Lung Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Genomic and Transcriptomic Profiles in Smokers and Never-Smokers Lung Squamous Cell Carcinoma Patients

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Purpose: Lung Squamous Cell Carcinoma (SCC) is a Non-Small Cell Lung Cancer (NSCLC) subtype with a strong clinical association with smoking habits and a very low incidence in never-smokers. Molecular profiling of SCC in never-smokers could unveil tumor vulnerabilities and new treatment strategies.

Patients and Methods: We considered a patient cohort of 17 former or current smokers (51.5%) and 16 never-smoker SCC patients (48.5%). TruSight Oncology® 500, investigating hotspots in 523 cancer-related genes, Tumor mutation burden (TMB) and micro-satellite instability (MSI), and RNA sequencing was performed on tumor tissue. Genomic and transcriptomic profiles were compared between smokers and never-smoker patients.

Results: The most frequently altered genes were *TP53* (67%), *CDKN2A* (20%) and *PIK3CA* (17%), with no substantial differences between groups, except for *TP53* which was more frequently mutated in smokers (86.7% vs 46.7%, p = 0.05), who also showed a higher TMB with respect to non-smokers (median 11 mut/Mb vs 5.5 mut/Mb, p = 0.028); all patients were stable for MSI score (median 1.87 vs 1.82, p = 0.87). Activating mutations in *EGFR* and *MET* were found in one and two never-smokers, respectively. Three smoker patients had simultaneous amplifications in *FGF3, FGF19* and *FGF4*. Enrichment analyses showed that cyclin-dependent protein Ser/Thr kinase activity and PI3K signaling pathways were affected in both groups, while cellular damage response was exclusively altered in never-smokers. Unsupervised hierarchical clustering on transcriptomes effectively identified different specific transcriptional subtypes between smokers and never-smokers. Gene set enrichment analysis highlighted that tumors from never-smokers are characterized by dysregulation in cell membrane potential and ion homeostasis across cell membrane pathways.

Conclusion: Genomic and transcriptomic profiles deeply differentiate SCC occurring in never-smokers with respect to SCC in smoker patients. Moreover, SCC could carry canonical NSCLC) activating mutations. Our data suggest that deep molecular analyses resolve tumor heterogeneity and may help with new algorithm-based treatment strategies for SCC.

Keywords: lung squamous cell carcinoma, smoking habits, next generation sequencing, transcriptomics

Introduction

Lung cancer (LC) is the most diagnosed and the most lethal malignancy worldwide, with almost 2.5 million new cases and 1.8 million deaths in 2022.[1] Non-Small Cell Lung Cancer (NSCLC) is the most prevalent histology, accounting for

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around 85% of LC cases; based on histologic features, NSCLC is further classified into other subtypes, of which adenocarcinoma (ADC, 70% of all NSCLC cases) and Squamous Cell Carcinoma (SCC, about 30% of NSCLC cases) are the most represented forms.[2] Even though they are both classified as NSCLC, SCC and ADC deeply differ in both biological and clinico-pathological features.[3] In fact, ADCs are mainly characterized by acinar, papillary, solid or mucinous patterns, with thyroid transcription factor (TTF-1) and napsin-A staining to support the diagnosis; more common in younger patients, in women, and it is more addicted to oncogene-activating mutations (eg *EGFR* mutations, *EML4-ALK* fusions), which are rarely detected in SCC.[3–5] Conversely, SCC histology is histo-pathologically classified into keratinizing, non-keratinizing and basaloid subtypes, with squamous cell patterns (supported by p40 and p63 staining) or keratinization aspects (supported by cytokeratin 5/6 staining).[3,6–8] Moreover, ADC and SCC deeply differ by transcriptomic profiling, highlighting different biologically relevant pathways involved in carcinogenesis and differentiation of tumor histology, and also molecular factors influencing patient prognosis, eg the availability of targeted therapies only for patients with ADC, which is often diagnosed with activating mutations.[9–11]

Smoking is the most common etiology for lung cancer, associated with around 80% of LC cases, as LC diagnosis is associated with both number of cigarettes/day and years of tobacco consumption.[12–14] Nonetheless, even though it could arise in smokers, ADC is more common in never-smoker patients, especially oncogene-addicted ADC, while SCC diagnosis is strongly associated with smoking habits.[5,15,16] While clinical and molecular features of both non-smokers and smokers ADC patients have been well elucidated, molecular profiles of SCC arising in never-smoker patients remain an uninvestigated field of research. As LC histology could influence patients' prognosis and therapy efficacy, outlining genomic and transcriptomic features of SCC in never-smoker patients could uncover tumor vulnerabilities, and specific signatures to help patient stratification.[17] Moreover, it could give insights on SCC tumor biology, as in the last years many indications are suggesting that SCC could be considered as an independent tumor histology, that deeply differs from ADC, even though still classified together.[3]

The main aim of the present study was to investigate the tissue molecular profile of non-smoker SCC patients using a comprehensive DNA next-generation sequencing (NGS) targeted panel and whole-transcriptome analysis and to compare it with that of a similar group of SCC patients with a history of smoking.

