MAP Kinase Pathways: Molecular Roads to Primary Acral Lentiginous Melanoma

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Abstract: The etiology and pathogenesis of lentiginous acral melanomas are poorly understood. Recent studies have postulated that DNA repair mechanisms and cell growth pathways are involved in the development of melanoma, particularly changes in the MAPK pathways (RAS, BRAF, MEK 1/2, and ERK 1/2). The aim of this study is to assess the status of the MAP kinase pathways in the pathogenesis of acral melanomas. The authors examined the components of the RAS–RAF–MEK–ERK cascades by immunohistochemistry in a series of 16 primary acral melanomas by tissue microarray. The expression of MAP kinase cascade proteins changed in most cases. The authors observed that 57.14% of cases were BRAF positive and that 61.53%, 71.42%, and 71.42% of cases were positive for MEK2, ERK1, and ERK2, respectively; RAS was not expressed in 92.31%, and all cases were negative for MEK1. The absence of RAS and positivity for MEK2, ERK1, and ERK2 were most seen in invasive cases with high thickness. These aspects of the MAPK pathway require further examination in acral melanomas between different populations. Nevertheless, the results highlight significant alterations in the MAP kinase cascades that are related to histological indicators of prognosis in primary acral melanomas.

Key Words: melanoma, acral, skin cancer

(*Am J Dermatopathol* 2015;37:892–897)

INTRODUCTION

Malignant melanoma is the most fatal type of skin cancer. Traditional melanoma classification has been based on histological subtype or anatomic location. However, recent evidence suggests that melanoma consisted of a group of diseases characterized by distinct molecular mutations. These mutations affect disease behavior but provide unique opportunities for targeted therapy.

Of the 4 major histological subtypes of cutaneous melanoma (superficial spreading, nodular, lentigo maligna, and acral lentiginous), acral lentiginous melanoma (ALM) is the least common. This subtype, first described by Reed,[1] represents approximately 2%–10% of all melanoma cases.[2–7] Despite its rarity, acral melanoma is the most common type of melanoma diagnosed in persons of darker skin color.[7] The pathogenesis of ALM remains poorly understood. ALM is thought to carry a worse prognosis when compared with other melanoma histological subtypes or other anatomic sites.[4,8–12] Additionally, it carries a high number of genomic alterations compared with other melanoma subtypes, and most of them account for a smaller proportion of genome.[13,14]

Several general features have been reported to be significant with regard to the progression of melanoma, such as alterations in tumor cell proliferation and cell cycle regulation, cell adhesion proteins, and tumor-associated angiogenesis.[15] Recent reports on the molecular events in melanoma have demonstrated a stepwise progression of genetic alterations in tumor-promoting events, resulting in aggressive forms of the neoplasm.[16–19] In this complex scenario, a molecular cascade is believed to be important in the pathogenesis of cutaneous melanomas—the MAP kinase pathway, which has been implicated in cell growth and survival.[16,17,19,20]

There is increasing evidence that activation of the MAPK pathway is associated with the development of melanoma. Five to thirty-six percent of primary melanomas have neuroblastoma RAS virus homolog (NRAS) mutations. Mutations in BRAF are observed in up to 80%–90% of melanomas, but they also exist in most benign melanocytic nevi.

Most studies have examined the MAPK pathway in cutaneous melanomas, but the key components in this important pathway have not been investigated with regard to melanogenesis in primary ALM. Additionally, there is relatively little data focusing on the frequency of BRAF mutations in acral melanoma, and there are only few studies that combine this with analysis of the cascade of RAS, BRAF, MEK 1/2, and ERK 1/2 proteins.[21] These were in Japanese populations where the overall frequency of cutaneous melanoma is lower than that in Western countries. This lack of knowledge undermines an emerging goal in cutaneous melanoma research to determine whether acral melanoma has different molecular pathways of tumor progression. Achieving this goal is critical for the development of tailored treatment of cutaneous melanoma.

To assess the status of the MAP kinase pathways in the pathogenesis of primary lentiginous acral melanomas, we

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The authors declare no conflicts of interest.

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examined the central components of these cascades by immunohistochemistry in 16 primary lentiginous acral melanomas by tissue microarray (TMA).

MATERIALS AND METHODS

Patients

The records of 16 patients who were diagnosed for acral melanoma in 2 hospitals in Brazil (Hospital das Clínicas, Medical School, University of São Paulo and AMO Oncological Clinic Care, Bahia, Brazil) over the past 5 years were reviewed. Clinical features such as gender, age, site of tumor, and presence of metastasis were assessed. The histopathology of all cases was reexamined.

