RESEARCH PROJECT

Dottorato di Ricerca (XXXIV ciclo) in **"Oncologia e Chirurgia Sperimentali"**
Università degli Studi di Palermo
(Dott. Valerio Gristina)

Project title:

Using exosomes as prognostic and predictive biomarkers to more comprehensively characterize and overcome resistance to standard treatments in advanced NSCLC patients: many a mickle makes a muckle?

Background:

A personalized therapeutic approach based on the detection of activating mutations in the kinase domain of the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) rearrangements correlated directly with sensitivity to EGFR tyrosine kinase inhibitors (TKIs) and ALK inhibitors, respectively. In addition, other important driver mutations found in NSCLC included kirsten rat sarcoma virus (KRAS), B-Raf proto-oncogene (BRAF) and ERBB2 (HER2) mutations, c-ros oncogene 1 (ROS1) and RET genes rearrangements, and MET amplifications. Among these, *ROS1* rearrangements as well as *BRAF* mutations have targeted therapies approved by international regulatory authorities(1). To date molecular testing is recommended as part of broad mutational analyses including all these oncogenic driver alterations with the final goal of identifying specific targets for which tailored agents are available in the context of clinical trials. So far both first- (erlotinib, gefitinib) and second-generation (afatinib) EGFR TKIs have been the standard first-line treatment options. Unfortunately, almost all patients would eventually develop drug resistance over time through secondary acquired mutations. Accordingly, a third-generation EGFR TKI (osimertinib) has been designed to target the T790M mutation which has resulted to be the most common mechanism of acquired or de novo resistance to TKIs (accounting for approximately 50-60% of pre-treated patients and with a variable frequency in treatment-naive patients) (2) showing improved efficacy in treated and, only recently, untreated EGFR mutated patients(3); conversely, other mechanisms of resistance (including HER-2 and/or MET amplification, PIK3CA and/or BRAF mutation, small cell lung cancer transformation)(4) were revealed to be relatively heterogeneous, only rarely occurring concurrently with T790M mutation and showing a much lower incidence. However, obtaining tissue re-biopsies for molecular analysis has resulted to be challenging in up to 50% of cases either due to patients comorbidities or insufficient and heterogeneous tumor tissue sampling(5), claiming for the enthusiastic development of noninvasive methods for molecular testing such as so-called liquid biopsy(6). Recently, the Food and Drug Administration (FDA) approved the first liquid biopsy companion diagnostic which relies on the molecular analysis of circulating cell-free DNA (cfDNA, released through cell death mechanisms such as necrosis and apoptosis in biofluids) using next-generation sequencing (NGS) technology. As a matter of fact, a primary challenge for the detection of circulating tumor DNA (ctDNA), the component of cfDNA released from tumor sites into the blood of cancer patients, has proved to be the low allelic frequencies mutation and low copy numbers of the mutation target. Thus, providing high specificity but only intermediate sensitivity as an alternative molecular test, a negative result of cfDNA does not represent tumor negativity for EGFR mutation and a tissue re-