Materials and Methods

Case Series

This was a retrospective study on SCC patients with a histologically confirmed diagnosis of lung SCC between 2004 and 2022 at the University Hospital of Parma, Santa Maria delle Croci Hospital of Ravenna and Morgagni-Pierantoni Hospital of Forlì, in Italy. Diagnostic haematoxylin-eosin slides were retrieved and reviewed by two expert pathologists to confirm SCC histology, prior to inclusion within the study. Patient demographics and clinical features, including tumor histology, age, gender, and smoking history were obtained through medical chart review. Formalin-fixed paraffin-embedded (FFPE) tumor specimens were retrieved from the Pathology Units and revised by dedicated expert pathologists to contain at least 50% of tumor cells. FFPE slides were provided for centralized analysis at Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori" for molecular analysis. The protocol was approved by C.E.ROM. Ethical Committee (study code IRST-B045). The present study was conducted in accordance with the Declaration of Helsinki 1964 and later versions, and all patients signed the informed consent.

Targeted Panel DNA Sequencing

The neoplastic area was scraped to collect the DNA. The tumor-derived DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified with the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

The TruSight™ Oncology 500 (TSO500) target panel (Illumina, San Diego, CA, USA) was applied to detect a wide range of genetic alterations, including single nucleotide variants (SNVs) and copy number variants (CNVs) in more than 500 genes, as well as genetic signatures as tumor mutational burden (TMB) and microsatellite instability (MSI). Fragmentation of 40 ng of gDNA was performed using the ME220 ultrasonicator (Covaris, Woburn, Massachusetts, USA). Libraries were

enriched for the 523 target genes in the panel following the manufacturer's instructions. After quantity and quantity controls, 8 final libraries for each run were pooled and loaded onto the NextSeq™ 550 sequencer (Illumina).

The analysis was performed with DRAGEN™ TruSight™ Oncology 500 Analysis v2 software starting from fastq files. This tool can identify low-frequency somatic variants in coding exons and other biologically significant regions in 523 DNA biomarker-related genes. The manufacturer's quality control criteria were used to determine whether a TSO500 result was valid, including median insert size ≥ 70 bp, median exon coverage ≥ 50 count, and percentage of exons with coverage of at least 100 count ≥80%. Variants with a variant allele frequency (VAF) greater than 5% were called. Variants frequently present in the normal population (GnomAD > 1%) were excluded (Karczewski et al, 2020). Pathogenicity of the variants was defined following ACMG guidelines (American College of Medical Genetics and Genomics).[18] Variants classified as "pathogenic" or "likely pathogenic" by Varsome (VarSome Clinical 11.7.5) were considered as clearly pathogenic and not retained in the analysis together with variants of uncertain significance (VUS). Alterations classified as "benign" or "likely benign" by Varsome were excluded.[19] The selected cutoff values were >20% of unstable microsatellite sites to define MSI and >10 mut/Mb to define high TMB.[20,21] The minimum number of usable microsatellites to determine MSI status was 40. The OncoPrint was built with the ComplexHeatmap R package.[22]

RNA Sequencing

Additional FFPE slides were scraped to extract RNA from tumor cells. FFPE RNA isolation kit (Roche Diagnostics, Vienna, AUT) was used to collect RNA, following manufacturer instructions. RNA quantification was performed with the Qubit 3.0 instrument using the RNA High Sensitivity kit (Thermo Fisher Scientific).

RNA libraries were generated starting from 500 ng of purified total RNA, using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus (Illumina) on an automated Hamilton STARlet platform (Reno, NV, USA). Briefly, the total RNA was depleted, fragmented and denatured. The two-strand cDNA was synthesized and ligated with anchors. Fragments were cleaned up and amplified to generate libraries. Library quantity and quality were assessed on Qubit 3.0 instrument and BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA). All libraries were sequenced on a single run on a NovaSeq 6000 platform (Illumina) with a NovaSeq 6000 S2 Reagent Kit v1.5, and two steps of 101 cycles of paired-end sequencing were performed.

Transcript-level read count was performed with kallisto v0.46.2, then raw counts were collapsed to gene-level with tximport v1.12.1, then Differential Expression Analysis (DEA) was performed with DESeq v1.22.1121–123. The same software was used to produce PCA plots, while for heatmaps and hierarchical clustering Seaborn v0.12.1 was used. Gene Set Enrichment Analysis (GSEA) was performed with the package GSEApy v0.9.16,124–126. For QC analyses, DESeq2's Variance Stabilizing Transformation (VST) was applied to raw read counts, and the 500 genes with the highest variance across the dataset were selected to perform PCA and hierarchical clustering; VST-transformed counts were also used as input for GSEA, after removing genes <1 across all samples. GSEA was analyzed by Gene Ontology (GO) Gene Function, v. 2018.