Tissue Microarray

To construct the TMA, hematoxylin and eosin–stained sections were analyzed, and a representative area of the tumor was marked on the slide. The tissue that corresponded to the selected areas was sampled from the donor block using a tissue microarrayer (Beecher Instruments, Silver Springs, MD). Each sample was arrayed once with a 1.0-mm-diameter core, spaced 0.2 mm apart. The sample consisted of 2 malignant cores from different areas of the tumor, placed coordinately in the array. Separate samples of normal acral skin were used as controls. After the array was completed, slides that contained serial 4-mm sections of the TMA block were prepared. Two slides, 40 mm apart, were used for the immunohistochemical study.

Immunohistochemistry

TMA slides were deparaffinized in xylene, hydrated in graded ethanol, and subjected to immunohistochemistry. Information on the primary antibodies, source, clones, antigen retrieval, and positive controls is shown in Table 1.

Immunohistochemical reactions were developed with "EnVision" alkaline phosphatase (Dako code 4018-1, Carpinteria, CA). Liquid Permanent Red (Dako, Carpinteria, CA) was used as the chromogen to distinguish the reaction (in red) from the melanin pigment (black or brown) of neoplastic cells. The slides were lightly counterstained with Carazzi hematoxylin and mounted with glass cover slips and resin.

Normal skin specimens were used as normal controls. Positive controls were performed as per the manufacturer's instructions (Table 1), and negative controls were obtained by incubating the slides with nonimmune serum. All immunohistochemical reactions were run in duplicate and analyzed on a conventional optical microscope that was equipped with a digital camera.

The results were evaluated by semiquantitative analysis of the stained area in each core as follows: negative when 30% of the core showed some positivity and positive when more than 30% of the core area was positive. Cytoplasmic staining for BRAF, MEK1, MEK2, ERK1, and ERK2 was also considered positive. For several cases, nuclear ERK1 and ERK2 staining was considered positive.

RESULTS

The clinical–demographic and histopathologic profiles of the cohort are summarized in Table 2. Patients' age ranged from 13 to 91 years, and the average age at the time of diagnosis was 57 years. There were 9 males (56.25%) and 7 (43.75%) females, and the melanomas primarily affected blacks/Afro-Brazilians (14 patients, 87.5%) versus whites (2 patients, 12.5%). Most patients (81.25%) had sole lesions. In situ melanomas were observed in 9 cases (56.25%). Invasive melanomas were observed in 7 cases (43.75%). Long distance metastases were noted in only 3 cases.

The expression of MAP kinase cascade proteins varied: BRAF was positive in 57.14% of cases, MEK2 was positive in 61.53% of cases, ERK1 and ERK2 were overexpressed in most cases (71.42% and 71.42%, respectively), RAS were almost absent (92.3% of cases were negative), and MEK1 was not expressed in any case. Low expression of RAS and positivity for ERK1 and ERK2 were most observed in invasive cases with high Breslow thickness and Clark level of II or higher.

Table 3 shows a separate analysis for the MAP kinase cascade proteins between the in situ ALM group and the invasive ALM group.

Acral melanomas histopathology and the results of the immunostaining of the key components of RAS–BRAF–MEK–ERK cascade by immunohistochemistry are illustrated in Figure 1.

DISCUSSION

Acral lentiginous melanoma distinguishes itself from the other subtypes for many features, both histological and clinical prognostic. This study extends current knowledge of the analysis of RAS/RAF/MEK/ERK signaling pathway in ALM.

Recent studies have attempted to examine the pathogenesis of melanoma by combining a molecular evaluation

TABLE 1. Information on the Primary Antibodies

Primary Antibodies	Source/Clones	Titration	Antigen Retrieval
RAS	Abcam F132-62	1:10	Citric buffer (pH 6.0)/pressure cooker
BRAF	Abcam 1H12	1:500	Citric buffer (pH 6.0)/pressure cooker
MEK1	Abcam E342	1:100	Citric buffer (pH 6.0)/pressure cooker
MEK2	Abcam Y78	1:150	Citric buffer (pH 6.0)/pressure cooker
ERK1	Abcam (ab57444)	1:300	Citric buffer (pH 6.0)/pressure cooker
ERK2	Abcam 5E8E1	1:500	Citric buffer (pH 6.0)/pressure cooker

