Allegato / *Annex B*

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

Borse DM 351/2022 **Scholaships DM 351/2022** Vedi scheda del corso e art. 2 del bando. *Refer to PhD Sheet and art. 2 of the Call.*	Selezionare una sola opzione. *Select only one option:* [ ]PA [ ]PC [ ]PNRR [ ]TDA
Borse DM 352/2022 **Scholaships DM 352/2022** Vedi scheda del corso e art. 3 del bando. *Refer to PhD Sheet and art. 3 of the Call.*	Indicare il codice della borsa DM 352/2022 per la quale si intende partecipare. *Insert the code of the DM 352/2022 scholarship to apply.* [PNNR._____]
Borse di Ateneo su tema vincolato ***Scholarships on specific research topic*** Vedi scheda del corso. *Refer to PhD sheet.*	Indicare il codice della borsa di ateneo con tema vincolato per la quale si intende partecipare. *Insert the code of the University scholarship on specific research topic:* [BA. _____]

Cognome / Surname	Cucinella
Nome / Name	Giuseppe
Titolo del progetto / Project title	Clinical evaluation of digital PCR to further stratity endometrial cancers with no specific molecular profile (NSMP)
Corso di dottorato / PhD	ONCOLOGIA E CHIRURGIA SPERIMENTALI
Firma del candidato/ Applicant's signature	

1 - Sommario / Abstract

ABSTRACT

Endometrial cancer (EC) is classified by molecular mutations in four groups according to the TGCA classification. Molecular classification identifies >50% of ECs as having 'no specific molecular profile/ NSMP' without mismatch repair deficiency, p53 abnormalities, or pathogenic POLE mutations. However, the NSMP group is characterized by either genetic or clinical heterogeneity lacking molecular specific signatures. Indeed, there is a need to classify further the NSMP based on new molecular features to understand the prognostic significance better and tailor the treatment. Recently, digital PCR has become a cost-effective and more accessible methodology able to assess the tumor genetic landscape. In this scenario, this study aims to further characterize the molecular profile of the EC among the NSMP group. Thus, we propose to perform additional molecular analysis of the most common hotspot genes using dPCR technique within the NSMP EC patients. Additionally, oncological outcomes of the molecular subgroups will be explored. This project might have a significant impact on clinical practice by further identify the EC patients with specific molecular targets for novel therapeutic approaches and ongoing clinical trials. We also anticipate that introducing the dPCR as a tool for the sub-stratification of NSMP EC patients will help the routine implementation of the molecular classification system.

2 - Descrizione del progetto / Project

BACKGROUND

Endometrial cancer (EC) is the most common gynecological cancer in high-income countries, and its incidence is rising globally [1]. Although most women with early-stage EC have a favorable prognosis, approximately 20% of patients have one or more high risk features associated with an increased risk of cancer-related death. Thus, accurate risk stratification is necessary to determine EC patients' eligibility for further treatment after the staging surgery [2]. A new molecular classification system defined by the Cancer Genome Atlas (TCGA) categorizes EC into four groups according to molecular profile, which includes ultramutated tumors with POLE exonuclease domain mutations, hypermutated tumors with microsatellite instability (MSI)/ DNA mismatch repair (MMR) deficiency, copy number-high tumors with TP53 mutations, and copy number-low tumors, which lack all of the alterations mentioned above [3]. Follow-up studies developed and validated a molecular classification system using surrogate markers, Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE), based on immunohistochemistry expression of p53 protein and mismatch repair (MMR) proteins and POLE sequencing [4-6]. According to this surrogate approach, tumors within the p53 wild-type group, which do not harbor mutations of neither POLE, MMR genes, nor abnormal p53, are also referred to as tumors with "No Specific Molecular Profile, NSMP" [4, 7].

ECs NSMP are the most common molecular subtype, accounting for approximately 50 % of all ECs [4]. Although the NSMP shows low-risk pathologic features (87% FIGO stage I, 93% low grade, 99% endometrioid histotype), the outcomes appear heterogeneous. The prognosis ranges from good to poor (poorer than the POLE-mutated group but similar to the MMR deficiency group) [4, 5, 8]. In this scenario, ECs NSMP remain the most challenging molecular subtype lacking a univocal prognostic significance. Indeed, the lack of molecular signatures does not allow identifying patients with a higher risk of recurrence who may benefit from more aggressive therapy. Yet, the behavior of ECs NSMP might be affected by other molecular features which are not included in the TCGA classification. This EC subgroup represents a heterogeneous set of tumors harboring mutations in PTEN, CTNNB1, and PIK3CA [3] and 1q32.1 amplification [9] with variable disease outcomes [10]. For example, carcinomas carrying a CTNNB1 mutation are associated with more aggressive behavior, especially in early stage endometrioid ECs [11-13]. Moreover, recent reports have suggested a prognostic role of the L1CAM status among NSMP tumors, showing a correlation with high grade, high stage, and poor outcomes [11, 14, 15]. Yet, a recent study confirmed the considerable molecular and clinical diversity of ECs NSMP through an in-depth assessment of the global genomic landscape of these tumors. The EC patients were clustered based on mutational and gene copy number alteration data with a strong association with meaningful clinical differences in oncological outcomes [10]. Although the interesting stratification of this group, the DNA next-generation sequencing (NGS) of 410–468 cancer-related genes used in this report by [10, 16] has cost prohibitive in routine clinical practice. Furthermore, in the era of personalized medicine, more accessible and alternative sources of genetic analysis are needed. In this view, digital polymerase chain reaction (dPCR) has been developed for the examination of genetic alterations in a wide variety of cancers [17-20] using a simple and practical technique [17, 21]. Therefore, better insights into the molecular signature of ECs NSMP through a cost-effective methodology are needed to improve risk prediction of recurrent disease, assist in adjuvant treatment decisions and develop novel treatment strategies.