Statistical Analysis

Data were summarized by median, first (IQ) and third (IIIQ) quartiles, for continuous variables and by means of absolute frequencies and percentages for categorical ones. The association between continuous variables and smoking status was assessed by the Wilcoxon–Mann–Whitney test whereas that with binary variables with the Pearson's $\chi 2$ test of the Fisher exact test, as appropriate. P-values for the association between pathogenic alterations and clinical data were adjusted using the Benjamini–Yekutieli method. However, since this is an exploratory study, we decided to report the unadjusted p-values in the text and complement them with the adjusted values. For this analysis, the odds ratio (OR) and corresponding 95% confidence intervals (CI) were also reported.

Results

Patients Clinico-Pathological Features

In this study, we collected RNA and DNA samples from all patients. However, we encountered some limitations: 2 DNA samples from smokers were quantitatively insufficient, and the quality of 5 RNA samples was not suitable for

transcriptome analysis (2 from smokers and 3 from never-smokers). Overall, adequate samples for at least one of the molecular analyses were available for 16 never-smoker SCC patients and 17 SCC patients who were current or former smokers (ie 6 current smokers and 11 former smoker patients).

Among the 33 patients, the median age at diagnosis of SCC was 71 years (IQ-IIIQ 63–76), male patients were 55%, and most of the patients had a stage I SCC (45% stage I, 30% stage II, 18% stage III, and 6% stage IV). Patient characteristics by smoking status are summarized in Table 1. Median packs/year (P/Y) for former and current smokers was 51 [IQ-IIIQ 40–74]. Specifically, median P/Y was 50 [IQ-IIIQ 30–85] and 53 [IQ-IIIQ 40–60] for former and current smokers, respectively, while for the 11 former smokers, the median time since cessation was 5 years (IQ-IIIQ 2–20). No substantial differences were present between the patients with and without smoking habits, except for a higher prevalence of stage I tumors among never-smokers as compared to current or former smokers. For paired comparison analysis, we considered molecular features of never-smoker patients with respect to patients with a smoking history (former and current smokers), as exposed to the risk factor of smoking habit for cancer occurrence.

DNA Targeted Sequencing

To investigate the presence of driver mutations and the molecular profile of never-smoker patients as well as to compare it with that of smokers or former smokers, a wide DNA mutation sequencing focused on 500 cancer-related genes, was performed. DNA sequencing analysis was performed on DNA with enough quantity and quality (n.15 patients with smoking habits [ie n.5 current smokers and n.10 former smokers] and n.15 never-smoker patients). By DRAGEN analysis software according to ACMG guidelines, overall 278 variants were identified, of which 82 were classified as pathogenic, and 22 CNV were found; considering only pathogenic variants, the most frequently mutated genes were *TP53*, 67% (87% [13/15] in current and former smokers patients, and 47% [7/15] in never-smokers), *CDKN2A*, 20% (27%, [4/15] in current and former smoker patients, and 13% [2/15] in never-smokers) and *PIK3CA*, 17% (27% [4/15] in current and former smoker patients, and 7% [1/15] in never-smokers. Interestingly, we found that one never-smoker patient had an activating insertion/deletion in exon 20 of *EGFR*, while further 2 never-smoker patients had a MET exon 14 skipping mutation (one exon 14 point mutation and one exon 14 splicing variant, respectively). Moreover, two patients (one smoker and one never-smoker) had an amplification of *EGFR*. We also found that tumors from 3 smoker patients had concomitant alterations in the *FGF* pathway, presenting simultaneous amplifications in *FGF3, FGF19* and *FGF4*. The spectrum of mutations with a frequency higher than 6% is reported in Figure 1.

Table 1 Patients' Characteristics and Clinical Associations

	Never-smokers (n=16)		**Current or former smokers (n=17)**		**P-value**
	n	**(%)**	**n**	**(%)**	
Sex					
F	8	(50.00)	7	(41.18)	0.611
M	8	(50.00)	10	(58.82)	
Age at diagnosis					
Median (IQ-IIIQ)	70.69 (64.8–76.95)		71.4 (60.27–75.53)		0.589
Stage					
I	9	(56.25)	6	(35.29)	0.335
II	5	(31.25)	5	(29.41)	
III/IV	2	(12.50)	6	(35.29)	
Pack-years[†]					
Median (IQ-IIIQ)	51 [40–74]				

Notes: [†]The median [IQ-IIIQ] pack-years were 50 [30–85] and 53 [40–60] for former and current smokers, respectively.

