Modulating Tumor Microenvironment: A Review on STK11 Immune Properties and Predictive vs Prognostic Role for Non-small-cell Lung Cancer Immunotherapy

Modulating tumor microenvironment: A Review on STK11 immune properties and predictive *vs* prognostic role for non-small cell lung cancer immunotherapy

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Short title

STK11 and immunotherapy in lung cancer

Opinion statement

The quest for immunotherapy (IT) biomarkers is an element of highest clinical interest in both solid and hematologic tumors. In non-small cell lung cancer (NSCLC) patients, besides PD-L1 expression evaluation with its intrinsic limitations, tissue and circulating parameters, likely portraying the tumor and its stromal/immune counterparts, have been proposed as potential predictors of IT responsiveness. *STK11* mutations have been globally labeled as markers of IT resistance. After a thorough literature review, *STK11* mutations condition the prognosis of NSCLC patients receiving ICI-containing regimens, implying a relevant biological and clinical significance.

On the other hand, waiting for prospective and solid data, the putative negative predictive value of STK11 inactivation towards IT is sustained by less evidence. The physiologic regulation of multiple cellular pathways performed by STK11 likely explains the multifaceted modifications in tumor cells, stroma and tumor immune microenvironment (TIME) observed in *STK11* mutant lung cancer, particularly explored in the molecular subgroup of *KRAS* co-mutation. IT approaches available thus far in NSCLC, mainly represented by anti-PD-1/PD-L1 inhibitors, are not promising in the case of *STK11* inactivation. Perceptive strategies aimed at modulating the TIME, regardless of *STK11* status or specifically addressed to *STK11*-mutated cases, will hopefully provide valid therapeutic options to be adopted in the clinical practice.

Keywords: Prognostic factor; Predictive factor; PD-1; PD-L1; NSCLC; LKB1.

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1. Introduction

The immunotherapy (IT) revolution in non-small cell lung cancer (NSCLC) has left unanswered fundamental questions on our actual understanding of the immune control of cancer [1,2]. Major drawbacks reside in the still limited population of patients responsive to IT and the partial success of combinatory approaches aimed at improving survival outcomes [3]. Thus, the identification of predictive biomarkers represents a priority in the actual strategies to optimize IT efficacy [4,5]. Ideal candidates to predict IT response should reflect the tumor - host interaction and its evolutionary changes following treatment. In this scenario the tumor immune microenvironment (TIME) and mutational status are pillars to guide comprehensive evaluation of NSCLC patients suitable for IT approach.

The characterization of TIME has been the object of intense investigations, mainly involving the assessment of PD-L1 status, tumor infiltrating lymphocytes (TILs) density and phenotype, and activating (e.g. interferon-γ, IFN-γ) or inhibitory (e.g. $CD38^{+}$, transforming growth factor-β) signaling pathways [6–8]. Specifically, cancer classification into "hot" (T cell-inflamed, PD-$L1^{high}$, $CD8^{+rich}$, IFN-γ signature) and “cold" (immune-excluded, characterized by PD-$L1^{low}$, immune suppressive phenotypes and TGF-β signature; immune-desert, with low $CD8^{+}$ infiltration) tumors has demonstrated prognostic and predictive potential [9,10] (**Figure 1**). Since cancer immunoediting and immune response rely on the cross talk between tumor and its microenvironment not only at the organ level, but also involving the peripheral circulation that provides immune cells, cytokines, growth factors and chemokines, great efforts have been addressed to circulating parameters [11] (**Figure 1**).

The notion that specific genetic mutations encompassing oncogenic drivers such as *EGFR* and *ALK* or the interferon (IFN)-γ - related signaling pathways [12] might impact on IT effectiveness has been consolidated in the last decades [13]. Reproducible and consistent evidence, mostly obtained in *KRAS*-mutant NSCLC, have suggested serine/threonine kinase 11 (*STK11*), coding for the liver kinase B1 (*LKB1*) protein, as a critical factor implicated in anti-tumor immune response, ultimately affecting the proneness to respond to IT [14].

While the biological heterogeneity and therapeutic responsiveness of *KRAS*-mutant (*KRASmut*) NSCLC represents a formidable challenge, it has been widely demonstrated that the coexistence of *STK11* mutations (*STK11mut*) confers an intrinsic resistance to IT [14,15]. *STK11* is a tumor suppressor gene whose mutations can be identified in nearly 15% of lung adenocarcinoma and in up to 30% of *KRASmut* tumors [16]. Acting as a tumor suppressor gene, *STK11mut* with functional significance inevitably lead to gene inactivation which, in addition to promote autonomous cell growth, conditions metabolic alterations and an immunosuppressive TIME [16,17].

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Thus, NSCLC carrying *STK11mut* may represent a suitable model to assess the contribution of both tumor intrinsic and immune microenvironmental variables to the negative outcome of IT. Negative predictive biomarkers for IT benefit may well play a similar or even superior role compared to positive one not only in sparing unnecessary treatment but also in the identification of defective pathways and their potential therapeutic targeting. By dissecting the multifaceted involvement of STK11 on cancer biology and immune evasion, the attempt of the present review is to shed light on the still controversial issue of compelling biomarkers predictive of the response to immunotherapy.

2. How to define STK11 deficiency

Located on the short arm of chromosome 19 (19p13.3), *STK11* gene spans 23Kb and is comprised of nine coding exons and one non-coding exon. The STK11 protein consists of 433 amino acids with an N-terminal domain containing a nuclear localization signal and a phosphorylation site with unknown function at Serine 31, a central kinase domain (residues 44-309), and a C-terminal domain.

