Genetic Analysis of Spitzoid Melanomas in Children

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A. 1. Study Purpose and Rationale

Melanoma is currently the sixth most common cancer in the United States with incidence rates increasing faster than for any other cancer.[i, ii] 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. However, despite these rising figures, melanoma of all types in childhood remains uncommon. Children represent 2% of melanoma patients in population-based studies [iii, iv] and melanoma accounts for only 3% of malignancies occurring in patients under the age of 20 years and 0.4% in pre-pubescent patients.[v]

The diagnosis of melanoma in this age group is also complicated by the presence of a benign melanocytic neoplasm called a Spitz nevus, which typically occurs in children and adolescents. Spitz nevi resemble a subset of melanoma referred to as "Spitzoid melanoma" or "melanoma with Spitz nevus-like features;" however, Spitzoid melanoma, like all other subsets of melanoma, is rare in children and occurs more commonly, though infrequently, in adults. Both Spitz nevi and Spitzoid melanoma present as solitary papules or nodules with or without pigmentation. Interestingly, the classic "ABCD (**A**symmetry, **B**order irregularity, **C**olor variation, **D**iameter > 6 mm) rule" for diagnosing melanoma does not apply to Spitzoid melanoma lesions and therein lays the difficulty in making a critical clinical diagnosis.[vi] Furthermore, although the gold standard for diagnosing melanoma is by histopathologic examination, studies detailing the histopathologic features of Spitzoid melanomas over the past few decades have failed to establish objective criteria for differentiating between these lesions and benign Spitz nevi, even when reviewed by expert pathologists.[vii] Subsequently, there are several reports in the literature of metastatic melanomas being initially misdiagnosed as Spitz nevi and resulting in fatal outcomes.[viii] Given the rarity of Spitzoid melanoma, the difficulty of diagnosis, and the potentially fatal outcomes of misdiagnosis, evaluation of these lesions presents a challenge to dermatologists, pathologists, and pediatric oncologists.

The biologic behavior of Spitzoid melanoma is poorly understood and the genetic basis of these tumors is largely unknown. Unlike other melanomas and melanocytic nevi which demonstrate mutations in key signal transducers including *RAS* and *BRAF* in the *RAS/RAF/MAPK* signaling pathway responsible for regulating cell growth, differentiation, and apoptosis, we and others reported the absence of common *RAS* and *BRAF* mutations in both Spitzoid melanoma and Spitz nevi.[ix, x] Therefore, it is unclear whether the Spitz nevus and Spitzoid melanoma are two distinct entities or they represent opposing ends of a biologic spectrum. Regardless, it appears that Spitzoid melanomas may have biologic behavior different from conventional melanomas and further elucidation of the molecular basis and biological nature of these tumors is required.

In order to provide insights into the pathogenesis of Spitzoid melanomas, in this study we will first search for chromosomal abnormalities in patients with Spitzoid melanoma and compare their chromosomal profiles with those of appropriately matched healthy controls. Second, we will evaluate the expression of downstream molecules (phosphorylated *MEK1, 2* and *ERK*) of the *RAS/RAF/MAPK* signaling pathway in Spitzoid melanoma and compare these levels with appropriately matched conventional melanoma controls. These studies will serve as the initial steps toward a molecular-based understanding of these tumors.

B. Study Design and Statistical Analysis:

a. Aim 1: Molecular cytogenetics of Spitzoid melanomas

Conventional melanomas demonstrate a variety of tissue chromosomal abnormalities. However, the cytogenetic analysis of blood samples of Spitzoid melanomas has not been previously reported and

many pediatric solid tumors, similar to certain childhood leukemias, arise from specific chromosomal deletions or translocations detected in blood samples. The prototype of this group is retinoblastomas which show cytogenetic aberrations of 13q in 10% of blood samples from cases.[xi] Other pediatric solid tumors which demonstrate cytogenetic aberrations include Wilms' tumor, neuroblastoma, Ewing's sarcoma, fibrosarcomas and primitive neuroectodermal tumors of the central nervous system. Recognizing that preliminary molecular studies suggest Spitzoid melanomas behave biologically different from conventional melanomas, we believe that cytogenetic analysis is a natural first step in elucidating the genetic basis of this disease. Moreover, although the likelihood of detecting a consistent abnormality in Spitzoid melanomas is low, the identification of a unique karyotypic pattern represents the basis for many diagnostic assays of childhood cancers. Furthermore, subtle findings from cytogenetic analysis allow identification of target regions suitable for more sensitive techniques, including comparative genomic hybridization on microarray chips and/or representation oligonucleotide microarray analysis, which detect amplifications or large and small homozygous and hemizygous deletions.

In this aim, we are proposing a case-control study with the cases being designated as blood samples from patients with Spitzoid melanoma. Samples of healthy controls will be group matched for age, gender, and race. Cytogenetic analysis of blood samples will be evaluated as a dichotomous, categorical variable where an exposure has occurred if any chromosomal abnormality is detected. Absence of a chromosomal abnormality will suggest an absence of exposure. This type of approach will require statistical analysis using a two group chi-square test on proportions. Review of the literature on retinoblastomas and Wilms' tumors suggests 10% of blood samples from patients with these tumors demonstrate a specific chromosomal abnormality in 13q or 11p13, respectively.[xi] Empirical evidence suggests that 1 in 1,000 healthy individuals demonstrate chromosomal abnormalities on cytogenetic analysis. Therefore, we will estimate that the proportion of cases with an exposure will equal at least 10% and that of controls will equal less than 0.5%. This design will require enrollment of 72 cases and 358 controls to demonstrate a statistically significant difference between the two groups with a power of 80% at an alpha of 0.05.

