1 Project Title: *

**Please provide a descriptive project title*

- Next-generation sequencing technologies for the characterization of non-acral sporadic cutaneous melanoma unrelated to common risk factors. A Melanostrum study.

Summary

Melanoma diagnosis and prognosis are currently based on limited clinico-histopathological parameters although tumors which are morphologically similar can behave differently. Moreover, the current classification system is unable to explain different progression models of advanced disease and to reliably identify those patients who will experience metastasis

Epidemiological data support at least two patterns to explain the development of non-acral sporadic cutaneous melanoma, the first associated with melanocytic proliferation and intermittent sun exposure and the second related to sun sensitivity and chronic sun damage. Although the majority of cutaneous melanomas follow these patterns of development, clinical experience indicates that a proportion of cutaneous melanomas arise through an alternate route. In a previous study, our results supported previous epidemiologic observations that recognized two different phenotypes at high risk of non-acral cutaneous melanoma: 1) the 'red-skinned red-heads' and 2) individuals with at least 50 melanocytic nevi and no histological signs of chronic sun damage in the surrounding apparently normal skin at the melanoma site. Besides confirming the previously defined patterns of melanoma development, we also identified a 'novel' group of melanoma patients with fewer known melanoma risk factors compared to patients from the first and second groups, the 'non-risk group'. In these patients, melanoma was less likely to be related to any of the two suggested divergent pathways: cumulative sun exposure (we excluded patients with either a personal history of outdoor working activity or recognized signs of chronic sun damage (i.e.; presence of actinic keratosis, solar lentigines, or a personal history of non-melanoma skin cancer) or nevus predisposition, and represented 6% of non-acral melanomas. The characteristics of this group suggest an unknown etiological pathway and possible susceptibility genes that affect the risk of melanoma beyond pigmentation or nevi-proneness (Nagore et al. 2012). These observations indicate that a large multicenter study for this additional subgroup would be of crucial clinical importance because their asymptomatic nature and a young age of disease affliction.

Based on these findings we propose a project, to characterize non-acral cutaneous melanomas that developed following a novel (unrelated to common risk factors) causal pathway by integrating new genetic technologies and clinical-pathological features. We aim to identify the clinical and molecular characteristics, as well as its clinical impact

(survival). Potentially, our study might help to improve diagnosis, to estimate biological aggressiveness and to identify predictive factors in this specific group of melanoma patients. In addition to its clinical relevance, an accurate characterization of non-risk melanoma patients could increase our understanding of cutaneous melanoma development and possibly improve new prevention strategies. Likewise our results will aim to a clinical and genetic classification system that may elucidate the mechanisms of melanoma heterogeneity and – most importantly- offer the possibility of individual risk or prognostic ascertainment as the next-generation sequencing (NGS) technology is becoming more and more available.

Project objectives

The hypothesis of the study is that non-acral melanomas that develop in the absence of common risk factors, i.e. high number of nevi and cumulative sun damage, display distinct genetic alterations, that are different from those presenting in melanomas with a suggestive association with known risk factors, and have a different clinical behavior and prognosis.

The main objective of the study is to identify and characterize clinically and biologically non-acral melanomas that do not fit into any of the two previously proposed pathways, i.e. chronic sun damage and nevus-prone ones.

The secondary objective is to know if these melanomas associate with an intrinsically different prognosis other than those belonging to the common pathways. To attain this objective we will study genetic alterations using NGS.

Background

Cutaneous melanoma is a malignant skin cancer originating from the uncontrolled growth of atypical melanocytes. Due to its propensity to metastasize, melanoma remains one of the most aggressive and treatment-resistant cancers. The incidences of cutaneous melanoma have increased over the last decades in white populations [1–2]. Cutaneous melanoma also represents an example of a multifactorial disease, with involvement of genetic, environmental and host factors. Ultraviolet exposure is widely accepted as the major environmental cause of cutaneous melanoma, but the relationship between risk and sunlight is highly complex [1–10]. The risk of cutaneous melanoma is modulated by environmental conditions and phenotype characteristics [1,3]. The main risk factors related to cutaneous melanoma are: exposure to ultraviolet radiation, a history of sunburn [1,2], multiple common and atypical nevi [2], fair skin type, red/blond hair and blue/green eye color [1–2], indicators of actinic damage [1], and a family history of cutaneous melanoma [3]. These epidemiological and genetic data suggest the existence of different patterns in the development of cutaneous melanoma [2,3]. It has been suggested that the pattern and amount of sunlight required to cause melanoma depends upon the characteristics of the host and the anatomic site of melanocytes [3]. This ‘divergent pathways model’ seeks to explain these additional complexities by proposing that cutaneous melanomas arise through at least two causal pathways, corresponding to key