OBJECTIVES

Primary Objective

The primary objective of this study is to describe the most common molecular mutations among NSMP ECs to refine the classification of patients with EC.

Hypothesis: NSMP ECs may be further stratified in different sub-classes with the incorporation of other molecular alterations.

We will focus on patients with EC NSMP according to the ProMisE system; thus, we will perform further molecular analysis through the application of dPCR.

Secondary Objective

The secondary objective of this study is to assess the oncological outcomes of patients with EC NSMP stratified by the most common molecular mutations.

Hypothesis: NSMP ECs clinicopathologic risk assessment might be improved by integration of additional molecular biomarkers predictive of individual tumor behavior.
We will assess the overall survival, cause-specific survival (i.e., death due to disease), and recurrence-free survival of these patients.

RESEARCH STRATEGY AND METHODS

Study Design

The present study is a multi-Institutional observational prospective study.
The patients with diagnosis of EC who will be surgically staged for primary EC at the ARNAS Civico, University of Palermo, Palermo, Italy, Fondazione Istituto G. Giglio di Cefalù-Gemelli Giglio Medical Partnership, Cefalù (PA), Italy, Azienda Ospedaliera Cannizzaro, Catania, Italy will be considered eligible.
This study aims to enroll 150 patients with EC in 36 months. This number can be considered appropriate based on both logistical/time issues. The cohort assessed to address the second objective will be restricted to patients with at least 12 months of follow-up. The molecular analysis will be carried out in a sample of tumors identified from the final specimen. The appropriate tumoral tissue will be collected to score the molecular/genetic expressions after the standard surgical procedure.
The first molecular analysis of each EC sample will be performed according to the ProMisE algorithm for the initial classification into one of the four molecular classes. Subsequently, all the NSMP ECs will be further analyzed through a panel of additional 8 genes in order to identify any possible molecular mutations. The NSMP EC patients will be classified according to the new molecular mutations identified. The possibility of stratifying patients into different risk classes (low, intermediate, high-intermediate, high) will be evaluated to predict the relapse and modulate the follow-up. The entire biomolecular analysis will be performed at the laboratory of the Tumor Immunology Unit, Department of Sciences for Health Promotion and Mother-Child Care "G. D'Alessandro," University of Palermo (Head of the Tumor Immunology Unit, Prof. Claudio Tripodo).

Study Population

Population-based cohort of consecutive patients who will be treated for primary EC at the centers involved in this study.

Inclusion

The following inclusion criteria will be applied:

- Endometrial cancer histologically proved
- Surgical staging including at least hysterectomy, bilateral salpingo-oophorectomy and lymph node assessment (SLN and/or LND)
- Signed informed consent

Exclusion

The following exclusion criteria will be applied:

- Patients who did not provide research authorization
- Patient age <18 years
- Patients with synchronous or metachronous second malignancies
- Patients with a diagnosis of non-epithelial uterine malignancies
- Patients who receive conservative (non-surgical) treatment (hormonal therapy, radiation therapy, chemotherapy)

Study Parameters-Data Collection

De-identified demographic and clinic-pathological data will be collected. Central pathology review will be performed. The gene mutations as results of the molecular analysis will be collected. Participating institutions will enter de-identified data into a secure central database. Patient anonymity will be maintained throughout the study.

Molecular Analysis

Tumor tissue samples from the final specimen after the surgical procedure will be collected for molecular analyses.

IHC staining

The IHC analyses will be tested for studying the MMR and p53 markers. The MMR proteins used for the analysis will include: MSH2, MSH6, MLH1, and PMS2. Mismatch repair protein deficiency (MMRd) is defined as loss of nuclear staining in at least one out of the four MMR

proteins. P53 aberrant protein expression (p53abn) is defined as either complete loss of nuclear protein expression or strong homogenous nuclear overexpression.

