Molecular Basis of Melanoma

Alexis L. Young

A. 1. Study Purpose and Rationale

Melanoma is currently the 5th and 7th most commonly diagnosed cancer in American men and women, respectively, with incidence rates increasing faster than for any other cancer.[i, ii] Currently it is estimated that 1 in 55 men and 1 in 82 women in the United States will be diagnosed with melanoma in his or her lifetime. The lifetime risk of developing invasive melanoma in the United States is currently 1 in 71 compared with an estimate of 1 in 600 in 1960. Despite the frequency with which melanoma is encountered, relatively few advances in the treatment of melanoma have been made and the mortality rate continues to rise. Several genes have been implicated in the pathogenesis of melanoma; however, its molecular basis is largely unknown. Mutations in the *B-RAF* (~50%) and *N-RAS* (~10%) genes leading to activation of the mitogen-activated protein kinase (MAPK) pathway (*RAS-RAF-MEK-ERK*) play a role in the pathogenesis of melanoma.[iii] This pathway, under physiologic conditions, is activated by mitogens, growth factors, and cytokines and regulates cell growth, survival, and differentiation. Constitutive activation of this pathway is observed in a variety of cancers, one of which is melanoma.

There are several subtypes of melanoma and melanocytic nevi that are classified based on clinical and histopathologic characteristics. Interestingly, mutation frequencies of *B-RAF* show differences among melanoma and melanocytic nevi subtypes. Although several genes and signaling pathways have been implicated in the pathogenesis of melanoma, the molecular basis of sporadic melanoma remains largely uncharacterized. We propose to study melanoma subtypes in which *B-RAF* and *N-RAS* are not involved, and identify molecules and signaling pathways that contribute to their pathogenesis.

In order to provide insights into the pathogenesis of melanoma subtypes, in this study we will evaluate the expression of downstream molecules (phosphorylated *MEK* and phosphorylated *ERK*) of the *RAS/RAF/MEK/ERK* signaling pathway in melanoma subtypes and compare these levels with appropriately matched conventional melanoma controls. Second, we will perform a candidate gene screening and look for activating mutations in oncogenes that signal through the *RAS/RAF/MEK/ERK* pathway. These studies will serve as the initial steps toward a molecular-based understanding of these tumors, not only for its importance in melanoma development, but also for discovering potential therapeutic targets.

B. Study Design and Statistical Analysis

Aim 1: Activation of the MAPK pathway in cutaneous melanoma subtypes

The MAPK pathway is a central signal transduction pathway, which transmits signals from multiple cell surface receptors that result in the activation of transcription factors in the nucleus. Activation of this pathway occurs through oncogenic mutations in RAS or RAF, specifically *B-RAF* and *N-RAS* in melanoma. Although a high percentage of melanomas harbor mutations in these genes leading to activation of the MAPK pathway, *B-RAF* and *N-RAS* mutations are not found in uveal and mucosal melanomas as well as in Spitzoid and desmoplastic melanoma. These data suggest that ERK activation occurs through mechanisms or pathways independent of RAS and RAF in some melanoma subtypes. In an attempt to gain insights into cutaneous melanoma subtypes, Spitzoid and desmoplastic melanoma, in this Aim, we will first evaluate whether activation of MEK and ERK, as in other cutaneous melanomas, play a role in these melanoma subtypes. The study groups will consist of desmoplastic, Spitzoid, and *B-RAF* wild-type cutaneous melanomas. *B-RAF* mutant melanoma samples will be used as controls.

