools.

3.4 Radiomic features enclose distinct prognostic profiles

~~To intersect TIME with radiomic images, we validated an internal pipeline where the tumor volume of interest (VOI) (Figure 3A) was used to extract scalable features, CT-RFs (n=841), grouped into one original and eight wavelet-related major classes (Figure 3B).~~

To estimate similarity matrix between CT-RFs, after an accurate process of feature extraction and preliminary analyses (Figure 3A, B), the generated heatmap displayed very clear block boundaries for CT-RFs belonging to same classes along the diagonal axis (R^2 = 1) or far from it indicating strong intra and weak inter-classes CT-RFs correlations (Figure 3C).

~~If the 841 CT-RFs are correlated variables, one could imagine using algorithms for dimensionality reduction. Thus, we applied principal component analysis (PCA) to CT-RFs extracted from 60 NSCLC and 13 normal tissues (10 uninvolved lymph nodes, 3 skeletal muscles). As shown in Figure 3D, two vectors contain nearly the 50% of data variance, sufficient to preliminary distinguish tumor versus normal samples.~~

~~To further confirm our PCA results, we applied on the same data set an unsupervised hierarchical clustering analysis and demonstrated that normal and tumor samples cluster in different branches, indicating that tissue heterogeneity may be intercepted by radiomic features (Figure 3E).~~

We then asked whether CT-RFs were differentially distributed within NSCLC subtypes and we calculated the mean of expression of each CT-RFs in ADC and SCC. As shown by circle plots (Figure 3D), the nine radiomic classes were equally represented in the two histotypes, suggesting that the detection of inter-tumor differences requires the integration of radiomics with more featured tissue characteristics such as the immune

microenvironment.

Next, following a supervised feature selection process, we documented that ~~o assess the impact of CT-RFs on survival outcome, we applied a Cox proportional hazard model on the 841 radiomic features considered as continuous variables. Matrix correlation analysis excluded redundant CT-RFs, narrowing to six the number of clinically relevant features (Figure 3E). Individual CT-RFs cut-off values obtained by CART Tree analysis segregated patients in two distinctive prognostic groups.~~ high values of 6 CT-RFs, wavelet-ClusterProminence, ClusterTendency, GrayLevelVariance, DifferenceVariance, Contrast and SumSquare, significantly correlated with a favourable prognosis (Figure 3E, F and Supplementary Figure S5). Moreover, wavelet-LLH_glcm_ClusterProminence emerged on uni- and multi-variate analysis as a potential radiomic predictor of clinical outcome (Supplementary Table S2), given that cases with high values of the feature had significantly increased OS (multivariate Cox regression analysis, HR:0.16, 95% CI 0.04-0.601, *P*=0.01) and DFS (HR: 0.32, 95% CI 0.16-0.43, *P*=0.001).

Finally, to assess the performance of our six-feature radiomic signature, we applied the same approach to CT images from 31 clinico-pathologically matched NSCLC representing the validation cohort. As apparent in Figure 3 E, F and Supplementary Figure S5, we confirmed the power of all 6 CT-RFs in segregating patients according to survival outcome. In addition, we validated the favourable impact of ClusterProminence of positive pixel values, which resulted in 26 months gain in OS (HR:0.11, 95% CI 0.03-0.40, *P*=0.001) (Figure 3F).

3.5 Identification of Radio-Immune Signatures

~~The first step toward our supervised analysis in search of a radio-immune signature was to exclude redundant radiomic features by a matrix correlation setting a threshold at 0.7. Then,~~ Following a stratification of patients according to high or low levels of single TIME

benchmark, we identified radiomic traits that discriminate specific immune parameters. Specifically, high PD-L1 levels were predominantly translated at CT imaging into high values of Cluster-related wavelet features, while NonUniformity-related CT-RFs were highly expressed in CD8+ rich TIME (Figure 3G, H).

Next, to establish a radiomic profile of hot and cold TIME (Figure 4A*i-ii*), we obtained 80 differentially expressed CT-RFs (Figure 4A*iii*), subsequently narrowed to ~~Following a correlation matrix analysis to exclude redundancy, we detected~~ 7 highly represented in hot (hCT-RFs) and 1 exclusively associated to cold TIME (cCT-RFs, Figure 4B). The ability of these signature-related CT-RFs to distinguish patients according to TIME category (P<0.01) was revealed by the area under the curve (AUC) of the receiver operator characteristic (ROC) curve and its confidence interval (Figure 4B).

