Allegato/Annex B

PROGETTO DI RICERCA / RESEARCH PROJECT
(max 5 pagine / max 5 pages)

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Titolo del progetto / Project title	YAP\TAZ expression as prognostic and predictive biomarker in PD-1\PD-L1 checkpoint blockade immunotherapy: a snowball effect in the chessboard of advanced NSCLC treatment?
Corso di dottorato / PhD	Oncologia e Chirurgia Sperimentali, Dipartimento Discipline Chirurgiche, Oncologiche e Stomatologiche
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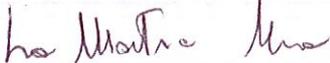
Abstract

Lung cancer remains the primary source of tumor-related deaths in both men and women worldwide. Non-small cell lung cancer (NSCLC) accounts for 80%–90% of lung cancers (1). Standard management including surgery, chemotherapy, and radiation therapy remains the most common management options for patients diagnosed with stage IV metastatic lung cancers, who benefit most of latter advancements in precision medicine. Lately, the immunotherapy revolution, specifically the development of immune-checkpoint inhibitors (ICIs), alike anti-programmed cell death receptor 1 (PD-1) and anti-programmed cell death ligand 1 (PD-L1), has dramatically altered the treatment landscape, leading to great progress in oncological therapies of thoracic malignancies. However, clinical responses to these therapies are variable being influenced by various factors. In the era of personalized medicine, biomarker-based evidence of molecular cancer pathway has become mandatory for the allocation of specific targeted therapies, as a more tailored approach to patient care. The development of predictive biomarkers is needed to optimize patient benefit, minimize risk of toxicities and guide combination approaches. Recently, the greatest focus has been on tumor-cell PD-L1 expression, despite PD-L1 testing alone is insufficient for patient selection (2). More recently, major emphasis has consequently been placed on YAP/TAZ research and the mechanisms of their regulation. The transcriptional co-regulators *Yes-Associated-Protein 1 (YAP)* and *Transcriptional coactivator with PDZ-binding motif (TAZ)* play an important role in cancer development and in multi-step metastasis process. *YAP\TAZ* are the main effectors of the Hippo signaling pathway and their key player function in sustaining proliferation, inhibiting apoptosis, promoting angiogenesis and suppressing immune system has been demonstrated in several recent studies (3). YAP/TAZ empower several of the key attributes of cancer cells, reinforce their control over the cell cycle by inducing other proto-oncogenic transcription factors, such as c-Myc. Furthermore, YAP/TAZ transcriptionally upregulate Bcl2-family members thus suppressing mitochondrial-induced apoptosis pathway, but can also overcome the alternative apoptosis cascade initiated by TNF-α _and FAS ligands (4,5).
Therefore, a non-invasive and reliable biomarker such as YAP\TAZ protein expression might be the key to a deeper understanding of the therapeutic regimens effect and the underlying molecular mechanisms of drug resistance in the interests of development successful anticancer treatments. In this scenario this study will contribute to early detection of lung cancer patients who could benefit most of immunotherapy, assessing *YAP\TAZ* protein expression as predective and/or prognostic biomarker.

Background

The advent of new therapeutic options for patients with advanced non-small cell lung cancer (NSCLC), such as anti—PD1 and anti-PD-L1 monoclonal antibodies, requires an increasing number of molecular tests for

clinically relevant biomarkers. Therefore, there is an urgent need to develop a better understanding of the immunobiology to establish methods for stratifying NSCLC patients who will respond well to immunotherapy. Over the past decade, the *Hippo signaling pathway* has emerged as a central player in regulating many aspects of tumor

biology. When the Hippo pathway is activated by upstream signals, MST1/2 kinases (homologs of Drosophila "Hippo") phosphorylate and activate LATS1/2 kinases. LATS1/2 subsequently phosphorylate transcriptional coactivators *TAZ and YAP* at key serine residues (S89 and S127, respectively) to inhibit their translocation into the nucleus, interaction with the *TEAD family* of transcription factors and activation of downstream genes (e.g. CTGF, CYR61). There is early evidence that the Hippo pathway can influence immune cell recruitment and activation as well as the anticancer immune response by its effector YAP\TAZ. After nuclear translocation, YAP and TAZ cooperate mainly with the TEAD family transcription factors to transactivate genes that regulate cell proliferation, differentiation, and apoptosis. As recent preclinical studies assess, YAP\TAZ contribute to the tumorigenesis of NSCLC, SCLC, breast cancer, malignant glioma and colorectal cancer cells by promoting cell migration, invasion, anchorage-independent growth, and epithelial–mesenchymal transition.