Germline *STK11* mutations are responsible for the Peutz-Jeghers syndrome, an autosomal-dominant hamartomatous polyposis syndrome [18]. In sporadic cancers, a wide range of somatic *STK11* genomic alterations were observed (single nucleotide variation, indels, hypermethylation of the promoter and homozygous deletions of LKB1) making challenging the development of a single assay capable of detecting all such alterations [19–21]. Hence, STK11 deficiency could be assessed with different screening strategy, mainly represented by next generation sequencing (NGS) technology for assessing *STK11* variants or immunohistochemical (IHC) approach to study LKB1 expression.

Both tissue and liquid biopsies have been used as testing material for detection of *STK11mut* [22–25]; the choice of NGS targeted panel must be carefully considered mostly for co-occurrence mutation analysis. In fact, in many daily routine practice NGS panel, genes of interest like *KEAP1* and/or *SMRCA4* are absent [26,27]. Usually, classification of mutations was categorized as disease associated on the basis of the designation in the NGS report (*i.e.* disease associated *versus* variant of unknown significance, VUS) querying the reference database e.g. cBioPortal, COSMIC and TCGA-Bioportal [22]. Currently, more than 400 unique mutations have been described for the *STK11* gene, among which ~70% promote the truncation of the protein and the other 30% represent missense mutations [28].

A correlation between the position of the *STK11mut* and their behavior as tumor suppressor *versus* oncogene was also observed. Pecuchet et al. described that disruptive mutations (nonsense,

frameshift and splice mutations) tended to be over-represented in exons 1 and 2 [29], data confirmed also in a dataset from *STK11* NGS across a variety of cancers in cBioPortal [30]. Truncating mutations could favor the use of alternative ATG initiation codon and the expression of a short isoform lacking the 124 N-terminal amino acid described as a putative oncogenic ΔN isoform [29].

On the contrary, no direct correlation between mutational status and loss of expression by IHC was observed making NGS technology a not exhaustive strategy to define LKB1 status [25,31,32]. For example, Skoulidis et al reported 17.3% of *STK11* wild-type tumors with the absence of LKB1 protein expression by IHC, thus confirming that genetic (*i.e.* mutational) LKB1 assessment might be insufficient to predict LKB1 functional status [25].

Studies on protein expression could be performed with different STK11/LKB1 rabbit monoclonal antibody: different clones were tested such as D60C5F10 (Cell Signaling Technology) [24] or Ley 37D/G6 (Abcam) [32]. The expression could be evaluated grading the cytoplasmic staining intensity, ranging from no discernible (0) to strong cytoplasmic staining (3+). Quantitative IHC for STK11/LKB1 can capture STK11/LKB1-deficient tumors in the absence of *STK11/LKB1* genomic alterations, representing a potentially reliable, simple, and cost-effective method to evaluate LKB1 loss.

Other approaches are emerging as useful tool to investigate alternative mechanisms responsible for LKB1 loss of function: studying the RNA level could highlight the effect mediated by epigenetic inactivation [33] or homozygous and intragenic deletions [20,34]. Chen et al. developed a NanoString-based assay, validated in multiple datasets and subsequently tested in a cohort of 150 lung adenocarcinoma patients, demonstrating high superior overall performance of STK11 signature studied by NanoString with respect to IHC and mutation status [31].

The unequivocal definition of LKB1 deficiency is actually an open issue: the presence of inactivating *STK11* mutations or the lack of LKB1 protein expression at IHC analysis are often discordant. Thus, an integrative analysis of LKB1 genetic alteration is timely and important to provide a better estimate of the incidence of this important tumor suppressor gene.

Thus far, both preclinical and clinical evidence regarding the impact of STK11 status on the immune context and response to IT in lung malignancies have been mainly documented through the analysis of *STK11mut*, while the evaluation of LKB1 expression being less represented across studies.

3. Biological implications of STK11 in NSCLC

3.1. Implication of STK11 and its genomic loss on cancer growth and metabolism

Under physiologic conditions, STK11 acts a master upstream kinase, directly phosphorylating and activating AMP-activated protein kinase (AMPK) and 12 related kinases with crucial roles in cell processes, such as metabolic regulation, DNA integrity, proliferation, polarity establishment and control of spatial orientation of the cellular structures, angiogenesis, and interaction with tissue milieu [35] (**Figure 1**). STK11-AMPK pathway serves as a cell metabolic checkpoint arresting cell growth in conditions of low intracellular ATP levels [35,36]. In addition, under exogeneous activation, STK11-AMPK signaling is able to induce p53 activity and interfere with cyclins/cyclin-dependent kinase interplay, thus blocking G1/S transition [37]. Moreover, whereas DNA damage occurs, this complex is able to localize into the nucleoplasm and preserves cells from genomic instability [38].

Based on these physiologic actions, STK11 has been described as tumor suppressor and experimental and clinical observations support the tumorigenic potential of its mutations. Evidence of the impact of *STK11mut* on cancer growth, aggressiveness, metabolism and angiogenesis [28] have been comprehensively reviewed in several reports [16,35]. Moreover, STK11 loss of function alters the regulation of PAK1 [39], FAK and CDC42 [40] thereby leading to epithelial-to-mesenchymal transition (EMT) and metastases [41,42].

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Importantly, the metabolic derangement produced by STK11 defects is translated in a redox imbalance. As a consequence of increased energetic and metabolic stress, STK11-deficient NSCLC cells generate elevated levels of reactive oxygen species (ROS) [43] (**Figure 1**). As the extent and type of ROS modulate basic biological processes in cancer cells including mutation, proliferation, DNA damage, autophagy and apoptosis [44], this phenomenon has several implications on the mechanisms responsible for the pathogenicity of STK11 loss of function. Moreover, the hypothesis has been advanced that this metabolic driven property may be linked to the close interaction of STK11 with signaling molecules instrumented to tightly control intracellular displaced DNA or chromatin fragments and their impact on immune surveillance (see section 3.3).