3.6 Prediction Models of Survival Outcome

In order to establish a prognostic score, we integrated predetermined risk factors from TIME (low CD8-to-CD3 and high PD-1-to-CD8) with qualitative CT imaging (no effect and spiculated margins) (Figure 5A*i*, *ii*). We first combined CD8-to-CD3 ratio and radiologic margins, documenting significantly reduced OS and DFS (Figure 5B*i* and Supplementary Figure S6) in the presence of at least one risk factor (OS=HR:2.66, 95% CI 1.03-6.89, P=0.035; DFS=HR:2.45, 95% CI 1.22-4.91, P=0.011). Next, PD-1-to-CD8 ratio was integrated with effect at CT imaging, revealing that high incidence of PD-1 receptor on CD8+ TILs combined with no effect had negative impact on OS (HR:16.86, 95% CI 2.27-25.72, P<0.001), while not reaching a statistical significance in terms of DFS (HR:1.81, 95% CI 0.92-3.58, P=0.07) (Figure 5B*ii* and Supplementary Figure S6). The discrimination ability of these two prognostic models was clearly apparent in the corresponding ROC curves (Supplementary Figure S7).

A similar prognostic model was generated by merging TIME with wavelet-LLH_glcm_ClusterProminence (Figure 5A*iii*), the CT-RF that individually exhibited the highest prognostic significance (Figure 3H). As documented by Kaplan Meier (Figure 5C*i-ii* and Supplementary Figure S5) and ROC (Supplementary Figure S7) curves, high PD-1-to-CD8 and low CD8-to-CD3 ratios merged with low ClusterProminence negatively affected OS (PD-1-to-CD8, HR:12.17, 95% CI 4.10-16.44, *P*<0.001; CD8-to-CD3, HR:8.76, 95% CI 2.05-17.45, *P*<0.001) and DFS (PD-1-to-CD8, HR:2.32, 95% CI 1.25-4.29, *P*=0.005; CD8-to-CD3, HR:1.72, 95% CI 0.89-3.31, *P*=0.080).

Finally, we integrated significant clinico-pathological (N status and pathologic stage), TIME (PD-1-to-CD8 and CD8-to-CD3) and CT-imaging features (CT-SFs: effect and margins; CT-RFs: wavelet-LLH_ClusterProminence) in multiparametric prediction models of overall survival. The statistical predictive power of the two obtained models, discerned by semantic or radiomic descriptors, was assessed by ROC curve. As shown in Figure 5B*iii*, combining CT-SFs with clinico-pathological and TIME parameters, we achieved high sensitivity and specificity (AUC=0.82, 95% CI 0.71-0.93, *P*<0.001). Furthermore, our CT-RFs-based model (Figure 5C*iii*) reached 0.91 AUC (95% CI 0.83-0.99, *P*<0.001), underlining the clinical relevance of a “radio-immuno-pathological” approach in the risk assessment of surgically resected NSCLC patients.

4. Discussion

Approximately 30% of NSCLC patients are diagnosed with early stage disease and undergo surgery with curative intent. The 5-year survival rates range from 30% among stage IIIA patients to 90% among those with stage IA, while the risk of recurrence dramatically accounts for 30% to 55% of disease relapse within 5 years from surgery [40,41]. This frustrating reality enforces the development of more accurate risk stratification models based on multi-omics approaches [42], further aiming at the identification of patients who

can benefit from adjuvant treatments including chemo- and immuno-therapy [40]. Moreover, the limited population of advanced NSCLC patients who can benefit from immunotherapy underscores the urgent demand of the definition of prognostic and predictive biomarkers. . In this regard, a promising approach able to predict the response to ICI in NSCLC has been proposed by the longitudinal assessment of radiomics signatures (delta-radiomics) [25].

In both resectable and metastatic settings, radiomics rapidly emerged as an innovative approach to achieve a patient- and tumor-specific management through diagnostic non-invasive tools. High throughput extracted imaging in addition to offer objective measurements of intra-tumor heterogeneity in NSCLC [6,16,43] when integrated with pTNM and tissue features may implement the actual risk stratification models [20,22,44–46]. The combination of TNM staging with radiomic features, describing density (Statistics Energy), compactness (Shape Compactness) and intratumor heterogeneity (Gray Level Nonuniformity), outperformed the prognostic value of TNM alone in early stage lung and head and neck cancers [22]. The same integrated prognostic index was subsequently validated in a cohort of stage IV disease [47].