associated epidemiologic factors with each [3]. One potential route is linked to chronic sunlight stimulation of skin which is particularly sensitive to sun exposure and is associated with photodamage and relatively a few nevi. The second route involves nevus-prone individuals, and is associated with melanocytic instability, and requires intermittent exposure to ultraviolet radiation [1–3]. The 'intermittent exposure hypothesis' proposes that cutaneous melanomas are caused by intense, acute, episodic exposures of melanocytes to solar ultraviolet radiation, help to explain the higher incidence of melanoma among indoor workers and the strong associations observed with sunburns and sunny vacations, but did not wholly explain the heterogeneous patterns of melanoma incidence and risk factor associations when analyzed by age and anatomical site [4]. Accordingly, melanomas arising through the 'nevus pathway' would require only modest sun exposure to develop, probably resulting largely from exposures experienced before adulthood, and their development is driven by host factors that manifest melanocytic instability such as histological evidence of nevus remnants adjacent to the tumor, tendency to present earlier in life, in individuals with high number of nevi and on body sites with high nevus counts like the trunk [3].

Melanomas localized on the palms, soles, and mucosal membranes show an increased rate of chromosomal aberrations and genetic alterations in KIT. Higher frequencies of personal and family histories of non cutaneous neoplasias have been described in acral melanomas, suggesting another specific path for acral melanoma [2].

On the other hand, although the development of most cutaneous melanomas is associated with sun sensitivity and melanocytic proliferation, clinical experience suggests that a proportion of non-acral cutaneous melanoma develops following a different pathogenic pattern. Our group previously described a third non-nevus-prone and non-sun-sensitive group of patients. This novel subgroup of melanoma cases, that represents 6% of all non-acral tumors, had fewer known risk factors for melanoma. These patients were predominantly women, developed melanoma more frequently at a very young age (under 25 years), and presented less multiple melanomas, which were mostly localized on the trunk and the legs. Altogether, the characteristics of this group suggest an as yet unknown etiological pathway and possible susceptibility genes that affect the risk of melanoma beyond pigmentation or nevi-proneness. For this reason larger studies into this subgroup of individuals are warranted to improve our understanding of pathogenesis and prevention strategies.

Finally, significant advances in melanoma genetics using the new next-generation sequencing (NGS) platforms have been attained. Novel emerging pathways have been identified in melanoma including receptor tyrosine kinases and protein phosphatases pathways (ERBB4, FLT1, PTK2B), G protein-coupled receptors and glutamate signaling (GRIN2A, GRM3, GRM8), transcriptional and chromatin modification signaling (MITF, TRRAP, SETDB1) and novel frequently mutated genes (*PPP6C, RAC1, SNX31, TACC1, STK19, BCLAF1, ALPK2, MYOCD, ZNF750, RXRA*) [10].The potential clinical applications of NGS technology for diagnostics and therapeutics aim to identify specific

changes in DNA by rapidly and simultaneously sequencing multiple gene targets within multiple samples.

References:

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7. Methodology *****

Patients and tumour samples:

In this study, primary formalin-fixed paraffin embedded (FFPE) tumours from 375 non-acral sporadic melanoma patients diagnosed at the Departments of Dermatology of the Instituto Valenciano de Oncologia, Institute of Dermatology, Catholic University of Rome, Italy, and University of L'Aquila, Italy, and XXX (all Melanostrum centres that want to participate) will be retrieved from the biobanks of the participating centers. Only tissues with 60% or more tumour cells will be included in the investigation. All patient's data will be obtained from the melanoma database of each institution. Ethical approval for the study has already been obtained from the institutional review board of the InstitutoValenciano de Oncologia and will be obtained according to each institution requirements. Written informed consent from all study patients will be obtained.

Methods:

Sample selection

First, all potential cases will be centrally re-examined by a single expert pathologist (Alessandro Di Stefani, Rome), who will confirm the diagnosis and the pathologic characteristics of melanomas and will select the tumor rich-area to be macrodissected for genetic analysis. The adjacent normal tissue will be also selected to be used as control. For the purpose of this study we will include only sporadic invasive melanoma patients with either superficial spreading, nodular o lentigo maligna melanoma. Patients with in situ tumors, acral and mucosal melanomas and patients with melanoma metastases from an unidentified primary tumor will be excluded from the analysis.

