Allegato/Annex B

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

Cognome/Surname	PERI
Nome / Name	MARTA
Titolo del progetto / Project title	Liquid NET: Prognostic and predictive role of molecular alterations in circulating tumor DNA in NET
Corso di dottorato / PhD	ONCOLOGIA E CHIRURGIA SPERIMENTALI/ EXPERIMENTAL ONCOLOGY AND SURGERY
Firma del candidato/ Applicant's signature	

1 - Sommario / Abstract

Liquid biopsies have gained increasing interest in the growing era of precision medicine. The integration of liquid biopsy platforms in neuroendocrine neoplasms (NENs) has been increasingly studied in recent years.
NEN represent a heterogeneous group of malignancies varying in biology and behaviour.
Genetic alterations in the mTOR pathway have been identified in NEN, providing a rationale for treatment with the mammalian target of rapamycin (mTOR) inhibitor.
Everolimus, a mTOR inhibitor, has shown effective in delaying progression of advanced well-differentiated NENs, but with a limited long-term efficacy, possibly due to a compensatory activation of the PI3K/AKT signalling.
To date few clear biomolecular predictive factors to Eve have been reported in NENs.
In this project we will focus on PIK3CA/Akt/mTOR pathway genomic alterations in circulating tumour DNA from advanced well differentiated NEN, comparing with tumor tissue analysis.
Moreover we will evaluate the clinicopathological significance and predictive role of PIK3CA/Akt/mTOR pathway alterations assessed both in tumor tissue and in liquid biopsy.

2 - Descrizione del progetto / Project

Background

NEN population
Neuroendocrine neoplasms (NENs) are rare tumors with an extremely variable behaviour and lacking established standard of care. These malignancies can arise from almost any endocrine cell in the body but predominantly originate from the gastro-entero-pancreatic (GEP-NENs) or bronchopulmonary tract. The NEN classification is mainly based on morphology into well-differentiated neuroendocrine tumours (NETs) and poorly-differentiated neuroendocrine carcinomas (NECs) (always G3). The high-grade forms are characterized by a proliferation rate (Ki-67) >20% and encompass a heterogeneous population of tumors with different biology, genetics and consequently treatments. For this reason, the WHO 2019 classification has established a separation of well-differentiated GEP-NET G3 from GEP-NEC population (1, 2).
The vast majority, 70–90%, of well-differentiated NETs tend to overexpress somatostatin receptors (SSTRs), which have been used as a specific marker in nuclear medicine imaging.
The management of NENs is complex and variable. In advanced disease treatment options include somatostatin analogues (SSAs), chemotherapy, peptide receptor radionuclide therapy (PRRT) and target therapy, including everolimus (EVE) and sunitinib.

Liquid biopsy in NEN
Liquid biopsies provide an easily accessible approach with increased ability to capture spatial and temporal intra-tumour heterogeneity compared to tumour tissue.

Blood-based assays for tumour diagnosis, screening and monitoring are therefore attractive, as they are faster to obtain, less invasive and can aid molecular profiling for therapeutic targets, monitor disease status and determine response to treatment in 'real time.'
Current treatment for patients with a NEN diagnosis has not yet been tailored to select patients on the basis of molecular alterations.
Minimally invasive liquid biopsy tests for advanced NENs and recent data from studies of the NETest have shown successful detection of tumor-specific transcripts in circulating RNA. A recent meta-analysis shows an accuracy of 90.2%–93.6% as a marker of natural history of
NET (3).
Circulating tumour (ct) DNA analysis has been shown to be feasible and demonstrated promise in identifying therapeutic targets in patients with NENs according to several case reports.
With the complexity of the classification, novel biomarkers are required to assist in clinical decision-making and ultimately improve patient outcomes. Identification of biomarkers that could be used to guide targets for therapy is an unmet need.
Recently, there has been an increasing interest in circulating tumor DNA (ctDNA) on the basis
of studies performed in a range of other cancers (4).
In a study conducted by Gleeson et al, alterations in mammalian target of rapamycin (mTOR) pathway genes were found to be the most frequent molecular events identified in pancreatic NET, together with MEN1, but it remains unclear whether these biomarkers possess predictive value (5).
In Zakka et al. were among the first to perform a population-based study to characterise
genetic alterations in patients with NENs using ctDNA (6). The most common mutations in circulating tumour DNA were found in TP53, KRAS, EGFR, PIK3CA (11%), BRAF, MYC, CCNE1 and PTEN (5%), demonstrating that ctDNA analysis is feasible in NEN patients and useful for longitudinal disease monitoring.
Current evidence of the utility of ctDNA in the management of patients with NENs is at an early stage, and it is worth noting that pathological alterations are more commonly detected in
NECs than in well-differentiated disease.
The CIRCAN-NEC pilot study investigated ctDNA mutations in the blood samples of 24 patients with a diagnosis of NEC, to assess the sensitivity of ctDNA in characterising genetic alterations, and their value in predicting response to chemotherapy. Preliminary results from the published abstract have demonstrated mutations in TP53, RB1, and KRAS, and suggested that ctDNA analysis is sensitive (7).
Although ctDNA is promising as a liquid biopsy and can identify targetable mutations, this is yet to be validated. Further large scale studies are recommended to assess the role of ctDNA in the management of patients with a NEN diagnosis, especially as predictive biomarkers (8).