However, less clear appears how radiomics can capture the complexity of tumor microenvironment. This represents a relevant issue since TIME is rapidly emerging as potential biomarker of response in the era of immunotherapy [26,27,48]. Important advances have been made in TIME characterization, focused on PD-L1 status, TILs density and phenotype, and activating or inhibitory signaling pathways [28,29]. Moreover, classifying cancers into T cell-inflamed or "hot" tumors (PD-L1^{high}, CD8^{high}, IFN-γ signature) versus non-inflamed or "cold" tumors (immune-excluded and immune-desert) [34], might predict survival and ICI response. In agreement with these observations, our “hot” or “cold” TIME, although differently assessed, defined prognostic classes of NSCLC and can be indeed distinguished by specific CT-RFs. Although aware that intercepting the heterogeneous and dynamic nature of TIME would require repeated biopsies, we proposed here an approach to non-

invasively translate tumor immune microenvironment into CT imaging features, ultimately providing a radio-immune signature.

Qualitative (semantic) description of CT tumor images, such margins, texture and effect, holds an established clinical relevance in NSCLC [39]. For instance, “spiculated margins” are a marker of aggressiveness with a negative prognostic impact on survival outcome [39,49]. Interestingly, in addition to confirm this evidence in our cohort of lung cancer patients, we observed that tumors exhibiting this specific CT-SF carry a TILs poor immune microenvironment. On the opposite, tumor effect on the surrounding parenchyma appeared to correlate with improved survival. A potential explanation of this unexpected result may relate to high PD-L1 levels and CD3+ TILs content in corresponding tissue samples. This finding refers to the idea that inflamed tumors exhibit a favorable prognostic impact when triggering tissue reactions able to counteract cancer invasiveness. Interestingly, a predictive radio-immune signature based on the abundance of TILs in the peritumoral area was recently documented in ICI treated NSCLC [25].

Thus, a tumor microenvironment reflecting a dynamic host immune response influences CT images, and this information can be detected by computed approaches.

A step forward from semantic CT analysis is radiomics, whose role has been repeatedly challenged against clinical and histologic standard as a predictor of biological behavior [20,22]. However, as emerging field, radiomics lacks standardized approaches both from platform and analysis perspectives, as larger populations scored with radio-immune analyses are warranted [5]. So far, most large-scale studies investigating radio-immune correlates were either conducted in a minority of NSCLC cases (n: 22/351,[20]; n: 18/75, [24]) or restricted to one (CD8+ TILs, [24]) or two (CD3 and PD-L1, [50]) TIME parameters. Conversely, we obtained informative insights on radio-immune profiles, by the integration of CT-RFs, established in training (n:60) and validation (n:31) sets of NSCLC patients, with multiple immunophenotypic characteristics. We demonstrated that cluster-

and non-uniformity-related CT-RFs identified an inflamed tumor immune contexture, characterized, respectively, by high PD-L1 levels and effector TILs subsets, validated markers of high intra-tumor heterogeneity [16,22].

Moreover, we established and validated the impact on survival of wavelet-LLH_glcm_Cluster Prominence, a feature considered as a measure of the skewness and asymmetry. Although the clinical significance of this CT-extracted feature has not been explored yet, PET-derived GLCM Cluster Prominence was directly associated with breast cancer tumor grade [51].

Limitations of the present study have to be acknowledged. First, the relatively small sample size that potentially attenuates our conclusive remarks. Nonetheless, the adequate patient follow-up (60 months) and the presence of a validation cohort may partially cover this issue. Another limitation is intrinsic to radiomic approach and it is related to the actual poor interpretation of high-throughput extracted data and lack of methodological standardization to reach validated and reproducible features with impact on patient survival. Additionally, patients were scanned by means of different CT scanners with different acquisition parameters, thus potentially yielding intrinsic variability in CT-RFs quantitative measurements. However, this limitation reflects daily medical practice which requires the use of multiple platforms to accomplish diagnostic demands.