Several other downstream regulated genes are also fundamental to decipher the multiple pathways involved STK11 onco-suppressive nature. Recently, a striking similarity in histologic and gene expression features of STK11- and salt-induced kinases (SIKs)-deficient tumors suggests a common tumor suppressive trait [45]. Moreover, *STK11mut* human adenocarcinoma cell lines and primary tumors display at high rate a SIK-deficient signature, thus opening new scenarios in

deciphering alternative mechanistic insights and potential therapeutic implications in this complex subset of NSCLC patients [45].

3.2. STK11 and tumor immune microenvironment

In the era of immunotherapy, the complex interplay between STK11 and TIME, has been the object of several preclinical and clinical investigations in both non-neoplastic diseases and cancer. Linking cellular bioenergetics with cell proliferation and metabolism, STK11 impacts on both tumor biology (see section 3.1) and T cell modulation [46], thereby encompassing multiple steps to shape TIME (**Figure 1**). In well-designed preclinical studies T cell selective knock out of STK11 reduced the progenitor pool of T effector cells [46,47]. Accordingly, transgenic STK11 ablation in mice prevented TCR-β-selected thymocyte differentiation and expansion via CD98 alterations, thus blunting the proliferative response of peripheral T cells [48].

Additional evidence on the involvement of STK11 in T effector and suppressor cell balance have been provided by its critical role in sustaining T regulatory cells (Tregs) function through the stabilization of FOXP3 [49]. In contrast, conditional knockout of *STK11* gene in dendritic cells (DCs) leads to excessive Treg cell proliferation involving IKK/IKBα-independent activation of NF-κB/OX40L pathway [50]. Moreover, knock-out of *STK11* in mice results in increased levels of pro-inflammatory cytokines and enzymes in bone marrow-derived macrophages upon lipopolysaccharide (LPS)-induced NF-κB activation [51].

In the last few years, the close interplay between *STK11mut* cancer cells and TIME has been deeply investigated in the context of NSCLC. Not surprisingly, also in reason of the physiologic implication of STK11 in phenotypic and functional integrity of the immune system, multiple TIME compartments are affected by defective mutation of the gene.

Inactivating *STK11* aberrations, mainly present in *KRASmut* adenocarcinoma, have arisen as major determinants of cold NSCLC TIME, dominated by low CD3+, CD4+ and CD8+ TILs, high Tumor Associated Neutrophils (TANs) and decreased levels of PD-L1 in spite of a high/intermediate TMB[16]. The negative effect exerted by STK11 on PD-L1 expression has been repeatedly reported [52–54] and appears to overcome PD-L1 mRNA stabilization promoted by *KRAS* [55].

In depth proteogenomic analysis of a series of nearly 100 lung adenocarcinomas revealed that, among the genomic landscape, the most significant impairment in immune activation was present in *STK11mut* tumors [56]. At transcriptional and post-transcriptional levels, severe reductions in DCs, NK T cells, and macrophages were coupled with a defined neutrophil degranulation signature.

Intriguingly, this functional defect was independent from the abundance of neutrophils. Importantly, when these downregulated immune features were entered in a deep-learning-based predictive algorithm, a highly accurate prediction of *STK11* mutational status from histopathological slides was attained. The predominant immunosuppressive TIME of *STK11mut* tumors is also supported by a retrospective analysis of 282 NSCLC documenting a reduced intratumor DC density [57]. Findings from a microarray-based gene expression analysis on a cohort of more than 400 lung adenocarcinoma showed that only *STK11mut* were associated with significantly lower immune surveillance signature characterized by NF-kB activity and T-cell receptor α-β chain expression [58].

On both mouse models and cell lines, STK11 loss resulted in an altered cytokine milieu with increased expression of proinflammatory CXCL7, G-CSF, IL-1β and IL-6 fostering neutrophils recruitment and T-effector cell suppression [59]. Compared to *KRASmut* tumors, *KRASmut*/*STK11mut* tumors displayed higher infiltration of TANs with enhanced expression of arginase 1 (ARG1) and IL-10, which exerted a negative effect on T lymphocytes balance leading to Tregs expansion and T-effector cells exhaustion. Moreover, STK11 inactivation conditioned lower levels of tissue PD-L1 expression in *KRASmut/STK11mut* tumors and in cultured murine and human cells lines [59].

Genetically engineered mouse models exploring the progenitor-specific etiologies of lung cancer histotypes, in addition to highlight the oncogenic boost of STK11, have shown the prominent ability of immune escape in tumors generated in mutant mice by downregulation of the antigen presenting machinery and TAN infiltration [60].

The critical impact of STK11 loss of function on lung cancer immune microenvironment is generally ascribed to epigenetic repression of stimulator of interferon genes (STING), causing insensitivity to cytosolic double-stranded DNA (ds-DNA) accumulation [61] (see section 3.3). An additional molecular mechanism contributing to the inert immune phenotype associated to *STK11mut* may reside in the oncogenic synergism between KRAS and MYC [62,63]. This co-operation induces high levels of CCL9 and IL-23, epithelial-derived signaling molecules involved in stromal reprogramming, thus triggering the recruitment of macrophages and the exclusion of adaptive and innate immune response by T and B lymphocytes and NK cells, respectively.

Consistently, the negative impact of STK11 aberrations on cancer immune background has been recently supported by the observation that both early-stage and advanced NSCLC harboring *STK11mut* display a lower expression of PD-L1 coupled with poor immune cell infiltration [54,64].

Thus, in keeping with its central role in shaping tumor immunobiology and immune contexture, *STK11* genomic alteration endows the tumor with multiple paths to escape the immune system, significantly affecting patient outcome and response to IT.

The complexity of STK11-driven immune features prompts the development of novel synergistic and highly personalized therapeutic approaches.