TABLE 2. Summary of Clinical Information and Histopathologic Analysis of 16 Cases of Acral Melanoma and Expression of MAP Kinase Cascade Proteins

Variable	Overall, %	BRAF	RAS	ERK1	ERK2	MEK1	MEK2
All patients	16 (100)	8 (57.14)	1 (7.69)	10 (71.42)	10 (71.42)	0	8 (61.53)
Sex							
Male	9 (56.25)	5 (62.5)	0	7 (70)	7 (70)	0	6 (75)
Female	7 (43.75)	3 (37.5)	1 (100)	3 (30)	3 (30)	0	2 (25)
Age, yr							
0–39	2 (12.5)	0	0	0	0	0	0
40–60	8 (50)	4 (50)	0	4 (40)	4 (40)	0	4 (50)
61–80	5 (31.25)	3 (37.5)	1 (100)	5 (50)	5 (50)	0	3 (37.5)
>80	1 (6.25)	1 (12.5)	0	1 (10)	1 (10)	0	1 (12.5)
Site							
Finger nail	2 (12.5)	0	0	1 (10)	1 (10)	0	0
Palm	1 (6.25)	0	0	0	0	0	0
Sole	13 (81.25)	8 (100)	1 (100)	9 (90)	9 (90)	0	8 (100)
Thickness, mm							
In situ	9 (56.25)	3 (37.5)	1 (100)	3 (30)	3 (30)	0	1 (12.5)
≤1.00	4 (25)	4 (50)	0	2 (20)	2 (20)	0	2 (25)
1.01–2.00	2 (12.5)	1 (12.5)	0	4 (40)	4 (40)	0	4 (50)
2.01–4.00	1 (6.25)	0	0	1 (10)	1 (10)	0	1 (12.5)
>4.00	0	0	0	0	0	0	0
Clark level							
I	8 (50)	3 (37.5)	1 (100)	2 (20)	2 (20)	0	0
II	2 (12.5)	2 (25)	0	2 (20)	2 (20)	0	2 (25)
III	3 (18.75)	2 (25)	0	3 (30)	3 (30)	0	3 (37.5)
IV	2 (12.5)	0	0	2 (20)	2 (20)	0	2 (25)
V	1 (6.25)	1 (12.5)	0	1 (10)	1 (10)	0	1 (12.5)
Ulceration							
Yes	3 (18.75)	0	0	2 (20)	2 (20)	0	1 (12.5)
No	13 (81.25)	8 (100)	1 (100)	8 (80)	8 (80)	0	7 (87.5)
Metastasis							
Yes	3 (18.75)	0	0	2 (20)	2 (20)	0	0
No	13 (81.25)	8 (100)	1 (100)	8 (80)	8 (80)	0	8 (100)

with histological and epidemiological techniques to identify its causal factors,[16,22–24] providing evidence that cutaneous melanoma develops through disparate molecular pathways that depend on the makeup of the host and the external environment to which the host is exposed.[16] In our study, acral melanoma affected primarily black patients (87.5%), which is consistent to literature information.

Our analysis of the MAPK pathway activation detected RAS protein in only 1 case (7.69%), which was in situ acral melanoma, as observed by Takata et al.[21] Our result is consistent with the previous reports showing rare NRAS mutations in melanomas arising from the mucous membrane or unexposed skin.[25–27]

TABLE 3. Analysis for the MAP Kinase Cascade Proteins in the in situ ALM Group and the invasive ALM Group

Protein	Overall, %	In Situ ALM, %	Invasive ALM, %
BRAF	8 (57.14)	3 (37.5)	5 (62.5)
RAS	1 (7.69)	1 (100)	0
ERK1	10 (71.42)	3 (30)	7 (70)
ERK2	10 (71.42)	3 (30)	7 (70)
MEK1	0	0	0
MEK2	8 (61.53)	1 (12.5)	7 (87.5)

The immunostaining was localized to the cell membrane and cytoplasm. Mutations in RAS genes have also been detected in 10%–37% of sporadic melanomas. Although some studies have linked NRAS to the progression of melanomas to metastatic disease, others consider this aberration an early event in the development of melanoma because oncogenic mutations are detected in congenital melanocytic nevi, melanomas in situ, and primary melanomas in the horizontal growth phase.[21,28] Most of our cases were negative for RAS, including lesions with distant metastasis.