DNA extraction

After confirming the IHC tumor makers, the DNA will be obtained from corresponding samples collected and maintained in the store buffer, at -80 ˚C. The DNA will be extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

POLE sequencing

Targeted primers were designed to cover the POLE exonuclease domain exons 9 to 14 and analyzed by Oxford Nanopore Technology (ONT) NGS sequencing. After quality control and purification of DNA, NGS sequencing will be performed according to the manufacturer protocols, using primers design to detect the Endometrial Carcinoma hot spot mutations, as reported by Leon-Castillo, A. et al [24-26].

Digital PCR (dPCR)

Digital PCR procedure will be performed using the QIAcuity 8, 5-plex, the QIAcuity PCR Kit, and the 24-well 26 K Nanoplates (Qiagen, Hilden, Germany) per manufacturer's instructions. Briefly, the dPCR reaction mixture will be assembled as follows: QIAcuity 4X Probe PCR Master Mix 10 µL, 10X Primer/Probe mix (0.8um Primer/0.4um probe) 4 µL, RNase-free water and DNA/cDNA template in a final volume of 40 µL. The primer/probes for detection of CTNNB1, KRAS, PIK3CA, PIK3R1, L1CAM, ARID1A, AKT1 and PTEN are placed within PCR amplification primers and target-specific probes, ideally positioned at or adjacent to the mutation sites for detecting either the mutant or wild type sequence. The workflow included (1) priming and rolling step to generate and isolate the chamber partitions, (2) the amplification step under the following cycling protocol: 95°C for 2min for enzyme activation, 95°C for 15s for denaturation, and 60°C for 30 s for annealing/extension for 40 cycles, and (3) the imaging acquisition step of all wells. Data will be analyzed using the QIAcuity Suite Software V1.1.3 (Qiagen).

STATISTICAL CONSIDERATIONS

Data will be summarized using standard descriptive statistics. In depth, qualitative data will be expressed by absolute and percentage frequency. Quantitative variables will be described either by mean and standard deviation (SD), if normally distributed, or by median and interquartile range (IQR), otherwise. The relationships between molecular subgroups and clinicodemographic characteristics for continuous variables will be assessed using Student t-test or Mann-Whitney U test, whereas categorical variables will be analyzed using chi-squared test for independence or Fisher exact test as appropriate. Using the date of the surgery as starting date, recurrence-free survival and overall survival will be estimated using the Kaplan-Meier method. Log-rank test will be used to compare differences in recurrence-free survival and overall survival among molecular classes. Univariate and multivariate logistic regression models will be used to evaluate the association between clinical and molecular factors and recurrence; associations will be summarized as odds ratio, corresponding with a 95% confidence interval. Based on univariate analyses, factors with a $p<0.20$ will be considered in multivariate model building through backwards and stepwise modelling. All statistical analyses will be performed with JMP (version 16). P values of <0.05 will be considered statistically significant.

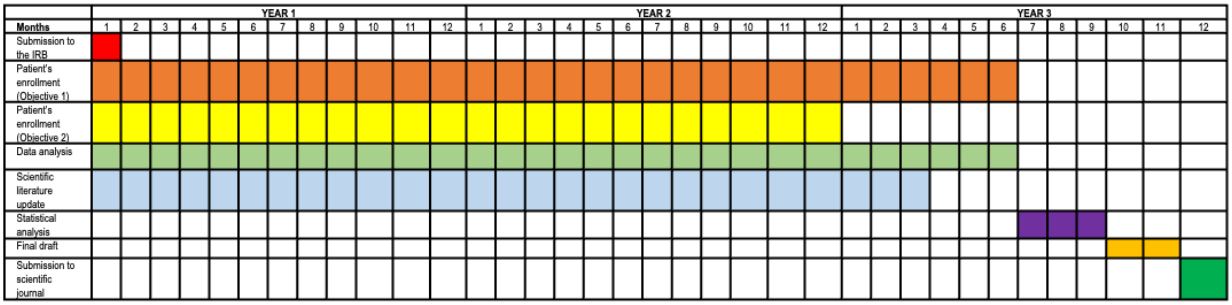
ETHICAL CONSIDERATIONS

The investigator will conduct the study ethical principles for medical research in accordance with the Declaration of Helsinki. All patients enrolled in this study will sign a written informed consent to allow anonymized data collection for research purpose. Each participating institution is required and responsible to obtain local Institutional Review Board (IRB) or ethics committee approval before collecting any data. The study will be not advertised, and no remuneration will be offered to eligible women to enter or continue the study.

CLINICAL IMPACT OF THE STUDY

The molecular definition of EC is bringing into clinical practice the opportunity of tailored precision therapy. We propose a novel approach that allows to identify molecular targets within NSMP ECs for which therapeutic drugs may exist. Several ongoing trials are testing the efficacy of different novel agents in patients with EC [22]. This approach will help partner with ongoing clinical trial enrolling NSMP patients for targeted therapy and immunotherapy approaches. Moreover, by confirming the reliability of dPCR in evaluating NSMP molecular expression, it would be possible to consolidate the feasibility of this technique in routine clinical practice, which is more widely accessible and cost-effective than DNA genome sequencing.

TIMELINE



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