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3.3. Defective STK11 Desensitizes the STING pathway to Promote Tumor Immune Evasion

Under normal conditions, the physiologic activation of AMPK by STK11 is not only involved in metabolic processes mainly converging to mammalian target of rapamycin complex 1 (mTORC1) inhibition, but also results in enhancement of STING [65] (**Figure 1**). The relevant role of STING in innate immunity resides in its ability to sense, through cyclic GMP–AMP synthase (c-GAS), cytosolic genomic ds DNA (gDNA) and, as more recently documented, also mitochondrial dsDNA (mtDNA) [66]. The immune signaling cascade triggered by STING activation ultimately results in increased expression of cytotoxic type 1 IFNs and T cell recruiting chemokines as well as PD-L1 [61]. This tightly regulated mechanism of immune surveillance is disrupted in *KRASmut*/*STK11mut* NSCLC and represents the molecular underpinning of the evidence that *STK11* aberrations confer an unfavorable TIME and condition an impaired immune response [65,67]. Specifically, STK11 loss of function is transduced in downregulation of AMPK-STING pathway and refractoriness to cytoplasmic dsDNA sensing which is further aggravated by the unrestrained inhibitory pressure of AMPK on epigenetic silencing enzymes (DNA (cytosine-5)-methyltransferase 1 [DNMT1], Enhancer of zeste homolog 2 [EZH2]) [61]. Strong supporting data on the clinical implication of these in vitro observations have been provided by the analysis of STING expression on a large series of NSCLC [68]. Indeed, *STK11mut* cases had the lowest levels of STING and immune gene expression, pointing to the derangement driven by genetic defects of this kinase in orchestrating the innate and IT induced immune response against cancer.

As deceptive STING plays a central role in the immunosuppressive trait of *STK11mut*ated lung cancer, several preclinical and clinical (NCT04096638, NCT03843359 and NCT04420884) attempts have been proposed to overcome IT resistance by local [69] or systemic cyclic dinucleotide (CDN) STING agonists. Acceptable safety and efficacy were obtained by MK-1454, a locally delivered CDN STING agonist, when combined with pembrolizumab in a phase I trial (NCT03010176). Preliminary evidence of STING activation by this approach were documented by increased serum levels of STING-associated cytokines [70]. Due to the limited applicability of

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intratumor injection in advanced tumors, more recently, an oral formulation of two non-CDN STING agonists has been explored, showing promising experimental results in terms of anti-tumor immunity and sensitization to IT [71,72]. With the potential of changing the actual therapeutic landscape, optimization of these approaches together with the adoption of patient- and disease-specific selective measures may open new venues in the overall management of cancer.

3.4. STK11 and tumor stromal-vascular network

In solid malignancies the contribution of stromal compartments typically exceeds that of neoplastic cells in the structural composition of the tumor mass. This intricate network of fibroblasts, mesenchymal cells, blood and lymphatic endothelial cells embedded in extracellular matrix physically and functionally sustains the multiple players regulating cancer fate. Compelling evidence of the relevance of STK11 in shaping tumor stroma arises from its interplay with TGF-β pathway and extracellular matrix (ECM) remodeling (**Figure 1**).

In addition to a direct biochemical interaction with TGF-β [73], in the context of gastrointestinal tumors, STK11 plays a crucial role in epithelial-stromal cross talk dictating multistep tumor formation. Specifically, the engagement of mesenchymal STK11 with TGF-β signaling in epithelial cells represents a determinant factor for the development of gastrointestinal polyps in humans and mice [74]. STK11 deficiency has also been shown to affect stromal TGF-β expression thus altering tumor suppression [75]. To the best of our knowledge, no such mechanisms have been shown to be operative in *STK11mut* lung cancer.

Lysyl oxidase (LOX) is a fundamental enzyme promoting collagen and elastin stabilization and cross-linking. NanoString-based quantitative mRNA/miRNA readouts in human lung adenocarcinoma, have shown LOX as a STK11 downstream regulated gene [76]. Defective STK11 increases LOX activity resulting in remarkable collagen deposition and formation of fibrotic foci in lung adenocarcinoma [77] which can be reverted by pharmacological inhibition of LOX enzymatic activity [78]. The alteration of ECM homeostasis with STK11 mutations is associated with phenotypic plasticity [78] and greater cancer cell proliferation and invasion [77]. Moreover, enhanced LOX activity was detected in serum from advanced lung cancer patients, strongly correlating with poor clinical outcome. Of note, upregulation of LOX expression by *STK11mut* also involves mTOR-HIF-1α pathway further underlining the role of STK11 in tumor angiogenesis, metastases, and glycolytic metabolism. The close link between STK11 and angiogenesis has been originally

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documented by the midgestation lethal phenotype associated with vascular abnormalities in transgenic mice carrying homozygous inactivating mutation of *STK11* [79].

More recently, the suppressive role of STK11 on angiogenesis was found to be mediated by its interaction with the angiogenic receptor neuropilin-1 (NRP-1) [80]. Indeed, STK11 loss of function in cancer cells from NSCLC patients unleashes NRP-1 trafficking and fosters its aberrant expression. Consistently, an inverse correlation between STK11 and NRP-1 expression was present in tumor samples at gene and protein levels [80].

From a therapeutic perspective, only one clinical observation is available regarding the impact of *STK11mut* on the effectiveness of anti-angiogenic agents. NSCLC patients with negative/weak STK11 status did not significantly benefit from the addition of bevacizumab to standard chemotherapy. Conversely, among intermediate/high STK11 cases, significantly fewer deaths were observed in those receiving bevacizumab [81]. Although the exact contribution of *STK11* alterations to the sensitivity to antiangiogenic drugs is uncovered, mechanistic insight has been provided in STK11-deficient PDX by the evidence of reduced AMPK activation and increased tumor necrosis as a result of bevacizumab administration [81].

4. Clinical implications of STK11 in NSCLC

STK11mut have been recently proposed as an important regulator of resistance to immune checkpoint inhibitors (ICI) in NSCLC (**Table 1**).