The overall frequency of BRAF in our primary acral melanoma samples was 57.14%, which is higher than those of previous reports of acral melanomas in European, Asian, and United States population.[21,29–32] The reasons for this could be accounted by ethnic differences between Brazilian patients and others that have been reported. In fact, little further information has become available about the frequency of BRAF in primary acral melanoma.

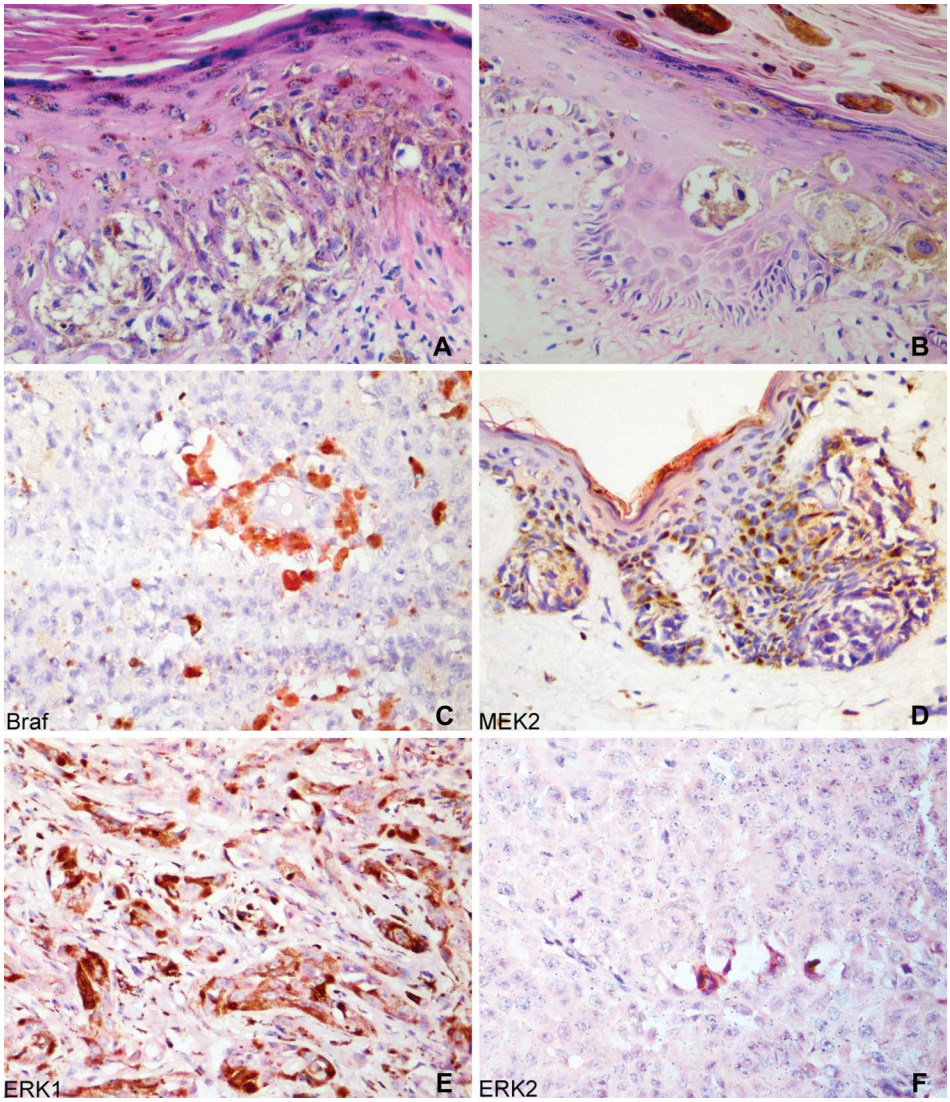

FIGURE 1. A and B, Acral melanoma: histopathologic aspects of nests of neoplastic melanocytes within the epidermis (original magnification, ×250 and ×150). C, Expression of BRAF in acral melanomas group of positive neoplastic cells surrounding a small lymphatic vessel (permanent red immunohistochemistry, original magnification, ×400). D, Expression of MEK2 in neoplastic melanocytes at the invasion front (permanent red immunohistochemistry, original magnification, ×250). E, Cytoplasmic expression of ERK1 widely distributed in neoplastic melanocytes (permanent red immunohistochemistry, original magnification ×400). F, Small groups of neoplastic melanocytes positive for ERK2 (permanent red immunohistochemistry, original magnification ×400).