mTOR pathway in NEN
mTOR is a conserved serine/threonine protein kinase and is a member of the family of the
PI3K-related kinases. As the name itself suggests, mTOR is inhibited by rapamycin (or sirolimus), a macrolide produced by Streptomyces Hygroscopicus, named after Rapa Nui (native language for Easter Island).
mTOR acts as the catalytic subunit of two large complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). These complexes have different upstream and downstream interactions, reflecting distinct roles in regulation of cell functions (9).
Everolimus is an oral mTOR inhibitor that selectively blocks mTOR complex (mTORC)-1, leading to an increase in mTORC-2 activity. This results in a positive feedback activation of AKT by phosphorylation on Ser473, and an inhibition of S6K negative feedback (10).
In the last decade, molecular studies pointed to several targets of the PI3K/Akt/mTOR pathway in NENs. Shah et al. found that, respectively 76 and 96% of 98 NENs tissues analyzed by IHC display constitutive AKT phosphorylation and activated ERK, a downstream target (8). Missiaglia et al. (11) demonstrated that the expression of two endogenous inhibitors of the mTOR pathway, PTEN and TSC2, were downregulated in a large proportion of tumors, respectively 35 and 60% of cases.
The activation of mTOR pathway, demonstrated by both p-mTOR and p-S6K positive IHC staining, was present in 33% and predict better outcomes in patients with NET treated with Eve (12).
Jiao et al. analyzed the exomic sequences of 10 sporadic panNENs and screened the most frequently mutated genes in 58 pancreatic NENs. Notably, 15% of the tumors showed mutations in mTOR pathway-related genes (the oncosuppressor PTEN, the negative regulators TSC2, and PIK3CA, and the catalytic subunit of phosphatidylinositol 3-kinase) (13).
In 2017 Scarpa et al. published a study on the whole genome sequencing of 98 pancreatic NETs in which they confirm the mTOR pathway activation in 15% of the analyzed samples. They identified mTOR pathway inhibitors alterations such as PTEN mutations (7.1%), TSC1 or TSC2 (2%) (14).
Inactivating mutations on negative regulators of the mTOR pathway (i.e., PTEN, TSC1, TSC2 and DEPDC5) were mutually exclusive, strengthening their role of driver mutations in panNETs (9).
The mammalian target of rapamycin (mTOR) is part of the PI3K/Akt/mTOR signaling pathway which has a central role in the oncogenesis of NETs.
In other malignancies, sensitivity to everolimus seems to be related to presence of TSC1/TSC2/MTOR alterations in tumor samples regardless of histology (15).

Previous studies have shown that the presence of certain PI3K and KRAS mutations may influence breast cancer cells' response to everolimus. Meanwhile, deletion of the KRAS mutation restores sensitivity to the therapy (16). Studies examining molecular predictors of response to mTOR inhibition also demonstrated increased efficacy in tumors with a higher percentage of phosphorylated (i.e., activated) mTOR, as assessed by immunohistochemistry (17, 18).
Other preclinical data have suggested that PIK3CA and PTEN aberrations and high p-Akt levels can predict sensitivity to rapamycin in NET cell lines, and this correlation was confirmed in NET patients (19).
Data from mutations in the mTOR pathway analyses could allow researchers to design confirmatory prospective clinical trials in which biomarkers are used to predict responses to mTOR inhibitors in NETs, eventually leading to better selection of patients for this type of treatment (20).

Objectives and expected results of the research project

The general aim of our study is to prospectively investigate novel circulating biomarkers in NET patients to demonstrate that ctDNA might be a novel alternative to tissue biopsies for molecular profiling, facilitating the future applicability.
The primary endpoint is to assess a descriptive comparison between PIK3CA/Akt/mTOR pathway genomic alterations detection in tumor tissue and in ctDNA.
The secondary endpoint is to explore the predictive role of PIK3CA/Akt/mTOR pathway alterations in ctDNA/tumor tissue and in IHC in NET patients treated with everolimus.
We will also monitor PIK3CA/Akt/mTOR related biomarkers during the course of therapy and correlate with clinicopathological characteristics and treatment outcomes, exploring the possible prognostic and predictive role of this biomarker in NET population.