Table 1. Impact of *STK11* mutations upon immunotherapy activity and efficacy in NSCLC patients.

Cohorts of pts	N of pts	Clinical setting	Treatment	ORR	mPFS (months)	mOS (months)	Ref
STK11mut *STK11wt*	6 17	Stage III – maintenance after CCRT (Sq+NonSq)	Pembrolizumab/durvalumab	- 	11.3 17.5 (p = 0.174)	-	[82]
MDACC cohort *STK11mut* *STK11wt*	 11 55	Stage IV, PD-L1 $\geq$ 1% (NonSq)	Anti-PD-1/PD-L1	 0% 34.5% (p = 0.026)	 1.7 19.3 (p = 0.0012)	 11.1 26.5 (p < 0.0001)	[25]
STK11mut *STK11wt*	31 96	Stage IIIB-IV	ICI	-	5.6 6.3 (p = 0.35)	8.6 12.1 (p = 0.035)	[83]
STK11mut *STK11wt*	6 26	Stage IV – 2nd-line (NonSq)	Nivolumab	-	1.2 4.8 (p = 0.07)	11.4 13.8 (p = 0.5)	[84]
STK11mut *STK11wt*	15 104	Stage IV (NonSq)	Durvalumab	6% 16% (X^2 = 0.05)	-	4.9 14.2 (p = 0.008)	[85,86]
STK11mut *STK11wt*	6 57	Stage IV - $\geq$ 3rd-line (NonSq)	Durvalumab	0% 25%	-	-	[85,87]
STK11mut *STK11wt*	23 97	Stage IV - $\geq$ 3rd-line (NonSq)	Durvalumab + tremelimumab	4% 25% (X^2 = 0.02)	-	6.7 15.6 (p = 0.001)	[85,88]
STK11mut *STK11wt*	1310	Nonsq	ICI	-	2.5 3.1 (p = 0.01)	-	[89]
STK11mut *STK11wt*	117 332	Stage IV – 1st-line (NonSq)	CPP	32.6% 44.7% (p = 0.049)	4.8 6.9 (p = 0.0012) HR in PD-L1+ 1.73, p=0.016	10.6 16.7 (p = 0.0083)	[90,91]
STK11mut *STK11wt*	137 122		CP	-	3.7 5.6 (p = 0.052) HR in PD-L1+ 1.00, p=0.99	-	
STK11mut *STK11mut*	102 120		CPP CP	-	4.8 4.3 (p = 0.75)	10.6 10.3 (p = 0.79)	
STK11mut *STK11wt*	40 230	Stage IV – 1st-line (Sq+NonSq)	ICI	41.2% 44.5%	4.0 4.8 (p = 0.4)	11.2 17.7 (p = 0.1)	[92]
STK11mut *STK11wt*	111 559	Stage IV – 2nd-line (Sq+NonSq)	ICI	24.7% 34.0%	2.2 3.0 (p = 0.0002)	6.3 12.0 (p = 0.0002)	
STK11mut *STK11wt*	288 1849	Stage IV – 1st-line (Sq+NonSq)	Chemotherapy	59.3% 65.7%	4.5 5.8 (p < 0.0001)	11.2 17.8 (p < 0.0001)	
STK11mut *STK11wt*	83 780	Stage IV – 2nd-line (Sq+NonSq)	Chemotherapy	33.3% 39.1%	4.0 4.3 (p = 0.7)	11.5 13.2 (p = 0.7)	
6 ICI cohorts *STK11mut* *STK11wt*	807	Stage IV (Non-sq)	ICI	 RR 0.71, p = 0.251	 HR 1.54, p = 0.002	 HR 1.57, p = 0.003	[93]

1 CT cohort *STK11mut* *STK11wt*	244		Docetaxel	-	-	HR 1.82, p = 0.006	
STK11mut *STK11wt* *STK11mut* *STK11wt*	32 272 28 266	Stage IV – 2nd/3rd line POPLAR and OAK trials (NonSq)	Atezolizumab Docetaxel	- -	- -	7.3 15.6 (p = 0.004) 4.8 10.2 (p = 0.001)	[94]
STK11mut *STK11wt* *STK11mut* *STK11wt* *STK11mut* *STK11wt*	55 257 51 271 41 268	Stage IV (Sq+NonSq)	Durvalumab Durvalumab + tremelimumab Chemotherapy	16.7 25.2 21.6 23.6 12.2 33.6	- - -	10.3 13.3 4.4 11.3 6.7 13.1	[95]

Abbreviations: CCRT, concurrent chemo-radiotherapy; CP, platinum-pemetrexed; CPP, pembrolizumab plus platinum-pemetrexed; ICI, immune checkpoint inhibitor; n, number; NonSq, non-squamous; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; pts, patients; Sq, squamous.

Skoulidis and collaborators firstly determined *STK11mut* as a negative predictive factor of response to immunotherapy in the clinical setting [25]. The authors found that the presence of *STK11mut* was associated with inferior clinical outcomes to PD-1 blockade in multiple independent cohorts of *KRASmut* NSCLC, including those treated with nivolumab in the CheckMate 057 phase III study [25]. In addition, they examined the impact of *STK11mut* on clinical response to anti-PD-1/PD-L1 in 66 PD-L1 positive non-squamous (NonSq) NSCLC patients, regardless of *KRAS* mutational status and PD-L1 expression levels. Impressively, none of the patients in the *STK11mut* group responded to ICI compared to 34.5% of the *STK11* wild-type (*STK11wt*) group ($p = 0.026$), despite including PD-L1 high expressing tumors. *STK11mut* patients had a dramatically shorter median progression-free survival (mPFS) and overall survival (mOS) with PD-1 axis blockade (PFS Hazard Ratio [HR] 4.76, $p = 0.00012$; OS HR 14.3, $p < 0.0001$). This effect was observed across both the PD-L1 high ($\geq$ 50%) and PD-L1 low (PD-L1 < 50%) groups [25].