BRAF can be expressed in the epidermis, sebaceous glands, fibroblast, and endothelium in a diffuse pattern; in melanoma, intense immunostaining is reported.[33] Additionally, BRAF expression can occur in up to 80% of primary cutaneous melanomas and in most benign melanocytic nevi, most of which do not progress to melanoma.[23] The presence of BRAF in nevi strongly suggests that BRAF activation is necessary but not sufficient for the development of melanoma.[15]

According to Uribe et al,[33] the absence of MAPK activation in the presence of BRAF expression, especially in congenital nevi and atypical nevi, cannot be explained by the absence of BRAF; instead, it is attributed to the existence of additional mechanisms that activate the pathway or inhibitory mechanisms that reduce the expression or activity of BRAF and other proteins that are associated with activation of MAPK phosphatases and inhibitors of RAF kinases.

Nevertheless, studies have reported that BRAF positivity is most common in melanomas that arise in sites where the skin is not chronically sun exposed. There is increasing evidence that BRAF expression is an early event in the development of melanoma, which might emerge through inaccurate DNA repair after UV-induced photoproduct formation.[16,17]

We observed that MEK2 was positive in 61.53% of our cases, most of them (87.5%) were invasive melanoma with high Breslow thickness. Only one of our in situ melanoma cases was positive for MEK2. In contrast, MEK1 was not present in any cases.

The overlapping and unique functions of these isoforms at each level of the RAS/RAF/MEK/ERK signaling pathway are only beginning to be determined. Often, no distinction is made between the functions of MEK1 and MEK2 or between ERK1 and ERK2, and few studies have examined the individual contributions of each isoform.[25,34,35] Additionally, only few studies evaluated the expression of MEK1/2 and ERK1/2 in ALM.[21]

The only MEK1 and MEK2 substrates that have been identified are ERK1 and ERK2, which are phosphorylated by

MEK1 and MEK2. Based on earlier genetic studies, MEK1 and MEK2 are functionally redundant in certain contexts.[32] Conversely, according to Emery et al,[35] whether the MEK dependency in BRAF mutant melanomas exists in other MAP kinase-driven contexts remains unknown. Preclinical studies suggest that certain NRAS mutant melanomas are sensitive to inhibition of RAF or MEK.

ERK1 and ERK2 were expressed in most of our cases (71.42%), which is similar to the 78% incidence of acral melanomas reported previously.[21] Like MEK2, most of our ERK-positive cases were also invasive melanomas with high Breslow thickness. Additionally, ERK1 was expressed cytoplasmically in most cases. According to previous reports, increased activity of ERK1/2 proteins has been implicated in fast melanoma cell growth, enhanced cell survival, and resistance to apoptosis.[15]

Cutaneous melanomas generally had stronger ERK1/2 expression (up to 50%–70% positive cells), but this staining was unrelated to the presence of BRAF mutations.[22,23,36,37] By multivariate analysis, the absence of cytoplasmic activation of ERK was an independent adverse prognostic marker, contradicting results that have failed to note any impact of p-ERK expression on survival.

In our study, all ERK1/2-positive cases were negative for RAS expression. This result suggests that the MAPK signaling pathway is constitutively activated in the majority of acral melanomas without NRAS mutations.[21] Similar results have already been reported in uveal melanomas. Uveal melanomas had no NRAS mutations, but constitutive activation of the MAPK pathway was demonstrated in 86% of samples (36 of 42) with immunohistochemistry,[38] suggesting that no NRAS mutations may be a prerequisite for the activation of the MAPK signaling pathway. Several alternative ways of activation of MAPK signaling have recently been demonstrated, including autocrine growth factor stimulation,[39] epigenetic inactivation of RAS association domain family protein 1 (RASSF1A),[40] downregulation of an ERK signaling inhibitor SPRY2,[41] loss or reduction of RAF kinase inhibitory protein (PKIP),[42] and overexpression of wild-type BRAF in part as a result of gene amplification.[43] Specific pathways leading to constitutive activation of ERK without RAS mutations in acral melanomas remain to be established.

These aspects of the MAPK pathway require further examination in acral melanomas between different populations. Nevertheless, our results highlight significant alterations in the MAP kinase cascades that are related to histological indicators of prognosis in primary acral melanomas. The study of known mutations and identification of new potential targets must continue in an effort to develop more effective therapies for this disease.

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