Materials
Inclusion criteria
We will include patients with histological diagnosis of NET with different primary origins in accordance with WHO 2019 classification and distant metastases (metachronous or synchronous) or locally advanced disease. Patients with diagnosis of well-differentiated NEN (including NET G3) will be included. Only patients with a measurable disease at baseline will be included.

Exclusion criteria
Patients with diagnosis of poorly differentiated (NEC), non-neuroendocrine malignancies,Non metastatic NET, mixed neuroendocrine-non neuroendocrine neoplasms (MiNEN) will be excluded. Patients with early stage, no radiological evidence of disease or a second cancer other than NET will be excluded.

Methods
Blood samples will be collected independently of line of therapy, before the cancer treatment, at the time of the best radiological response and at the time of disease progression.
Demographic, pathological and clinical information will be collected from the medical record and then they will be included into an anonymized database. A focused analysis will be performed in patients that receive everolimus.
All samples will be obtained after receiving written informed consent from the patients.

Analysis on tumor issue
Representative tissue FFPE blocks will be selected for IHC and slides will be read out for percentage of positive tumor cells.
Immunoistochemical (IHC) staining score well be performed from formalin-fixed, paraffin-embedded (FFPE) material.
The IHC profile will be used for PTEN, pAKT (Ser473), pmTOR (Ser2448), p70S6-kinase (S6K), p4-EBP1, TSC2 (9).
Tumor DNA was then extracted from microdissected tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.
Moreover we will search for PIK3CA (exons 7, 9 and 20), PTEN and TSC2 mutations by PCR-based direct sequencing.

ctDNA analysis
Blood samples will be sent to the laboratory within six hours after collection. Total blood samples were shipped by road at room temperature. These samples were centrifuged for 10 minutes at 1600 g and the cell pellet was discarded. The supernatant was then centrifuged at 6000 g for 10 minutes and the resulting plasma was stored in 2 ml cryotubes and kept at -80°C until the next use.
To determine the concentration of DNA, two blinded independent complementary assays were carried out. First, the quantification of double-strand DNA was assessed using a Qubit 2.0 Fluorometer and the

Qubit dsDNA HS Assay Kit (Life Technologies, Q32854, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration was expressed in ng/mL and then converted to ng/µL. We also quantified amplifiable DNA by quantitative PCR (qPCR) for PIK3CA (exons 7, 9 and 20), PTEN and TSC2 mutations using the Quantifiler Human DNA Quantification Kit (Applied Biosystems, PN4344790F, Foster City, CA, USA) according to the manufacturer's instructions, with the hTert gene (human telomerase reverse transcriptase).

Statistical Analysis

A total of 30 patients will be considered for this pilot study. This number can be considered appropriate according to the prevalence data of these rare tumors and to timeline issue.

The match between ctDNA and tissue was defined as the total number of matching alterations with the denominator being the total number of alterations detected in paired patient samples.

The overall percent agreement between ctDNA mutation and IHC profile on tumor tissue was defined as the number of concordant cases divided by the total number of evaluated cases.

Response rate (RR), overall survival (OS) and progression-free survival (PFS) will be used to evaluate the treatment outcomes. RR was defined according to response evaluation criteria in solid tumors (RECIST) version 1.1.

The OS will be defined as the time interval from date of advanced disease diagnosis to death from any cause or to last date of follow-up. The PFS will be calculated from the start of treatment to the date of radiological disease progression or death from any cause.

Patients will be separated in two groups: PIK3CA/Akt/mTOR pathway alterations positive (group 1) and and PIK3CA/Akt/mTOR pathway alterations negative (group 2).

The difference between the categorical variables will be calculated using Fisher's exact test, while Student's t-test was used for continuous variables.

The survival curves will be estimated using the Kaplan–Meier method, providing median and p values, with the use of the log-rank test for comparisons. Univariate and multivariate analysis for the most significant variables will be performed using a logistic regression model. All analyses will be considered as statistically significant with a p value ≤0,05. Statistical analyses will be performed using the SPSS Statistic software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

Timeline

The enrolment phase will last 30 months, the data will be analysed in 6 months (see Gantt chart).

N°	Project activities	MONTHS																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Patients' enrolment																		
2	Blood sample collection																		
3	Tissue sample analysis																		
4	Data collection from the medical record																		
5	Blood sample analysis																		
6	Scientific literature update																		
7	Bioinformatic analysis																		
8	Statistic analysis																		

N°	Project activities	MONTHS																	
		19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
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Expected results/outcomes

- We want to facilitate the future applicability of liquid biopsy in NET setting
- We aim to demonstrate the feasibility and reliability of ctDNA analysis for PIK3CA/Akt/mTOR pathway alterations
- We hypothesize that PIK3CA/Akt/mTOR pathway genomic alterations, both in tumor tissue and in ctDNA, have a predictive role for prognosis and response to targeted therapies

3 - Bibliografia / References

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