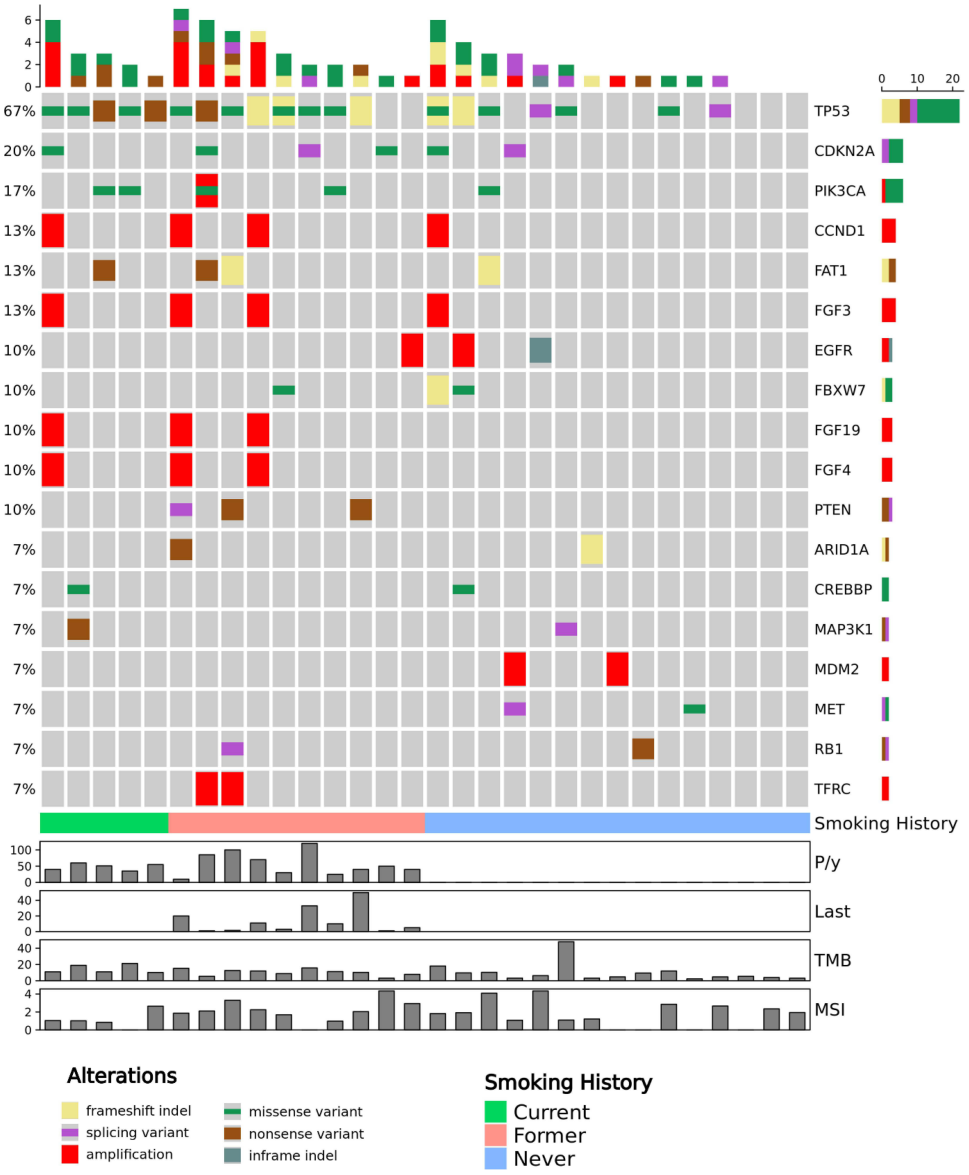

Figure 1 Oncoprint plot for gene alterations in the patients' cohort. Altered genes at a higher frequency than 6% are reported, ordered by number of mutations per patient. Columns represent individual samples and are divided by smoking habits. The different colors represent the different alterations, as indicated in the figure legends. The bar plots at the top and right of the oncoprint indicate the count of events found respectively in each sample and in each gene. In the lower part of the oncoprint, the smoking habit is reported for each sample.

No associations were found between patients' clinical features and pathogenic mutations. With regard to *TP53*, it was found altered in 13 out of 15 smokers or former smokers (86.7%) with respect to 7 out of 15 never-smokers (46.7%, OR: 7.43, 95% CI: 1.23–45.01), showing a borderline statistical significance (p = 0.05) that disappeared after adjusting for multiple comparisons (adjusted p = 1.00).

Overall, smoker or former smokers patients had a higher rate of pathogenic alterations with respect to never-smokers (67.1% vs 32.9%, p = 0.004), also resulting in a higher TMB for these patients (median 11 mut/Mb, IQ-IIIQ 8.8–15.3, vs 5.5 mut/Mb, IQ-IIIQ 3.2–14.4, p = 0.028) (Figure 2A and B). MSI assessment revealed that all patients were classified as microsatellite stable, with no differences between smokers or former smokers and never-smoker patients (median 1.87 IQ-IIIQ 1.0 −2.7 vs median 1.82 IQ-IIIQ 0–2.7, p = 0.87) (Figure 2C).