The negative influence of *STK11mut* on ICI response was further confirmed by Biton et al [84]. Among different genetic alterations which constituted a tumor immune profile (e.g. *EGFRmut*, *TP53mut*), *STK11mut* alone was associated to shorter mPFS in a small cohort of 32 NonSq NSCLC patients treated with second-line nivolumab compared to the counterpart [84]. Concordantly, in a larger cohort of 1310 ICI treated individuals, mPFS was 2.5 months in *STK11mut* patients compared to 3.1 months in *STK11wt* group ($p = 0.01$) [89]. Jure-Kunkel et al also confirmed the negative impact of somatic *STK11mut versus STK1wt* in advanced Non-Sq NSCLC patients enrolled in two independent trials evaluating durvalumab monotherapy (CP1108/ATLANTIC), and in a durvalumab plus tremelimumab trial (D4190C00006), both in terms of shorter OS and significantly reduced overall response rate (ORR) [85]. Similarly, in exploratory analysis from the phase I/II MYSTIC trial evaluating durvalumab +/- tremelimumab in treatment-naïve NSCLC patients, *STK11mut* patients had a significantly shorter OS compared to the *wt* cohort [95]. Other research groups supported *STK11mut* as marker of poor response to ICI in NSCLC [83], even in the maintenance setting after concurrent chemoradiotherapy for stage III disease [82]. Moreover, in different studies *STK11mut* has been associated with hyperprogression to ICI, in terms of an accelerated tumor growth with worsening clinical status [96,97].

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Of interest, *STK11mut* also defined a group of patients with apparently inferior clinical outcomes to the chemo-immunotherapy combination with platinum-pemetrexed plus pembrolizumab in the first-line setting [90]. Interestingly, in this study, patients who harbored *STK11mut* did not benefit from the addition of pembrolizumab to chemotherapy, both in terms of mPFS (4.8 *versus* 4.3 months, HR 1.13, 95% CI 0.83-1.54, $p = 0.75$) and mOS (10.6 *versus* 10.3 months, HR 1.03, 95% CI 0.71 to 1.49, $p = 0.79$) compared to platinum-pemetrexed alone [90]. Nonetheless, a recent report on

patients enrolled in KEYNOTE-189 documented a better outcomes with pembrolizumab plus chemotherapy regardless of the presence of STK11 or KEAP1 mutation [98].

The notion that *STK11mut* may confer innate resistance to ICI in NSCLC was mainly drawn from retrospective studies that lacked the chemotherapy control group. In this regard, different reports explored the significance of *STK11mut* in both ICI and chemotherapy cohorts [92,93,95]. When focusing on chemotherapy, patients with *STK11mut* achieved worse outcomes to different chemotherapeutic regimens than *STK11wt* patients, across different treatment lines, to a similar extent for ICI [92,93,95]. In a large real-world cohort of 2137 advanced NSCLC patients treated with frontline chemotherapy (65.6% platinum-based), mPFS and mOS were shorter in *STK11mut* patients with regard to *STK11wt* (mPFS 4.5 *versus* 5.8 months, HR 1.4 [95% CI, 1.2-1.6], $p < 0.0001$; mOS 11.2 *versus* 17.8 months, HR 1.4 [95% CI, 1.2-1.6], $p < 0.0001$), with the same detrimental effect seen in ICI cohort [92]. When comparing anti-PD-1/PD-L1 efficacy to platinum-based chemotherapy in a treatment naïve cohort of 2276 patients, there was no association between *STK11mut* and both mPFS and OS (mPFS anti-PD-1/PD-L1 *versus* chemo, HR 1.05, 95% CI 0.76-1.44; mOS anti-PD-1/PD-L1 *versus* chemo, HR 1.13, 95% CI 0.76-1.67) [99]. Pooling data from POPLAR and OAK trials (randomizing pretreated patients to receive either atezolizumab or docetaxel), *STK11* status confirmed its prognostic role in non-squamous histology. Compared to *STK11wt* group indeed, median estimations of OS in *STK11mut* were halved in both immunotherapy and chemotherapy arms[94]. Moreover, in a recent post-hoc analysis of patients enrolled in IMpower150 study focused on *KRASmut* patients, *STK11mut* and/or *KEAP1mut* cohort achieved numerically shorter mPFS and mOS than the *wt* group, regardless of the treatment arm [100]. These results might remodulate the value of *STK11mut* as prognostic rather than predictive factor. However, further prospective research is needed to confirm these findings.

Other retrospective studies tried to assess whether *STK11mut* negative impact on ICI efficacy was either independent or conditioned from *KRAS* mutational status (**Table 2**). Shire et collaborators found that outcomes of *KRASmut/STK11mut* patients were similar to those in patients with *STK11mut* only, suggesting no additional deterioration in the double mutants [92]. On the contrary, other reports demonstrated that co-mutations in *STK11* and *KRAS* were associated with worse mPFS and mOS than *STK11mut* only patients treated with different regimens, including ICIs [83,101]. Still, a single-center retrospective study did not show significant differences in mPFS between *STK11mut/KRASmut* and *STK11mut* only NSCLC patients, but oppositely – among ICI-treated patients – double mutants appeared to have a better prognosis (mOS 20.7 *versus* 13.6 months, $p = 0.049$) [102]. Lastly, in a retrospective analysis which included 1261 patients treated with ICIs, *STK11mut* was found to confer resistance to ICI in *KRASmut* but not *KRASwt* NSCLC patients [103].

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Of note, the recent clinical development of KRAS G12C inhibitors (namely sotorasib and adagrasib opens new scenarios in the treatment of *KRASmut* NSCLC. Albeit limited to early evidence, *STK11* status does not affect negatively affect KRAS G12C inhibition, and *STK11* mutations have been associated to higher response rates to adegrasib [104,105].