The expression of downstream molecules in the *RAS/RAF/MEK/ERK* pathway will be evaluated by immunoblotting. Expression levels of phosphorylated *MEK* and phosphorylated *ERK* will be evaluated individually as dichotomous, categorical variables where an exposure will be characterized by expression

levels of each gene greater than one fold of the negative control and levels less than one fold that of the negative control will designate a lack of exposure. This type of approach will require statistical analysis using a two-group chi-square test on proportions. Since previous studies in conventional melanomas have demonstrated upregulation of phospho-*MEK,* and phospho-*ERK,* as well as activating mutations in *RAS* and *B-RAF*, we will assume that 80% of our controls will demonstrate an exposure. In contrast, given that preliminary studies failed to show common *RAS* and *B-RAF* mutations in Spitzoid and desmoplastic melanomas, we postulate that only 30% of our cases will demonstrate elevated expression of phospho-*MEK* and phospho-*ERK*. This design will require enrollment of 18 cases and 18 controls of each melanoma subtype to demonstrate a statistically significant difference between the two groups with a power of 80% at an alpha of 0.05. Should such an outcome be reached, we could postulate that Spitzoid and desmoplastic melanomas develop through a different signaling pathway than that of conventional melanomas. Conversely, if we fail to demonstrate such a difference, we could postulate that downstream molecules in the *RAS/RAF/MAPK* signaling pathway are upregulated by different upstream activators such as PKC or PKA. Either outcome would provide insight into the molecular basis of these tumors.

Aim 2: Candidate Gene Screening in Cutaneous Melanoma Subtypes

We have shown that *B-RAF* and *N-RAS* mutations are not found in Spitzoid melanomas. Similarly, desmoplastic melanomas lack mutations in *B-RAF*. In this Aim, we will screen these melanomas for mutations in genes that are involved in MAPK signaling. These genes will be compared to wild type controls.

Aim 3: Chromosomal Aberrations in Cutaneous Melanoma Subtypes

Chromosomal imbalances, particularly amplifications and deletions, are common in solid tumors, including melanoma. In human melanoma, conventional cytogenetic methods have detected specific chromosomal numerical aberrations, most prominently LOH of 1p, deletion of 6q22-27, amplification of 7, LOH or deletion of 9p21, and LOH of 10q24-26.[iv,v] In an attempt to identify chromosomal regions that contain tumor suppressor genes or oncogenes for melanoma, in this Aim, we will examine Spitzoid, desmoplastic, and B-RAF wild type cutaneous melanomas for DNA amplifications, or large and small homozygous and hemizygous deletions by array-based comparative genomic hybridization. The melanoma subtypes under investigation will be compared to B-RAF mutant cutaneous melanomas.

Evidence of chromosomal aberrations by array-based comparative genomic hybridization will be evaluated individually as dichotomous, categorical variables where an exposure will be characterized by evidence of DNA copy number changes. This type of approach will require statistical analysis using a one-group chi-square test on proportions. We would expect that 0% of our controls will demonstrate similar chromosomal aberration, excluding area 7q that is responsible for *B-RAF* mutations. In order to allow for sampling variability in our analysis, we will accept an exposure level of less than 5% in our control group. In contrast, for Spitzoid, desmoplastic, and B-RAF wild type cutaneous melanomas, we will postulate that 50% or more of our cases will demonstrate corresponding chromosomal aberrations different from those seen in the control group. This design will require enrollment of 22 cases of each melanoma subtype and 95 controls to demonstrate a statistically significant difference within the groups with a power of 80% at an alpha of 0.05. Should such an outcome be reached, we could postulate that Spitzoid, desmoplastic, and B-RAF wild type melanomas develop through a different chromosomal aberration than that of B-RAF mutant melanomas. Conversely, if we fail to demonstrate such a difference, we could postulate that we included an inadequate number of samples or that our technique was not sensitive enough to detect the specific DNA copy changes. High-resolution comparative genomic hybridization (CGH)-based microarrays were developed to increase the resolution of chromosomal studies and to provide a comprehensive assay by using large-insert clones as the target for analysis. New technology allows for the detection of DNA copy changes as small as 1 Mb.[vi] If the DNA copy number changes in Spitzoid and desmoplastic melanoma are smaller than 1 Mb, then array-based comparative

genomic hybridization will not be sensitive enough to provide evidence for exposure. Either outcome would provide insight into the molecular basis of these tumors.