Patients will be assigned to three mutually exclusive groups, initially defined according to the pattern of cutaneous melanoma development, according to the following criteria:

1- *Nevus-prone*: patients with at least 50 nevi (greater than 2 mm) and no histological signs of chronic sun damage in the surrounding apparently normal skin at the melanoma site. The latter will be assessed according to the classification previously described (Landi, Bauer et al. 2006).

2- *Chronic sun damage*: patients in which the melanoma is located on chronically or intermittently sun-exposed skin and with histological signs of chronic sun damage (with the presence of moderate or severe solar elastosis). These patients can have only lentiginous/flat nevi but less than 10 common palpable nevi (greater than 2 mm) and no clinically atypical nevus.

3- *Non-risk*: individuals with less than 10 nevi (any size), and skin type higher than II. To better define this group, we will include only patients who, in addition to these features, will lack any of the following characteristics: a past personal history of severe sunburns at the site of primary melanoma, a personal history of non-melanoma skin cancer, solar elastosis at the apparently normal surrounding skin and solar lentigines at melanoma site.

Patients who simultaneously fulfilled the criteria of the three previous groups will be excluded from the analyses. So far, we have already identified at the participant centers 150 cases belonging to the first group, 150 to the second and 75 to the third.

Sample processing

DNA extraction and preparation. DNA will be extracted from FFPE section of a total 50 µm of thickness, using QiaCube® (Qiagen) automatic extractor and GeneRead DNA FFPE Kit® (Qiagen). This will allow both extracting the genetic material and repairing the DNA artefacts due to formalin fixation.

The DNA samples will be subjected to a target sequencing for the analysis of a panel of 26 genes, using the Illumina Technologies, with a MiSeq® sequencer (Illumina®), using the MiSeq Reagent V2 500 cycles Kit (Illumina®). The gene panel includes 26 genes which were selected because of their involvement in carcinogenesis and melanogenesis and are listed hereafter: *CDKN2A, NRAS, BRAF, TP53, ATRX, DAXX, PIK3R1, PPP6C, PTEN, NF1, PIK3CA, KIT, AKT, MITF, MAPK2K, ROS1, CDK4, CDK1, ARID2, HRAS, RAC1, RB1, GNA1, GNA11, IDH1 and KRAS.*

The following protocol will be used to analyze the panel of somatic mutations by massive sequencing in the Mi-SeqIllumina:

1. Quantification of the DNA extracted using Quant-iTPicoGreen kit Assay kit from Life Technolgies in QuantiFluorfluorimeter (Promega).

2. Evaluation of the DNA quality for the NGS IlluminaMiSeq with the Illumina QC FFPE Kit in a Real time PCR System (7500 Fast Applied BioSystems).

3. Multiplex PCR. The gene panel will be amplified using an amplicon approach with a total of 633 amplicons using a GeneRead DNAseq Custom Panel V2 (Qiagen®).

4. Perform the amplicons library of each sample with TruSeq Custom Amplicon Kit Assay (Illumina). Prior to sequencing, fragment ends will be repaired and adaptors will be ligated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs). In addition, a PCR reaction will be performed in order to create a unique barcode for each sample using the NEBNext® Multiplex Oligos for Illumina® (New England Biolabs).

5. Normalize and mix libraries marked for a single sequencing experiment.

6. Perform sequencing on the Mi-SeqIllumina Sequencer.

7. Analyze the results with IlluminaAmplicon Viewer software.

In addition, *TERT* promoter mutations will be analyzed by Prof. Kumar group in Heidelberg according to previously described protocol (Horn S et al, Science 2013;228:959).

Data Analysis

Subsequent bioinformatics analysis using MutSigCv algorithm will help to prioritize and distinguish “driver” mutations from neutral “passenger” mutations, to rank “driver” alterations according to their frequency and characteristics in order to determine the most common affected molecular pathways.

Common polymorphisms present in public databases, such as the Exome sequencing project (ESP) and The 1000 Genomes Project will be excluded. Rare variants (frequency <0.001 in ESP or 1000 Genome) of each of the 60 genes will be compared between tumor and normal tissue samples to identify the somatic lesions linked to melanoma. We will compare results with the melanoma TCGA exome sequencing data. All unpublished “driver” mutations identified in this study will be validated by traditional Sanger sequencing.