Recently, the critical role of the Hippo pathway in cancer development has been increasingly recognized (6-8). Dysregulated signaling by the Hippo pathway has been reported in several cancer types such as breast, liver, lung, prostate, gastric, and colorectal tumors (9-11). Extensive studies have experimentally established its tumor suppressor function. For example, STK3/STK4 loss in a mouse liver leads to uncontrolled cell proliferation and differentiation (12,13) and YAP/ TAZ overexpression leads to tissue overgrowth and cancer. Moreover, the prognostic impact of YAP\TAZ was verified on the protein level in 4 independent cohorts, comprising altogether 130 evaluable SCLC cases and presented at ASCO Annual Meeting 2020, demonstrating that SCLC-Y subtype is enriched for T-cell inflamed phenotype and long-term survival, and may predict for clinical benefit of immunotherapy (14).

So far, a comprehensive molecular portrait of the Hippo pathway and its effectors YAP\TAZ in cancer has not been characterized, resulting in important knowledge gaps for utilizing this pathway in cancer medicine and new oncology treatments.

As a proof-of-concept, based on these evidences our final goal is to test whether the impact of YAP\TAZ mRNA transcript levels were translated into protein expression differences which could be assessed as predictive and/or prognostic biomarkers by IHC and liquid biopsy in routine clinical diagnostics.

Main objective

This proof of principle study aims to elucidate the diagnostic accuracy of YAP\TAZ in tissue-blood matched samples of treatment-naive lung cancer patients. This explorative study, including the systematic assessment of tumor biopsies at baseline and the monitoring of treatment-induced changes in blood profile, would provide new key information on the potential biomarkers of early progression detecting strategies to overcome drug resistance. Additionally, we will investigate the YAP\TAZ expression level and, in case of pathway deregulation, analyze the potential epigenetic aberrant methylation of the promoter region of these genes.

Secondary objectives

- o To evaluate the role of deregulated YAP\TAZ as potential biomarker stratifying patients for ICIs treatment response in lung cancer

- o To evaluate a comprehensive molecular characterization of patients with lung cancer by a novel multiplex digital colour-coded barcode hybridization technology (NanoString Technologies) for mRNAs and ncRNAs (miRNAs, siRNAs, rRNAs, etc.)

Materials and Methods

Primary endpoint

I. Prospectively evaluate the assessment of YAP\TAZ in lung cancer tumor tissue specimens and in plasma cfDNA at baseline and during oncological treatment

II. Compare tumor tissue molecular analysis with cfDNA by liquid biopsy validating the results in terms of sensitivity, specificity, negative and positive predictive power.

Secondary endpoints

I. Correspond median Overall Survival (OS), median Progression Free Survival (PFS), Time To Progression (TTP) and Overall Response Rate (ORR) to the expression level of YAP\TAZ in tumor tissues and peripheral blood of NSCLC and SCLC patients undergoing ICIs treatments

II. Correlation of YAP\TAZ expression with tumor features such as organ-specific site of metastatic spread

Inclusion criteria

o Signed written informed consent
o ECOG PS ≤ 2
o Patients with histologically- or cytologically- confirmed NSCLC and SCLC
o Stage III B-C/Stage IV disease (according to 8th edition of IASCL TNM staging system)
o Treatment-Naïve pts, eligible for anti-PD-1\PD-L1 therapy

Exclusion criteria

o ECOG PS ≥3
o > 1h from blood withdrawal and plasma recovery
o Prior systemic oncological treatment

Study Design

This is a prospective cohort study including patients with advanced NSCLC who are treatment-naïve and are candidate to receive ICIs regimens. Furthermore, we will also analyze the molecular profile of the tumor to demonstrate whether or not there is concordance between plasma cfDNA and tissue DNA and RNA mutation profile.