C. Study Procedures

Aim 1: Activation of the MAPK pathway in cutaneous melanoma subtypes

The clinical information will be obtained either by the Principal Investigator or by the referring physician. Following informed consent, medical history and pedigree information will be obtained, and the patients will undergo a complete skin examination. Prior to recruitment, the patients will have already been diagnosed with melanoma and will have chosen to undergo surgical excision of their tumors by their primary care provider. Frozen tissue samples of melanoma subtypes will be obtained from the surgically excised tumors and transported to Dr. Celebi's laboratory in the Department of Dermatology, Vanderbilt Clinic 15-202, where they will be stored for future studies.

To date, we have collected 33 Spitzoid and 18 desmoplastic melanoma paraffin-embedded tumor specimens. Fresh frozen tumor cells will be homogenized, and the cells will be lysed in low salt Nonidet P-40 buffer (10mM Hepes, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA) supplemented with 1 mM dithiothreitol, and protease and phosphatase inhibitors. Cell lysates will be fractionated by SDS-PAGE electrophoresis and immunoblotted with antibodies against MEK, phosphor-MEK, ERK, and phosphor-ERK (Cell Signalling, Beverly, MA). A melanoma cell line, CRL 1675, will be used as controls and will be grown in the absence and presence of 10 micromol/L MEK inhibitor UO126 (Promega). All these procedures will be performed in Dr. Celebi's laboratory in the Department of Dermatology, Vanderbilt Clinic 15-202.

Aim 2: Candidate Gene Screening in Cutaneous Melanoma Subtypes

Spitzoid and desmoplastic melanoma samples will be screened for activating mutations in K-*RAS, H-RAS, A-RAF*, and *C-RAF*. Tumor cells will be isolated by laser capture microdissection and genomic DNA from tumor tissue will be isolated as described by us previously. The candidate genes will be amplified by PCR using specific primers and directly sequenced. In order to maintain sensitivity, we do not expect to laser capture more than 10% of normal cells.

Aim 3: Chromosomal Aberrations in Cutaneous Melanoma Subtypes

We will study Spitzoid and desmoplastic melanoma tumor samples for chromosomal aberrations using array-based comparative genomic hybridization (CGH), a tool to quantitatively measure DNA copy number changes in cancer and to map those changes directly onto the human genome. The following groups of tumor samples will be compared:

1. Spitzoid melanoma
2. Desmoplastic melanoma
3. *N-RAS* and *B-RAF* wild type cutaneous melanoma
4. *N-RAS* and *B-RAF* mutant cutaneous melanoma

Tumor cells will be isolated with laser capture microdissection and DNA will be extracted using standard techniques. 1-2 microg of DNA will be sent to the Cytogenetics Core in the Department of Pathology at Dana Farber Cancer Center for evaluation of chromosomal gains or losses using microarrays with 1 Mb intervals over the entire genome.

First, we anticipate that these studies will detect DNA copy number differences between these melanoma subtypes. We will then design future studies that will include large numbers of tumor specimens that will enable for statistical analysis. Second, we anticipate that these studies will lead to the identification of chromosomal loci that accompany tumor suppressor genes or oncogenes involved in the

pathogenesis of Spitzoid and desmoplastic melanoma. Our future studies will be directed to screen candidate genes in these loci and eventually identify genes involved in the development of these tumors.

D. Study Drug or Devices

Not applicable.

E. Study Questionnaire

Not applicable.

F. Study Subjects

We will identify, with the help of our collaborators, a unique cohort of melanoma subtypes with specific histopathologic features of Spitzoid, desmoplastic, and *B-RAF* wild-type cutaneous primary and metastatic cutaneous melanomas. In this series, all patients will have already chosen to undergo surgical excision of their previously diagnosed melanoma. All subjects will be evaluated by the Principal Investigator or the referring physician where a complete skin evaluation will be performed and medical history and pedigree information will be obtained. Following informed consent, we will obtain sample tissue from the surgically excised lesions.