biospy may be still necessary. In the era of personalized medicine, emphasis has been recently placed on exosomes for their role in facilitating early detection and diagnosis while improving treatment outcomes. Exosomes have been defined as 30-100 nm extracellular stable vesicles consisting of nucleic acids and proteins, released by a wide range of living cell types and found in various bodily fluids, participating in communication between cells and thereby contributing to tumor growth, metastasis, angiogenesis and drug resistance. Hence, mutations such as T790M can be found using a single step isolation of both cfDNA and exosomal RNA/DNA (exoNA), so combining these sources of nucleic acids would increase sensitivity reducing the number of false negatives and generating approximately 10-fold more copies of both T790M and EGFR activating mutations. In addition to the increased sensitivity of mutation detection, it has been shown that mutations on exoNA would correlate better to overall survival compared with cfDNA mutation analysis(7) whereas exosomal proteins (e.g. integrins) uptaken by organ-specific cells would prepare the premetastatic niche and therefore could be used to predict organ-specific metastasis(8). Likewise, circulating microRNAs (18-25 nucleotides small non-coding RNAs [miRNAs] that may play important roles in gene regulation) have been described as ubiquitous players involved in early NSCLC diagnosis. Several studies have reported that, following the release of exosomes by NSCLC cells during cisplatin stimulation, the sensitivity of cells to cisplatin was decreased, probably mediated by the exchange of microRNAs between exosomes via cell□to□cell communication(9). Unlike circulating miRNAs and cfDNA, an important feature of exosomes is that nucleic acids in the lumen of these vesicles are protected from nucleases present in plasma and other biofluids by a lipid bilayer membrane, which allows for isolation of intact and good quality RNA, DNA and proteins. Furthermore, a growing body of evidence highlighted the enrichment of miRNAs with important roles in tumorigenesis in exosomes (exomiRs, such as miR-21 and miR-24) when compared to circulating plasma in NSCLC patients(10). Moreover, another paradigm shift in the treatment of NSCLC has been the introduction of immunotherapy with immune checkpoint inhibitors (ICIs) that disrupt co-inhibitory T-cell signalling such as programmed cell death-1 (PD-1) or its ligand PD-L1, showing a clear clinical benefit with increased overall survival (OS) versus standard chemotherapy, both in the second-line setting (nivolumab, pembrolizumab and atezolizumab) and as first-line treatment in PDL-1>50% selected patients (pembrolizumab)(11). Albeit durable responses and improved survival rates have been commonly observed suggesting a long-lasting immunologic memory in large subsets of patients treated with ICIs, some patients would experience primary or secondary immune escape which has not been comprehensively explored and seemed to differ from the traditional static drug resistance of most anticancer therapies. Namely, NSCLC appeared to present more frequently with specific immune-inflamed findings(12). Indeed, escape mechanisms that target the tumor cell recognition and killing in the final steps of cancer immunity cycle are hypothesized to apply to inflamed malignancies. Notwithstanding, combining agents that block cancer-derived soluble factors inhibiting T-cell priming (such as IL-10, VEGF, prostaglandins, TGF-β, adenosine, and indoleamine 2,3-dioxygenase [IDO]) signaling with PD-L1:PD-1 inhibition have shown additive antitumor activity in preclinical models(13). To date, a crucial immune escape mechanism in immune-inflamed tumors seemed to be the adaptive expression of PD-L1 on neighboring tumor-infiltrating immune cells and, in some cases, on tumor cells, induced by to the local production of IFN-γ by CD8+ T cells after the recognition of cancer-associated antigens(14) and therefore serving as a negative feedback regulator of cytolytic activity. Intriguingly, other mechanisms of immune escape considered in these tumors have focused on the reduced recognition of cancer cells by immune cells

(loss, downregulation or alteration of the MHC-I) along with the coexpression of additive inhibitory checkpoints (B- and T-lymphocyte attenuator, lymphocyte-activation gene 3 protein [LAG-3], T-cell immunoglobulin domain, mucin domain-3 [TIM-3], and the recently described T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain [TIGIT]) and the presence of immunosuppressive cells in the tumoral microenvironment (tumor-supporting M2-like macrophages, Myeloid-derived suppressor cells [MDSCs], TGF-β, IDO)(15).

Main objective:

This study aims to further elucidate the role of exosomal nucleic acids (in particular mutational DNA), miRNAs and proteins as new and reliable biomarkers when compared to cfDNA and tissue biopsy in treatment-naive NSCLC patients and in NSCLC patients who have received first-line treatment based on chemotherapy, EGFR TKIs and ICIs. This explorative study, including the systematic assessment of tumor biopsies at baseline and the monitoring of treatment-induced changes in blood profile, would provide key information on the potential biomarkers of early progression identifying strategies to overcome drug resistance.

Secondary objectives:

- To investigate the role of alterated expression level of specific exosomal miRNAs (exomiRs) and proteins as potential biomarkers of early progression in NSCLC;
- To evaluate if specific exosomal proteins would correlate with metastatic propensity and/or predict likely sites of metastasis in NSCLC patients.