The reshaping of TIME by KRAS G12C inhibitors has been documented in preclinical models, and the potential impact of STK11 deficiency represents a topic of crucial interest, also in view of potential treatment combinations including KRAS targeted agents and immunotherapy [106].

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Table 2. Impact of *STK11* mutations upon immunotherapy activity and efficacy in NSCLC patients according to *KRAS* status.

Cohorts of pts	N of pts	Clinical setting	Treatment	ORR	mPFS (months)	mOS (months)	Ref
STK11mut *STK11/KRASmut* *STK11*/TP53mut *STK11/KRAS*/TP53mut	18 19 18 7	Stage IV – 1st-line (Sq+NonSq)	Different treatments (51 platinum-doublet; 6 targeted-therapy; 5 ICI)	-	5.1 (p = 0.048) 2.4 4.3 (p = 0.043) 13.0 (p = 0.03)	16.1 (p < 0.001) 7.1 28.3 (p < 0.001) 22.0 (p = 0.025)	[22]
SKT11mut *STK11/KRASmut*	37 36	Stage IV	Different treatments	-	No differences	11.9 (p = 0.028) 20.3 Pts receiving ICI 13.6 (p = 0.049) 20.7	[102]
*STK11-KEAP1*wt *STK11-KEAP1*mut *STK11mut* *KEAP1*mut *STK11-KEAP1*wt *STK11-KEAP1*mut *STK11mut* *KEAP1*mut	 2276 	Stage IV – 1st-line (NonSq)	Anti PD-1/PD-L1 Platinum chemotherapy 	-	- HR 0.8 [0.48-1.47] HR 1.33 [0.93-1.9] HR 1.71 [1.2-2.45] - HR 1.07 [0.73-1.57] HR 1.32 [1.04-1.68] HR 1.53 [1.22-1.93]	- HR 0.88 [0.43-1.81] HR 1.43 [0.91-2.26] HR 1.71 [1.04-2.81] - HR 0.96 [0.61-1.52] HR 1.19 [0.89-1.6] HR 1.49 [1.14-1.95]	[99]
*STK11wt/KEAP1*wt *STK11mut/KEAP1*wt *STK11wt/KEAP1*mut *STK11mut/KEAP1*mut *STK11wt/KEAP1*wt, PD-L1+ *STK11mut* and/or *KEAP1*mut, PD-L1+	74 24 14 27 - 	Stage IV – 1st-line (NonSq)	CPP	44.6% 33.3% 28.6% 7.4% - -	- HR 0.36 [0.2-0.65], p = 0.0008 HR 0.99 [0.59-1.69], p = 0.84	- - -	[91]
DFCI/MGH cohort *KRASmut/STK11wt* *KRASmut/STK11mut* *KRASwt/STK11wt* *KRASwt/STK11mut* *MSKCC/MDACC cohort* *KRASmut/STK11wt* *KRASmut/STK11mut* *KRASwt/STK11wt* *KRASwt/STK11mut* *Combined cohort* *KRASmut/STK11wt* *KRASmut/STK11mut* *KRASwt/STK11wt* *KRASwt/STK11mut*	620 189 55 320 56 641 209 83 283 66 1261 398 138 603 122	Stage IV (NonSq)	ICI	 34.9% 12.7% 20.6% 25.0% 30.1% 10.8% 17.3% 22.7% 32.4% 11.6% 19.1% 23.7%	 6.4 1.8 2.9 3.2 3.6 2.0 2.7 2.3 4.8 2.0 2.8 2.5	 19.8 8.0 11.2 14.4 16.9 5.9 14.1 9.1 17.3 6.2 12.4 13.0	[107]
Group 1	166	Stage IV – 1st-line (Sq+NonSq)	ICI				[92]

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KRASmut/STK11mut *KRASwt/STK11wt*				-	4.1 4.4	10.0 16.3	
Group 2 *KRASmut/STK11mut* *KRASwt/STK11wt*	427	Stage IV – 2nd-line (Sq+NonSq)	ICI	-	2.2 2.8 (p < 0.005)	6.9 12.0 (p < 0.005)	
Group 3 *KRASmut/STK11mut* *KRASwt/STK11wt*	1493	Stage IV – 1st-line (Sq+NonSq)	Chemotherapy	-	4.5 5.9 (p < 0.005)	11.7 18.2 (p < 0.005)	
Group 4 *KRASmut/STK11mut* *KRASwt/STK11wt*	650	Stage IV – 2nd-line (Sq+NonSq)	Chemotherapy	-	4.4 4.3	11.3 13.2	
SU2C cohort *KRASmut/STK11mut* (KL) *KRASmut*/TP53mut (KP) *KRASmut/STK11wt*/TP53wt (K-only) *KRASmut/STK11mut* *KRASmut/STK11wt*	 54 56 64 54 120	Stage IV (NonSq)	ICI	 7.4% 35.7% 28.6% (p < 0.001) -	 1.8 3.0 2.7 (p = 0.0018) 1.8 2.7 (p < 0.001)	 6.4 16.0 16.1 (p = 0.0045) 6.4 16.0 (p = 0.0015)	[25,108]
CM-057 cohort *KRASmut/STK11mut* (KL) *KRASmut*/TP53mut (KP) *KRASmut/STK11wt*/TP53wt (K-only) *KRASmut/STK11mut* *KRASmut/STK11wt*	 6 7 11 3 17	Stage IV – 2nd-line (NonSq)	Nivolumab	 0% 57.1% 18.2% (p = 0.047) -	 2.0 5.1 2.1 (p = 0.62) 4.2 5.5 (p = 0.22)	 - -	
STK11mut/KRASmut *STK11mut/KRASwt*	14 63	Stage IIIB-IV	ICI	-	3.0 5.1 (p = 0.56)	5.3 11.4 (p = 0.13)	[83]
KRASmut *KRASwt* *KRASmut/STK11mut* and/or *KEAP1*mut *KRASmut/STK11wt/KEAP1*wt	80 74 71 235 234 226 34 38 29 46 36 42	Stage IV – 1st-line (NonSq)	ABCP ACP BCP ABCP ACP BCP ABCP ACP BCP ABCP ACP BCP	- - - -	8.1 (HR 0.42) 4.8 (HR 0.80) 5.8 8.4 (HR 0.65) 6.8 (HR 0.82) 7.0 6.0 (HR 0.49) 3.2 (HR 0.88) 3.4 15.2 (HR 0.36) 7.4 (HR 0.64) 6.9	19.8 (0.50) 11.7 (HR 0.63) 9.8 18.9 (HR 0.98) 19.5 (HR 0.90) 18.2 11.1 (HR 0.60) 7.9 (HR 0.87) 8.7 26.2 (HR 0.43) 21.0 (HR 0.43) 10.7	[100,109]