Based on our findings, the integration of radiomic, immunophenotypic and clinico-pathological parameters may implement the actual risk stratification models, especially for surgically resected NSCLC cases that harbor a more aggressive course independently from pTNM. It is our intent to extend this patient-centered non-invasive approach to the prevalent cohort of unresectable and advanced NSCLC, with the aim to predict response to treatment.

5. Conclusion

In closing, qualitative and quantitative analysis of CT images integrated with specific TIME features may provide new risk assessment models potentially able to outperform the prognostic value of standard pTNM staging system in NSCLC.

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Disclosure

All Authors have nothing to disclose.

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Table 1. Explanatory prognostic factors in Cox proportional Hazards models.

OS	Univariate[a]				Multivariate[b]			
	HR	CI (95%)	χ^2	p value	HR	CI (95%)	χ^2	p value
Sex	2.059	0.878-4.826	2.875	0.090	1.056	0.389-2.869		0.915
Age	0.971	0.925-1.020	1.401	0.237				
Smoking	0.933	0.509-1.709	0.051	0.821				
Histotype	0.642	0.292-1.408	1.243	0.269				
Staging	1.789	1.104-2.899	5.847	**0.018**	1.024	0.291-2.333		0.716
N status	1.875	1.125-3.126	6.192	**0.013**	1.823	1.016-3.270		**0.044**
PD-L1	1.308	0.557-3.069	0.383	0.537				
CD3	0.722	0.213-2.442	0.277	0.600				
CD8	0.625	0.265-1.475	1.174	0.283				
CD8/CD3	0.426	0.174-1.039	3.736	**0.050**	0.671	0.334-2.825		0.056
PD-1	0.906	0.356-2.307	0.043	0.906				
PD-1/CD8	2.955	1.290-6.669	7.203	**0.010**	3.779	1.490-9.585		**0.005**
Shape	0.771	0.346-1.718	0.407	0.525				
Texture	3.397	1.124-10.272	5.299	**0.030**	1.563	0.254-9.633		0.630
Efffect	0.411	0.181-0.193	4.840	**0.033**	0.276	0.114-0.670		**0.004**
Structure	1.143	0.494-0.249	0.098	0.755				
Margins	1.939	0.883-4.257	2.821	0.093	4.076	1.541-10.780		**0.005**

DFS	Univariate[a]				Multivariate[b]			
	HR	CI (95%)	χ^2	p value	HR	CI (95%)	χ^2	p value
Sex	2.205	1.134-4.291	5.702	**0.020**	0.945	0.403-2.217		0.897
Age	0.928	0.886-0.971	2.545	0.080				
Smoking	0.906	0.562-1.461	0.165	0.685				
Histotype	0.272	0.144-0.515	10.036	**0.003**	0.418	0.207-0.844		**0.015**
Staging	1.320	0.891-1.956	1.935	0.166				
N status	1.522	1.037-2.232	4.741	**0.032**	1.114	0.737-1.682		0.609
PD-L1	1.043	0.539-2.019	0.015	0.901				
CD3	1.391	0.607-3.186	0.613	0.436				
CD8	0.739	0.394-1.387	0.892	0.347				
CD8/CD3	0.330	0.166-0.657	10.892	**0.002**	0.430	0.204-0.907		**0.027**
PD-1	0.864	0.422-1.768	0.161	0.688				
PD-1/CD8	1.284	0.660-2.467	0.546	0.461				
Shape	1.017	0.558-1.853	0.003	0.956				
Texture	2.615	1.091-6.271	5.007	**0.031**	2.185	0.693-6.890		0.182
Efffect	0.809	0.444-1.474	0.480	0.809				
Structure	1.100	0.590-2.048	0.090	0.765				
Margins	1.321	0.715-2.439	0.790	0.374	2.023	1.046-3.912		**0.036**

OS: overall survival; DFS: disease free survival; Sex (Male = 0, Female = 1), Age (continue variable), Smoking status (0 = negative smoking history, 1 = positive smoking history), Histotype (ADC = 0, SCC = 1), Staging, N status, PD-L1 expression, CD3, CD8, CD8/CD3, PD-1 expression, PD-1/CD8 (continue variables). Statistical results with $P<0.05$ are bolded.

[a]Univariate analysis is carried out without any adjustment. [b]Multivariate analysis is carried out considering parameters statistically significant in univariate model.

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