Tumor and adjacent normal tissue will be obtained by systematic biopsy at baseline and stored as formalin-fixed paraffin- embedded (FFPE) sample. Paired whole blood samples will be collected (in K2 EDTA tubes) before and at 1 and 3 months during the course of treatment, according to a standardized protocol and stored frozen. Clinical parameters will be also collected before and at 1 and 3 months during the course of the treatments.

Firstly, YAP\TAZ and other related genes alterations (EGFR, ALK, ROS-1,BRAF, KRAS, *PI3KCA, HER-2, ERRC1, MET, RET, NRAS, PT53, TMB, PDGFR*) will be assessed at baseline using a targeted NGS assay lung cancer mutation panel kit in tumor tissue samples, validating and correlating the data in plasma cfDNA analysis throughout time.

Secondly, specific proteins (such as PDL-1, PD-1, TIM-3, VEGF, IDO, TNF-α and TGF-β) will be evaluated using specific ELISA assays in plasma as potential prognostic and/or predictive biomarkers of drug resistance in NSCLC and SCLC patients who have experienced clinical and/or radiological progression of disease; likewise, expression of specific proteins (such as integrins) will be evaluated for eventually dictating organ-specific metastasis.

Lastly, material from both RNA and protein preparations will be combined for hybridization with nCounter probes (NanoString Technologies) and digital imaging, in order to develop a YAP/TAZ target gene signature exhibiting consistent prognostic power. Indeed, when applied to biological samples, nCounter RNA Protein profiling offers information on both RNA and protein expression using the same digital platform. Such tools enable stratification of patients based on the expression profiles of immune-related genes and may facilitate the identification of better immunotherapeutic adjuvants, such as novel combinations of checkpoint inhibitors. Expression data from this panel has potential to promote a better understanding of the interactions between the host immune system and tumor microenvironment and to identify novel methods to predict and improve patient

outcome. Unfortunately, the increasing numbers of biomarkers that are needed for a comprehensive molecular characterization of patients with lung cancer are frequently difficult to perform by sequential single gene mutational assays using the small amounts of nucleic acids extracted from a cytological smear.[4][6] This limit can be overcome by multiplex digital color-coded barcode hybridization technology (NanoString Technologies, Seattle, Washington, USA) which gives the possibility to simultaneously analyze in a single-tube multiplexed fashion a broad spectrum of clinical relevant biomarkers on different biological specimens, across all levels of biological expression. This approach provides a method for direct detection of targets with fluorescent molecular barcoded probes without the need of reverse transcription and/or amplification.

Finally, YAP\TAZ mRNAs, ncRNAs and proteins data will be normalized and compared to healthy controls. All the entire analysis will be performed in the Lab of Prof. Antonio Russo (Section of Medical Oncology, Palermo, University Hospital).

Statistical analysis

A total of 80 patients will be considered for this pilot study. This number can be considered appropriate according to both logistic/timeline issues. Sample size is in accordance with the systematic review of Billingham SA et al. (15), in which the median number of patients enrolled in pilot and/or feasibility studies is around 30 and a number of 80 as planned is at the upper quartile or beyond. The Mann–Whitney U test will be used for intergroup comparisons of two independent samples, while the Fisher's exact test for categorical values. A paired Wilcoxon test will be used to compare the median cfDNA plasma levels before and after therapy with nivolumab. Survival analysis on the basis of the median cut-off values will be performed using the Kaplan–Meier method, providing median and p values, with the use of the log-rank test for comparisons. Univariate and multivariate analyses will be performed using the Cox proportional hazards and logistic regression models. A p value < 0.05 will be considered significance. All the statistical analyses will be performed using SPSS statistics software, version 20 (IBM, Armonk, NY, USA).

Timeline

Months				YEAR 1													YEAR 2												YEAR 3						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Patients' enrollment																																				
Samples collection																																				
Scientific Literature Update																																				
Bioinformatic analysis																																				
Statistical analysis																																				
Article publication																																				

WP1: Patients' enrollment
WP2: Samples collection
WP3: Scientific literature update
WP4: Bioinformatic analysis
WP5: Statistical analysis
WP6: Article publication

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