Abbreviations: ABCP, atezolizumab, bevacizumab, carboplatin, paclitaxel; ACP, atezolizumab, carboplatin, paclitaxel; BCP, bevacizumab, carboplatin, paclitaxel; CCRT, concurrent chemo-radiotherapy; CP, platinum-pemetrexed; CPP, pembrolizumab plus platinum-pemetrexed; ICI, immune checkpoint inhibitor; n, number; NonSq, non-squamous; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; pts, patients; Sq, squamous.

5. Summary

Unveiling the contribution of molecular underpinnings to cancer outcomes and therapeutic response is critical to reach the goal of personalized medicine in the current immunotherapy-driven scenario of NSCLC treatment. Through its principal substrate AMPK, STK11 acts as master kinase playing a crucial role in basic cellular processes, such as metabolism, DNA integrity, proliferation, cell polarity and angiogenesis.

STK11 loss of function is translated in defective networking within the tissue milieu and promotes cancer growth, aggressiveness, epithelial-to-mesenchymal transition (EMT) and metastases. Moreover, *STK11* mutations significantly affect immune cell function and tumor-host immune homeostasis, leading to a cold, non-T cell-inflamed, tumor immune microenvironment (TIME). The mechanistic underpinnings of *STK11*-mediated immune suppressive TIME, featured by low effector cells and PD-L1 levels together with increased tumour-associated neutrophils and altered tumour cytokine/chemokine composition, mostly reside in epigenetic silencing of stimulator of interferon genes (STING).

Hence, it is conceivable to understand how and to which extent *STK11* genomic aberrations, fostering the tumor immune escape processes, critically impact on patient outcome and response to (immuno)therapy, harbouring a predictive and/or prognostic role that needs to be deciphered. With the support of a thorough literature review, *STK11* mutations harbors a prognostic rather than predictive role in NSCLC patients treated with ICIs. In STK11-deficinent lung tumors, the development of novel potential teatment strategies aims at harnessing the immune system to turn immune-cold tumors in immune-reactive diseases, eventually improving outcomes of NSCLC patients.

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Figure legend

Figure 1. Schematic representation of *STK11*$_{mut}$ -driven inhibitory and activating molecular pathways.

The lack of activation of the key substrate AMPK resulting from STK11 deficiency is translated in multiple deranged immunomodulatory and stromal signaling consisting of:

-**impaired immune response:** through indirect SAM- or direct EZH2 and DNMT1-mediated epigenetic silencing of STING.

-**immunosuppressive TIME**: through dampening STING-triggered production of cytokines, as IFNβ, and chemokines (CXCL10, CCL5 and CCL3) which in an autocrine or paracrine fashion can activate DCs for antigen presentation, including MHC, and cross-priming of anti-tumor T cells. These changes in cytokines and chemokines milieu also result in suppressed PD-L1 expression and reduced recruitment of T and B lymphocytes, DCs and TAM. Conversely, STK11 defective mutations increases the expression of proinflammatory cytokines/chemokines (IL-1β, IL-6, G-CSF, CXCL7, CXCL3 and CXCL5) promoting the recruitment of ARG-1 secreting TAN and expansion of Tregs.

-angiogenesis and extracellular matrix (ECM) remodelling: disruption of the canonical STK11/ AMPK/ TSC 1-2/mTOR/raptor pathway, in addition to alter the metabolic status, activates HIF1α-mediated angiogenic boost, through VEGF and Ang2, and collagen cross-linking through upregulation of LOX. Unleashing of NRP1 and activation of NOX1 are two additional pro-angiogenic routes taking place from deregulation of STK11/AMPK axis.

AMPK: Adenosine mono phosphate Activated Protein Kinase; SAM: S-adenosyl methionine; EXH2: Enhancer of zeste homolog 2; DNMT1: DNA (cytosine-5)-methyltransferase 1; IFNβ: Interferon β; DCs: Dendritic Cells; MHC: Major Histocompatibility Complex; PD-L1: Programmed Death Ligand-1; TAM: Tumor Associated Macrophages; ARG-1: Arginase-1; G-CSF: Granulocyte-Colony Stimulating Factor; TAN: Tumor associated Neutrophil; Treg: T regulatory cell; TSC 1-2: tuberous sclerosis complex 1-2; mTOR: mammalian target of rapamycin; HIF1α: Hypoxia Inducible Factor α; VEGF: Vascular Endothelial Growth Factor; Ang2: Angiopoietin 2; LOX: Lysil Oxidase; NRP1: Neuropilin-1; NOX1: NADPH oxidase 1.

► activation; ⊣ inhibition; ▪▪▪▪ missed upon STK11$_{mut}$; ▬ induced by STK11$_{mut}$

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