All melanomas characterized by specific mutation profiles will define a molecular subtype which will be correlated with epidemiological and clinico-pathological features of the lesions and/or patients in order to identify potential morphological and molecular signatures of each subgroup. Linear regression analyses of melanoma risk factors, including number of nevi, presence of dysplastic nevi, pigmentation characteristics and patterns of ultraviolet radiation exposure in relation to molecular patterns will be conducted to identify exposure-specific melanoma subtypes.

The following additional clinico-pathological information, collected on a dedicated clinical form using a detailed questionnaire which will be performed during dermatological examination, will be available for each patient:

- Demographic and epidemiological data: patients’ age at diagnosis and sex, site of primary melanoma, past personal history of severe sunburns, personal history of chronic sun exposure.

- Phenotypic characteristics (hair and eye color, presence and absence of clinically atypical nevi, signs of sun damage, number of nevi)

- The following histopathological data of melanoma will be considered: clinico-pathological variant, Breslow thickness, ulceration, tumor mitotic rate, presence of remnants of adjacent nevus, regression, predominant cell type and solar elastosis (Landi, Bauer et al. 2006).

Statistical analysis will be performed to confirm the association of molecular melanoma subtypes with the epidemiological and clinico-pathological data. This will provide an integrated morphologic-genetic model for melanoma characterization. Contingency tables and Chi-squared test (or Fisher test where appropriate) will be used to compare differences between the distributions of each variable according to the predefined group (nevogenic vs. chronic sun exposure vs. non-nevus-prone and non-sun-sensitive). Univariate and multivariate logistic regression models will be used to calculate ORs and their corresponding 95% confident intervals.

Survival analyses will be performed for disease-free and melanoma specific survivals with the corresponding events defined by the date of the first recurrence of death due to melanoma. Patients without the event will be censored at the date of the last known date. Kaplan-Meier curves, log-rank test and uni- and multivariate Cox regression analyses will be performed for this purpose.

The collaborative group is composed by experts in different fields of melanoma management from each Department involved in the project: Dermatology, Molecular Biology and Pathology. Each participant has actively participated in research projects related to melanoma and the institute includes all the technologies needed to develop the project.

Organizational structure of the team and the role of collaborators or participating centers (to be completed by all Melanostrum participants):

- Clinical data collection: all centers.
- Clinico-Pathological review of representative slides from all melanomas: Dr. Alessandro Di Stefani and Prof. Ketty Peris
- DNA extraction from paraffin-embedded samples and quality evaluation: Drs. García-Casado, Traves and Fernández.
- Mutational screening to identify melanoma molecular subtypes: Drs. García-Casado and Fernández.
- TERT promoter mutation analysis: Prof. Rajiv Kumar
- Validation of NGS-detected "driver" mutations by Sanger sequencing: Dr. Cristina Pellegriniand, Prof. Maria Concetta Fargnoli
- Statistical analysis for survival and database management: Dr. Nagore, Dr. Manrique
- Bioinformatic analyses (NIH??: to be answered by Tere): Dr. Joaquin Dopazo.
- Project coordination: Edu and Tere

Evaluation criteria and definition of success/failure *****

Criteria or benchmarks for success:

- Identification of selected genes and/or molecular pathways defined according to a novel pre-defined causal clinico-epidemiological pathway. Potentially, these molecular alterations might be useful for the development of targeted therapies.
- Clinical application of the molecular classification integrating epidemiological, clinico-pathological features with the genetic profile to identify predictive clinical factors for a better patient's management and eventually for screening campaigns.

Discuss potential problems or alternative strategies:

The possible problems and limitations in our project could include the non population-based nature of the study. The self-reported personal and family medical history could be solved by reviewing the patients past medical history, and the potential heterogeneity in the pathological criteria, specifically solar elastosis, will be solved by reviewing the histopathological samples by one expert dermatopathologist (Alessandro Di Stefani).

On the other hand the strengths of our project include the large sample size, the uniform collection of data from incident melanoma cases, the physician-assessed clinical and phenotypic characteristics, and the homogeneity of diagnosis and referral procedures.

Proposed deadlines beginning (to be discussed at Melanostrum)

- 01/01/2018

Proposed deadlines ending (to be discussed at Melanostrum)

- 31/12/2019

11. Work plan & timing (to be discussed at Melanostrum)

The study will be divided into 4 phases, distributed along a 2-year period.

Phase Nº 1. Identification of cases (Valencia, Rome and L'Aquila and other Melanostrum centres), selection of samples and histopathological review (Rome) (months 1-6).