During the consent procedure, subjects will be informed of the molecular analysis of their tissue sample. Additionally, we will ask permission to use their samples in future research related to melanoma subtypes. The results of the tests will not be shared with the individuals unless a chromosomal aberration is identified that is associated with a known disease condition and/or will change the patient's management.

G. Recruitment

The subjects will be recruited from the clinical practices of dermatologists at New York Presbyterian Hospital. The consent will be obtained by the by the referring physician.

H. Confidentiality

Each subject will be assigned a random number as a code. The fresh frozen tissue samples and DNA samples in addition to any other data obtained from a subject will be identified by his or her code. All patient information and patient files will be kept in the Principal Investigator's office located at VC-15-202 in a locked cabinet. Only the Principal Investigator has access to these cabinets.

I. Potential Conflict of Interest

None.

J. Location of Study

Patients will be evaluated and tissue samples will be obtained at the Columbia Presbyterian Medical Center, Irving Pavilion 12th floor Dermatology office suites. All molecular studies will be conducted in Dr. Celebi's laboratory.

Array-based comparative genomic hybridization (CGH) will be performed in the Department of Pathology at Dana Farber Cancer Center where all specimens will remain de-identified.

K. Potential Risks

There will be no treatment administered during this study. As a result, the sole potential physical risk associated with this study includes surgically-related complications such as bleeding or infection at the surgical site. If bleeding is present, the physician will ensure that it is stopped before the patient leaves the facility. Infection is a rare complication that can be avoided by using sterile technique.

Additional risks may involve insurance companies using the information obtained from molecular testing to deny coverage to applicants. We will not share the data obtained in this research protocol with the patients. However, should a positive test result arise that is associated with a known condition or disease, the subject will be given the option to undergo independent testing and/or consultation with a specialist physician and/or genetic counselor. However, genetic consultation and counseling are not provided throughout this study.

In rare instances, unexpected information about a subject such as an unknown diagnosis may arise. During the consent procedure, we will ask subjects whether they wish to be informed of such unexpected findings.

L. Potential Benefits

This research may not directly benefit subjects; however, a molecular-based understanding of Spitzoid and desmoplastic melanoma will provide further insight into the clinical disease. The potential findings from this study will be important in developing possible therapeutic targets.

M. Alternative Therapies

Given that this protocol does not involve therapy, an alternative would be to refuse to enroll in this study and resume routine follow up.

N. Compensation to Subjects

None.

O. Cost to Subjects

Patients will not be billed for participation in this study.

P. Minors as Research Subjects

Approval from the Department of Pediatrics Committee on Human Investigation will be obtained.

Q. Radiation or Radioactive Substances

Not applicable.

A. References

[i] Rigel, DS, Friedman, RJ, Kopf, AW. The incidence of malignant melanoma in the United States: issues as we approach the 21st century. J Am Acad Dermatol 1996; 34:839.

[ii] Jemal, A, Tiwari, RC, Murray, T, et al. Cancer statistics. CA Cancer J Clin 2004; 54:8.

[iii] Gill, M, Celebi, JT. B-RAF and melanocytic neoplasia. J Am Acad Dermatol 2005; 53:108-14.

[iv] Funk JO, Schiller PI, et al. p16INK4a expression is frequently decreased and associated with 9p21 loss of heterozygosity in sporadic melanoma. J Cutan Pathol 1998; 25(6):291-6.

[v] Cachia AR, Indsto JO, et al. DKN2A mutation and deletion status in thin and thick primary melanoma. Clin Cancer Res 2000; 6(9):3511-5.

[vi] Shih IeM ,Wang TL. Apply innovative technologies to explore cancer genome. Curr Opin Oncol. 2005 Jan;17(1):33-8.