Finally, we performed a GO enrichment pathway analysis comparing gene expression between never-smokers and smokers or former smokers. In both groups of patients, replicative senescence pathways, regulation of cyclin-dependent

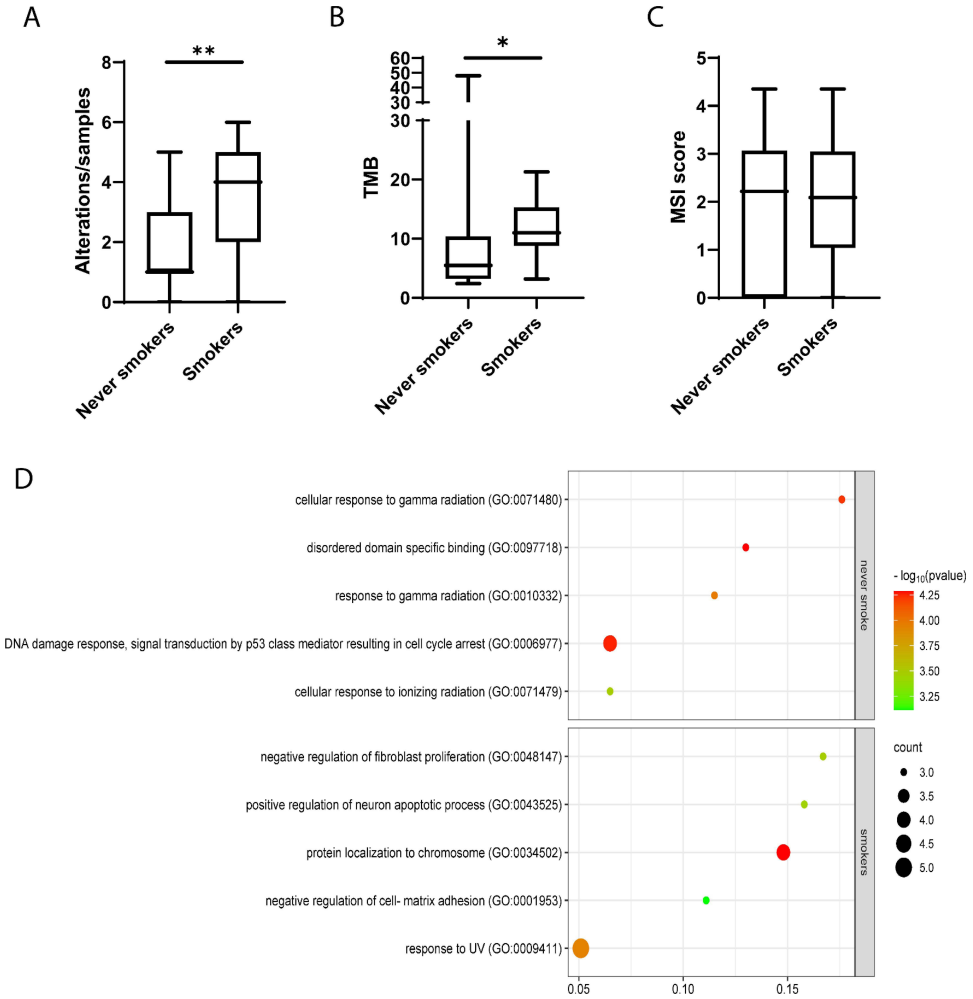

Figure 2 Box plots and Pathway enrichment bubble plot. The box plots comparing the rate of pathogenic alterations (**A**), tumor mutation burden (**B**) and microsatellite stability (**C**) between never-smokers and smokers or former smokers. Box Plots were obtained using GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA). *p-value: 0.028 **p-value: 0.004. (**D**) The plot showed only the differentially enriched pathways between the 2 groups of smoker and never-smoker patients. In the scatter plot are presented the top 5 enriched GO pathways. Pathway enrichment bubble was generated using SRplot.[23]

protein serine/threonine kinase activity pathways, cell cycle and several PI3K signaling pathways were enriched with alterations. Only patients with current or past smoking habits showed alterations in the regulation of fibroblast proliferation, neuronal apoptotic process, cell-matrix adhesion, MAPK cascade, base excision repair and UV response pathways. On the other hand, never-smokers showed several pathways of radiation response, DNA damage response, and signal transduction by p53 class mediator enriched (Figure 2D and Supplementary Table 1).

Transcriptome Analysis Marks the Difference Between Smoker and Never-Smoker Patients

A whole-transcriptome analysis in RNA from patients' tumors was performed. Total mRNA analysis was performed on tissue mRNA with at least >20 million successfully sequenced reads, ie 13 never-smoker and 15 smoker or former smoker patients. By considering only differentially expressed genes with a fold change > 2 and an adjusted p-value < 0.05, unsupervised clustering, and identified 277 genes for which mRNA expression was able to effectively discriminate never-smoker patients from smoker and former ones (Figure 3A and Supplementary Figure 1).