Identification of FFPE blocks from the cases fulfilling the selection criteria, preparation of one Haematoxylin-eosin (H&E) stained section.

Centralized histopathological review in Rome. Selection of feasible cases for NGS and selection of tumor areas to guide further macro-dissection.

Preparation of 5 unstained FFPE 20 um slides and the marked H&E slide to be sent to Valencia for NGS.

Clinical, epidemiological and survival data will be retrieved from each database and merged in a common database.

Phase Nº2. DNA extraction from FFPE samples and quality evaluation (Months 7-10) (Valencia).

DNA extraction by macrodissection of neoplastic areas and corresponding adjacent histologically normal tissue from the unstained FFPE slides guided by H&E selection areas.

Assessing of DNA quantity and quality for NGS feasibility with IlluminaMiSeq.

An aliquote of the DNA will be sent to Heidelberg for *TERT* promoter mutations.

Phase Nº 3. Mutational screening to identify melanoma molecular subtypes (Months 11-18) (Valencia, L'Aquila, Heidelberg)

A melanoma-specific mutation panel consisting of 26 highly frequently mutated genes will be tested in each sample by high-throughput sequencing using the IlluminaTruSeq Custom AmpliconTechnology (Illumina) and a MiSeq Sequencer platform. The genes, with clinical relevance and potentially actionable targets, have been generated through a comprehensive literature search and interrogation of different bioinformatics sources as the Catalogue of Somatic Mutations in Cancer (COSMIC) and The Cancer Genome Atlas (TCGA) database. Missense mutations, small deletions or insertions in coding regions and UTR regions and known regions affected by CNV will be considered.

Bioinformatics analysis (MutSigCv algorithm) will help to prioritize and distinguish "driver" mutations from neutral "passenger" mutations, to rank "driver" alterations according to their frequency and characteristics in order to determine the most common affected molecular pathways. Common polymorphisms present in public databases will be excluded. Rare variants (frequency <0.001 in ESP or 1000 Genome) of each of the 26 genes will be compared between tumor and normal tissue samples to identify the somatic lesions linked to melanoma. We will compare results with the melanoma TCGA exome sequencing data.

All unpublished "driver" mutations identified in this study will be validated by traditional Sanger sequencing in L'Aquila.

TERT core promoter will be studied in Heidelberg.

Phase Nº 3. Statistical and data analysis (Months 19-24) (Valencia/NIH)

Statistical analysis will be performed to confirm the association of molecular melanoma subtypes with the epidemiological and clinico-pathological. This will provide an integrated morphologic-genetic model for melanoma characterization. Contingency tables and Chi-squared test (or Fisher test where appropriate) as well as Univariate and multivariate logistic regression models will be used to assess differences between the 3 defined groups.

Survival analyses will be performed for disease-free and melanoma-specific survivals by Kaplan-Meier curves, log-rank test and uni- and multivariate Cox regression models. For this purpose, age, sex, site of primary tumor, Breslow thickness, ulceration, tumor mitotic rate, vascular invasion, microscopic satellite, regression and tumor infiltrating lymphocytes as well as sentinel lymph node status will be used as co-variates.

Significance of the project / originality

The identification of melanoma subtypes with specific genetic/morphologic profiles obtained by integrating new molecular technologies and clinico-pathological findings can lead to new guidelines for screening and management of melanoma patients.

A better knowledge of the genes and/or signaling pathways involved in different melanoma subtypes and in different progression stages of the disease can have an important scientific and socio/economic impact defining models of melanoma progression and tumor aggressiveness and helping in the identification of potential targets for treatment of advanced disease. Moreover, the identification of mutational profiling related to specific morphologic features and exploration of unknown signaling pathways and somatic gene mutations involved in melanoma initiation and progression can help predicting the clinical outcome and disease prognosis.

With the recent progression of technology, a melanoma-specific mutation panel might be developed and possibly translated into a clinical setting after being tested and validated in an elevated number of melanoma cases. This panel is for use with the MiSeq technology. The final validated panel might represent a cost-effective, sensitive, rapid and high-throughput tool for diagnostic improvement and treatment strategies in melanoma. A correct detection of cancer mutations is a critical step in the new personalized cancer therapy approach, in which tailored drugs are administered to patients according to their tumor “mutation profile”.

Further step

The fulfill the objectives of the study, we aim to start with a prospective fresh tissue collection of cases belonging to the target group according to the before mentioned criteria, to perform, as for Tere’s proposal, deep sequencing and to compare them with available public databases (i.e. TGCA).