Materials and Methods

Primary endpoint:

To compare tumor tissue molecular analysis with cfDNA and exosomes from peripheral blood validating the results in terms of accurate diagnostic concordance

Secondary endpoints:

- Time to progression (TTP), to be correlated with the expression level of exosomal miRNAs and proteins in tumor tissues and peripheral blood of NSCLC patients undergoing standard treatments, for the identification of potential biomarkers of drug resistance;
- Correlation of exosomal proteins with known NSCLC tumor features such as metastatic propensity and organ-specific site of metastatic spread

Main inclusion criteria:

Signed Written Informed Consent; Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) of ≤ 2; Patients with histologically- or cytologically-documented NSCLC with Stage IIIB/Stage IV Disease (according to Version 8 of IASCL TNM) or with recurrent or progressive disease following multimodal therapy (radiation therapy, surgical resection, or definite chemoradiation therapy for locally advanced disease) who are treatment-naive and eligible for chemotherapy-based regimens, EGFR TKIs and ICIs;

Main exclusion criteria:

Patients with ECOG PS ≥ 3; Patients who received prior therapy with ICIs or EGFR TKIs.

Study design

This is a prospective cohort study including patients with advanced NSCLC who are treatment-naive and are candidate to receive chemotherapy-based regimens, EGFR TKIs and ICIs. Tumor 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. Tumor mutations will be assessed by lung cancer mutation panel kit. Clinical parameters will be also collected before and at 1 and 3 months during the course of the treatments. Initially, EGFR mutation analysis (also including T790M) and other related gene alterations (ALK, *PI3KCA, HER-2, KRAS, ERRC1, BRAF, MET, RET, ROS-1)* will be tested at baseline using a targeted NGS assay lung cancer mutation panel kit in tumor tissue samples, validating and monitoring the data in plasma exosomes and cfDNA analysis throughout time. Secondly, EGFR T790M mutation will be analyzed in patients who are suspicious for first-line EGFR TKIs-resistance using droplet digital PCR in plasma exoNA (exosomal DNA and RNA along with present cfDNA) that will be co-isolated from the samples using Exolution Plus extraction technology (Exosome Diagnostics). Thirdly, alterated expression level of exosomal miRNAs will be evaluated using The TaqMan® Array Human MicroRNA Card Set v3.0 platform in plasma exoNA as potential prognostic and/or predictive biomarkers of drug resistance in NSCLC patients who have experienced clinical and/or radiological progression of disease. The quality yield and distribution of miRNAs were analyzed using the Agilent 2100 bioanalyzer with Small RNA Chips. Lastly, specific exosomal proteins (such as PDL-1, PD-1, TIM-3, VEGF, IDO and TGF-β) will be evaluated using specific ELISA assays in plasma exoNA as potential prognostic and/or predictive biomarkers of drug resistance in NSCLC patients who have experienced clinical and/or radiological progression of disease; likewise, expression of specific exosomal proteins (such as integrins) will be evaluated for eventually dictating organ-specific metastasis. Finally, exosomal miRNAs 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.(16), in which the median number of patients enrolled enrolled in pilot and/or feasibility studies is around 30, and a number of 80 as planned is at the upper quartile or beyond.

Timeline:

Time	Work Packages	Activites
1ST YEAR	WP1-WP2	Scientific Literature Update, Patients' enrollment, Sample collection
2ND YEAR	WP3-WP4	Scientific Literature Update, Patients' enrollment, Sample collection

3RD YEAR	WP5-WP6	Data analysis, Article Publication

Future Perspectives and Public Health Benefit

While a fraction of NSCLC patients who have not previously received a given treatment fail to respond (intrinsic or innate resistance), the vast majority of patients subsequently become refractory and eventually succumb to disease progression even if after an initial response (secondary or acquired resistance). Therefore, using noninvasive and reliable biomarkers such as exosomes could be the key to a deeper understanding of the therapeutic regimens effect and of the underlying molecular mechanisms of drug resistance in the interests of the development of successful anticancer treatments. In this scenario this study will contribute to early detection of NSCLC patients who have experienced drug resistance to conventional treatments favoring the identification of exosomes as potential prognostic and/or predictive biomarkers which may be used by clinicians to select patients for new treatment strategies.

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