By GSEA in GO Gene Function pathway enrichment analysis, we identified 3 enriched pathways in never-smoker patients with respect to smoker ones, and one pathway linked to these pathways had a trend (ie GO:0099604, GO:0005272, GO:0005217, GO:0005248), as shown in Figure 3B and Table 2. In particular, all these pathways were related to cell membrane potential and ion homeostasis across cell membranes, especially calcium and sodium channel activity, suggesting that dysregulation in membrane potential homeostasis could have a role in cancer onset and progression.

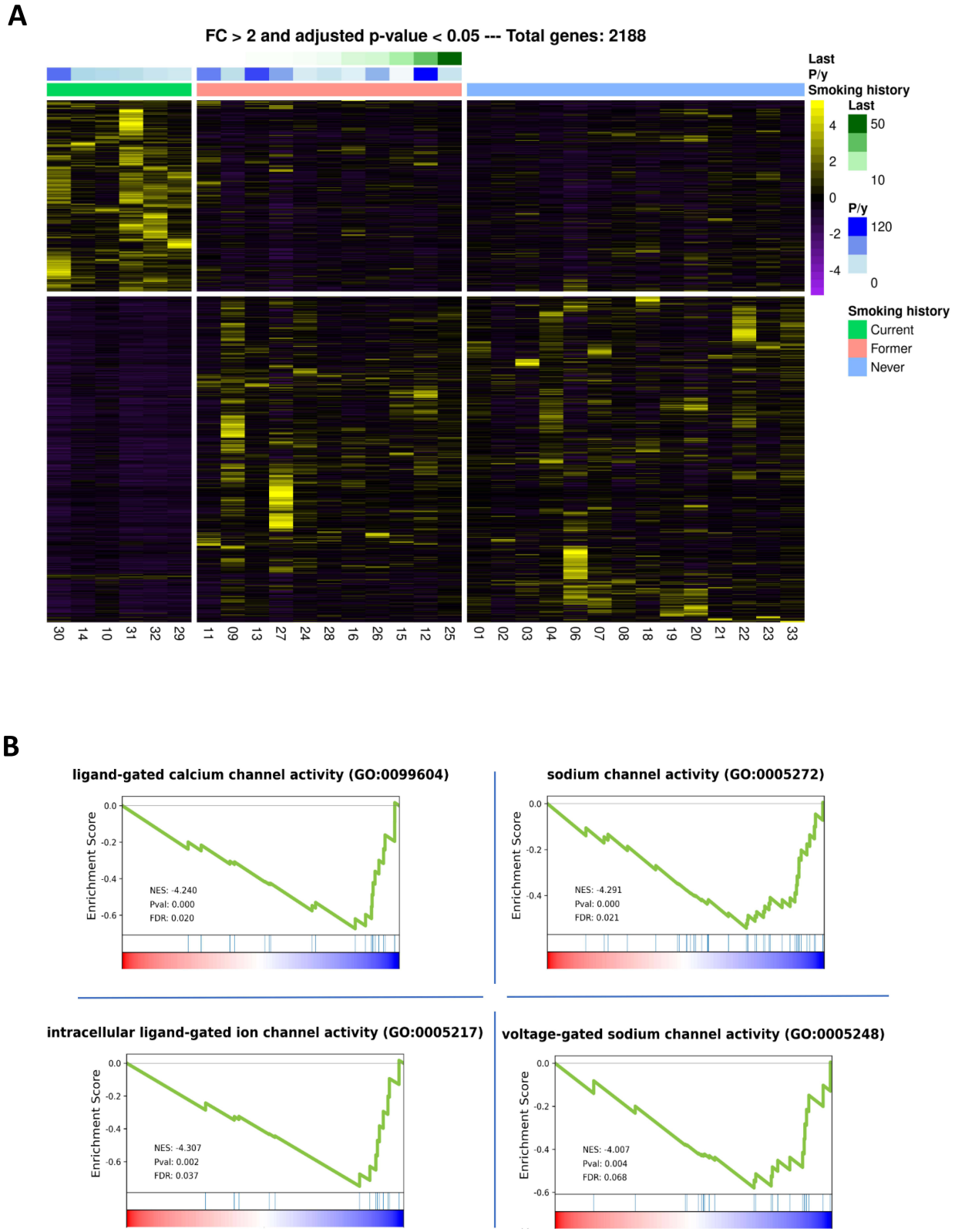

Figure 3 Heatmap performed by unsupervised clustering in the whole patient cohort and Gene Set Enrichment Analysis. (**A**) Considering a fold change > 2 and p ≤ 0.05, 2188 genes were found differentially expressed between the clinical subgroups of smokers, former smokers and never-smoker patients. Genes (rows) and patients (columns) are ordered by hierarchical clustering based on gene expression, using the Euclidean distance method and average linkage clustering for both genes and samples. (**B**) The analysis showed the transcriptome for pathways differentiating smokers patients versus never-smoker patients. A negative or positive Normalized Enrichment Score (NES) indicates an enriched pathway in the never-smokers group or in the smokers group, respectively. FDR: False Discovery Rate.