Since no previous studies in the literature have addressed this aim, a potential complicating factor in this design is that an exposure is characterized as "any" chromosomal abnormality. Ideally, we hope to identify a consistent, specific abnormality among cases similar to the aberrations described in retinoblastomas and Wilms' tumors. However, if we fall short of this goal, subtle cytogenetic findings will still be valuable in guiding future genetic studies.

b. Aim 2: Activation of the *RAS/RAF/MAPK* signaling pathway in Spitzoid melanoma

In this aim, we are proposing a case-control study with the cases being designated as frozen tissues samples of Spitzoid melanoma with lymph node metastasis. Control samples of conventional melanoma lymph node metastasis will be individually matched for age, race, and gender. Molecular studies will be employed to evaluate the expression of downstream molecules in the *RAS/RAF/MAPK* signaling pathway. Expression levels of phosphorylated *MEK1, 2* and *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. The baseline expression level will be established by a human melanocyte cell line negative control. Realtime PCR will be used to quantify levels of expression for categorization. Since previous studies in conventional melanomas have demonstrated upregulation of *MEK1, 2* and *ERK,* as well as activating mutations in the upstream regulators *RAS* and *BRAF*, we will assume that 80% of our controls will demonstrate an exposure. In contrast, given that preliminary studies failed to show common *RAS* and *BRAF* mutations in Spitzoid melanomas, we will postulate that only 30% of our cases will demonstrate elevated expression of *MEK1, 2* and *ERK*. This design will require enrollment of 18 cases and 18 controls 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 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.

C. Study Procedures

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. A 10 to 15 cc blood sample will be collected by venipuncture. The examination and blood collection will be performed only once and will require approximately 1 hour.

3 cc of blood will be transferred to Dr. Dorothy Warburton's laboratory in the Department of Genetics and Development, Babies Hospital South Room 406, for preparation of metaphase chromosomes and for fluorescent it situ hybridization (FISH) analysis. The remaining blood sample will be used to isolate DNA in Dr. Celebi's laboratory in the Department of Dermatology, Vanderbilt Clinic 15-202, and will be stored for future studies.

Frozen tissue samples of conventional and Spitzoid melanomas with lymph node metastasis will be obtained. The tissue will be homogenized and the cells will be lysed in low salt Nonidet P-40 buffer supplemented with 1 mM dithiothreitol and protease inhibitors. The lysates will be fractionated by SDS-PAGE and immunoblotted with antibodies against ERK, phospho-ERK, MEK 1, 2 and phospho-MEK 1, 2. These antibodies are commercially available (Santa Cruz). All these procedures will be performed in Dr. Celebi's laboratory in the Department of Dermatology, Vanderbilt Clinic 15-202.

D. Study Drug or Devices

Not applicable.

E. Study Questionnaire

Not applicable.

F. Study Subjects

We have identified a unique cohort of Spitzoid melanomas occurring in prepubescent children with histopathologic features of Spitzoid melanoma and metastasis to the regional lymph nodes consistent with stage III disease. In this series, all except one child are alive with metastatic disease. Currently, we have obtained and studied tissue specimens from 12 cases and in this study we propose to collect blood samples from these patients to perform molecular cytogenetic analysis in search of chromosomal aberrations. Additional blood samples for Aim 1 will be provided by collaborators and/or collected prospectively.

Control tissue specimens of conventional melanoma lymph node metastasis individually matched for age, race, and gender will be retrieved from the archives of Dermatopathology Laboratory at Columbia University.

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, a blood sample will be collected and transported to the laboratory.

During the consent procedure, subjects will be informed of the cytogenetic analysis of their blood sample. Additionally, we will ask permission to use their samples in future research related to Spitzoid melanoma. 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 as well as Dr. Celebi's national collaborators. The subjects will either be provided with a phone number to schedule an appointment with the Principal Investigator or the referring physician will provide pertinent clinical information and the blood sample to the Principal Investigator. The consent will be obtained by the Principal Investigator during the visit or by the referring physician.

H. Confidentiality

Each subject will be assigned a random number as a code. The blood 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 blood samples will be obtained at the Columbia Presbyterian Medical Center, Irving Pavilion 12th floor Dermatology office suites or at the pediatric outpatient facilities of the GCRC. FISH analysis will be performed in Dr. Warburton's laboratory and all other molecular studies will be conducted in Dr. Celebi's laboratory.

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 rare phlebotomy-related complications such as bruising, bleeding, or infection at the injection site. Bruising, if present, is self-limiting and usually resolves within 1-2 weeks of venipuncture. 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 cytogenetic 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 melanoma will provide further insight into the clinical disease. The potential findings from this study will be important in developing diagnostic assays in addition to generating animal models for Spitzoid melanoma.

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.

R. References

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