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Table 2 Gene Set Enrichment Analysis of Transcriptome for Pathways Differentiating Smokers Patients versus Never-Smoker Patients. p-value and FDR are Specified for Each Pathway

GO term	Pathway	*p*-value	FDR
GO:0099604	Ligand-gated calcium channel activity	<0.0001	0.020
GO:0005272	Sodium channel activity	<0.0001	0.021
GO:0005217	Intracellular ligand-gated ion channel activity	0.002	0.037
GO:0005248	Voltage-gated sodium channel activity	0.003	0.063

Abbreviations: GO, Gene Ontology; FDR, False Discovery Rate.

Discussion

Molecular profiles of SCC arising in never-smoker patients remain an unexplored area of research. In this study, we performed a wide molecular characterization including genomic and transcriptomic analyses to highlight which molecular features distinguish SCCs arising in never-smoker patients from the ones arising in patients with a history of smoking. The results of this study reveal distinct transcriptomic profiles between SCC in never-smoker and smoker patients. While the most commonly altered genes were similar between the two groups, including *TP53, PIK3CA*, and *CDKN2A*, there were notable differences observed. Based on the mutational profiles of the entire cohort, the most altered genes are comparable to the typical SCC profile.[24]

Molecular profiling of patients with NSCLC led to the identification of activating targetable mutations, and targeted therapy was demonstrated to confer a great benefit for patients carrying these mutations, even in the adjuvant setting.[25–27] On the other hand, these mutations are exclusively present in lung ADC patients, while for lung SCC, only the SQUIRE trial demonstrated that the addition of necitumumab to gemcitabine confers a survival benefit for patients with higher *EGFR* expression.[28] Interestingly, in our case series, we identified 3 targetable mutations in 3 different never-smoker patients (ie *EGFR* exon 20, *MET* exon 14 skipping and *MET* non canonical variant), highlighting that about 20% of never-smoker SCC patients could carry potential targetable mutations. On the other hand, we observed multiple FGF pathway alterations in 3 smokers, suggesting possible therapeutic implications also in this set of patients. In addition, in the genomic landscape of patients with SCC, the majority of mutated genes are *TP53* and *CDKN2A* (90% and 70%, respectively), as reported in the review of key literature data performed by Roy S. Herbst et al.[29] This finding is confirmed in our small cohort in which we were able to identify *TP53* and *CDKN2A* as the most altered genes (67% and 20%, respectively).[24] *CDKN2A* encodes for p16 and p14 proteins, and the loss of p16 leads to a disruption of the RB pathway, making *CDKN2A* a known tumor suppressor gene altered in lung SCC, whose mutations have been demonstrated to be associated with SCC patients' prognosis when co-occurring with *TP53* mutations.[30,31] As expected, smokers had a significantly higher rate of *TP53* mutations as they are associated with different squamous cell cancer histologies, especially in patients with smoking habits.[32–34] As a consequence, smoker patients also carried a significantly higher rate of pathogenic alterations (67.1% vs 32.9, p = 0.004), resulting in a higher TMB (median 11 mut/Mb vs 5.5 mut/Mb, p = 0.028) for the accumulation of DNA damage and of gene alterations.[35,36] The clinical significance of TMB in patients with SCC was highlighted as it has been demonstrated to be a strong predictor of better clinical outcomes, particularly in patients treated with single-agent immune checkpoint inhibitors.[37] The landscape of mutations in driver and non-driver genes showed an urgent need for molecular evaluation of patients with SCC, allowing the improvement of their management and treatment options. By GO enrichment analysis, both groups showed enrichment of pathways related to PI3K, replicative senescence and regulation of cyclin-dependent kinases, suggesting that dysregulation of cell cycle control and escape from senescence are critical events in SCC pathogenesis, regardless of smoking status, as also demonstrated by the induction of apoptosis and senescence following the pharmacological inhibition of CDK4/6 in several types of solid tumors.[38–40] Several PI3K signaling pathways were also enriched with gene alterations in both smokers and never-smokers. The PI3K/AKT/mTOR pathway is a key regulator of cell growth, proliferation, and survival of many cancers, including SCC. Targeting this pathway may represent another therapeutic opportunity for patients with SCC, and several clinical trials are nowadays evaluating PI3K target molecules (NCT:

NCT02785913; NCT: 03065062). Defects in PI3K/AKT/mTOR signaling pathways were associated with enhanced cellular responsiveness to irradiation and chemotherapy, which balance the effect of mutations, which can be translated into second events, which tend to undermine the balancing mechanism against tumors.[41,42]

RNA analysis demonstrated that lung SCC also exhibits distinct transcriptomic characteristics based on smoking history, suggesting that both genomic and transcriptomic changes play critical roles in shaping the tumor microenvironment and influencing treatment outcomes. By unsupervised clustering analysis, we successfully discriminated between never-smoker and smoker patients based on their transcriptomic profiles, leading to the identification of 277 genes that were significantly associated with smoking status. Interestingly, the analysis revealed a specific subgroup of smoker patients whose transcriptomic features were more closely aligned with those of never-smokers; in terms of expressed proteins, this association was already seen, as reported by Jacqueline M. Vink et al.[43] Interestingly, former smokers were clustered as an intermediate transcriptomic profile between current and never-smokers, suggesting that smoking cessation is directly reflected in tumor transcriptomics plasticity of gene expression. Then, this plasticity underscores the potential of gene expression profiles after quitting smoking, which could influence tumor behavior and treatment responses.[44,45]

On tumor RNA, we investigated the tumor-enriched pathways by GSEA, identifying key pathways related to cell membrane potential and ion homeostasis, particularly involving calcium and sodium channel activity, while a fourth pathway linked to these was reported with a strong trend (GO:0099604, GO:0005272, GO:0005217, GO:0005248). These pathways are crucial in maintaining cellular homeostasis and signaling. Ion channels are known to be involved in lung carcinoma, exhibiting different expression patterns compared to normal tissue.[46] In particular, sodium channels are transmembrane proteins that regulate action potentials, neuronal excitability, and ion transport,[47,48] and the targeting of these channels could lead to the identification of new potential biomarkers and therapeutic targets.[49] The significant function of calcium signaling in lung cancer has also been documented. The expression levels of certain specific Ca^{2+} channels and calcium-binding proteins are modified in lung cancer, thereby influencing cell proliferation, metastasis, apoptosis, and tumor development.[50,51] Recent research highlights the crucial role of Mitofusin-2 (*MFN2*) in regulating mitochondrial function and calcium homeostasis in lung adenocarcinoma, suggesting its potential as a therapeutic target.[52] Additionally, the selective T-type Ca^{2+} channel blocker KYS05047 has demonstrated anti-proliferative effects in A549 lung cancer cells, indicating that targeting calcium signaling pathways could be an effective strategy for treating this malignancy.[53] The transcriptomic profiles are an emerging effective tool to better characterize tumor at a molecular level, providing new insights for new treatment strategies, and could be included in prognostic and predictive tools to patients stratification. In the context of lung cancer, this evidence has been achieved for Small-Cell Lung Cancer molecular subtyping, and the translation of this approach to other lung cancer subtypes orphans of targetable markers could revolutionize treatment algorithms.[54] In summary, ion channels, particularly sodium and calcium channels, play a crucial role in various aspects of lung cancer pathogenesis, including tumor invasion, progression, and treatment-related side effects, underscoring their potential as valuable biomarkers and therapeutic targets for LC. On the other hand, several studies have reported ion channel overexpression in SCC, but the ion channel overexpression in never-smoker SCC patients is not fully understood.[46,55] Understanding these mechanisms may provide insights into targeted therapies and improved patient management strategies.

The major study limitations are related to the retrospective nature of patient enrolment, and to the number of patients enrolled, which needs to be larger in confirmation studies. On the other hand, SCC is not commonly diagnosed in never-smokers, limiting the enrolment power. Moreover, smoking habits, in terms of time from smoking cessation for former smokers and pack/years could influence molecular features, as also highlighted by our transcriptomic profiling; further studies including a higher number of patients and considering clinical variables of different smoking habits are warranted to confirm the first results of this exploratory study.

Conclusion

In this study, we showed that about 20% of never-smoker SCC patients carry a potentially targetable alteration, suggesting the importance, in this subset of patients, of a molecular characterization as for ADC patients. We also confirmed the involvement of the PI3K/AKT/mTOR pathway in SCC independently of smoking habits, reinforcing the

utility of PIK3 inhibitors development as a potential treatment strategy in this disease. Moreover, we highlighted that SCCs arising in never-smokers display peculiar transcriptomics profiles, suggesting that smoking habits influence tumor phenotype.

Abbreviations

ADC, adenocarcinoma; CNVs, copy number variants; FFPE, Formalin-fixed paraffin-embedded; GO, Gene Ontology; LC, IQ, first quartiles; IIIQ, third quartiles; Lung cancer; MSI, microsatellite instability; NGS next generation sequencing; NSCLC, non small cell lung cancer; SCC, Lung Squamous Cell Carcinoma; SNVs, single nucleotide variants; TTF-1, thyroid transcription factor; TMB, Tumor mutation burden; TSO500, TruSight™ Oncology 500; VAF, variant allele frequency; VST, Variance Stabilizing Transformation; VUS, variants of uncertain significance.

Ethics Approval and Consent to Participate

All methods used in our studies involving human participants adhered strictly to the ethical guidelines of our institutional research committee, aligning with the principles outlined in the 1964 helsinki declaration and its subsequent modifications, or equivalent ethical norms. The protocol was approved by C.E.ROM. Ethical Committee (study code IRST-B045) All patients considered in the present study signed written informed consent.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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