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PREMALIGNANT QUIESCENT MELANOCYTIC NEVI DO NOT EXPRESS THE MHC CLASS I CHAIN-RELATED PROTEIN A

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Abstract The MHC class I chain-related protein A (MICA) is an inducible molecule almost not expressed by normal cells but strongly up-regulated in tumor cells. MICA-expressing cells are recognized by natural killer (NK) cells, $CD8^+$ $\alpha\beta$TCR and $\gamma\delta$TCR T lymphocytes through the NKG2D receptor. Engagement of NKG2D by MICA triggers IFN-γ secretion and cytotoxicity against malignant cells. Although most solid tumors express MICA and this molecule is a target during immune surveillance against tumors, it has been observed that high grade tumors from different histotypes express low amounts of cell surface MICA due to a metalloprotease-induced shedding. Also, melanomas develop after a complex process of neotransformation of normal melanocytes. However, the expression of MICA in premalignant stages (primary human quiescent melanocytic nevi) remains unknown. Here, we assessed expression of MICA by flow cytometry using cell suspensions from 15 primary nevi isolated from 11 patients. When collected material was abundant, cell lysates were prepared and MICA expression was also analyzed by Western blot. We observed that MICA was undetectable in the 15 primary nevi (intradermic, junction, mixed, lentigo and congenital samples) as well as in normal skin, benign lesions (seborrheic keratosis), premalignant lesions (actinic keratosis) and benign basocellular cancer. Conversely, a primary recently diagnosed melanoma showed intense cell surface MICA. We conclude that the onset of MICA expression is a tightly regulated process that occurs after melanocytes trespass the stage of malignant transformation. Thus, analysis of MICA expression in tissue sections of skin samples may constitute a useful marker to differentiate between benign and malignant nevi.

Key words: melanoma, MICA, skin

Resumen ***Los nevos melanocíticos premalignos quiescentes no expresan la molécula* MHC class I chain-related protein A**. *MHC class I chain-related protein A* (MICA) es una molécula casi ausente en células normales pero sobre-expresada por células tumorales, que promueve el reconocimiento por células citotóxicas naturales (natural killer o NK) y por linfocitos T $CD8^+$ $\alpha\beta$ y $\gamma\delta$ a través del receptor NKG2D, lo que dispara la secreción de IFN-γ y la citotoxicidad contra las células malignas. Aunque la mayoría de los tumores sólidos expresan MICA y esta molécula constituye un blanco durante la inmunovigilancia contra tumores, tumores de alto grado expresan bajos niveles de MICA en superficie celular debido al clivaje inducido por metaloproteasas. Asimismo, los melanomas se desarrollan luego de la neotransformación maligna de melanocitos. Sin embargo, se desconoce la expresión de MICA en estadios premalignos (nevos melanocíticos). En este trabajo analizamos la expresión de MICA en 19 nevos primarios de 11 pacientes mediante citometría de flujo. Cuando el material fue suficiente, también analizamos la expresión de MICA por *Western blot*. En ninguno de los 15 nevos primarios (intradérmicos, *junction*, mixtos, lentigo y congénitos) ni en muestras de piel normal, lesiones benignas (queratosis seborreica), premalignas (queratosis actínica) y cáncer benigno basocelular, detectamos expresión de MICA. Contrariamente, un melanoma primario de diagnóstico reciente mostró intensa expresión de MICA en superficie celular. Nuestros resultados indican que el inicio de la expresión de MICA ocurre una vez que la célula ha traspasado el estadio de transformación maligna, por lo que el análisis de su expresión en secciones de piel podría constituir un marcador útil para diferenciar nevos benignos de melanomas malignos.

Palabras clave: melanoma, MICA, piel

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Neoplastic transformation is the result of the accumulation of a minimal number of mutations in normal cells that confer loss of cell cycle control, resistance to apoptosis, self-perpetuation capacity. In some cases, these tumor cells also acquire the ability to invade distant tissues (metastasis). This complex process occurs in immunocompetent hosts where the immune system sculpts the tumor phenotype due to the immunological pressure exerted on the tumor cells1. Cytotoxic cells such as natural killer (NK) cells, $CD8^+$ $\alpha\beta$TCR and $\gamma\delta$TCR T lymphocytes critically contribute to anti-tumor immunity in part due to expression of the NKG2D receptor[2]. Different ligands for NKG2D (NKG2DLs) have been described in humans, such as the MHC class I chain-related proteins A and B (MICA and B), the glycosylphosphatidylinositol (GPI)-bound surface molecules UL16 binding protein (ULBP)-1, -2 and -3, and the retinoic acid early inducible gene transcripts (Raet)-1E and G[3]. Many NKG2DLs are expressed on tumor cells of different origins[4]. In particular, MICA has been detected in melanoma cells[5]. Expression of some NKG2DLs is induced by the DNA damage response[6] and other oncogenic pathways that are constitutively active in tumor cells[7-9]. Also, MICA expression is regulated by NF-κB[10]. However, high grade malignant tumors express less MICA than low grade tumors[11] which is due to proteolytic shedding driven by tumor-secreted metalloproteases[12, 13]. It has been observed that malignant melanomas, which arise from neotransformed melanocytes, usually express MICA[5, 14]. However, benign nevi (junctional, compound, and intradermal nevi) retain melanin and form nests of cells that occasionally progress to malign melanomas but the expression of MICA in these preneoplastic lesions remains unknown. Therefore, we addressed the expression of this NKG2DL in 19 samples from benign quiescent nevi obtained from 11 patients. The characteristics of these nevi are given in Table 1. Also, one sample of a patient with malignant melanoma (grade II/III) was obtained at the moment of diagnosis. Procedures were followed in accordance with the Declaration of Helsinki and had the approval by the Institutional Ethical Committee *(Hospital de Clínicas)*. Written informed consent was obtained from each patient. Patients were selected according to the clinical diagnosis. Dermatoscopic analysis confirmed that the biopsed sample was a benign quiescent nevus. Briefly, 3 mm diameter punches were obtained from skin with nevi. Samples were divided in two. One part was used for the confirmation of the diagnosis by the pathologist. The other was used for the preparation of single cell suspensions by mechanical disruption of the tissue samples, and processed for flow cytometry analysis. When a sufficient number of cells were recovered, cell lysates were also obtained for SDS-PAGE and Western blot analysis. Also, the human melanoma cell lines IIB-MEL-IAN and A375N were used[14]. Cells were cultured in 10% fetal bovine serum (NatoCor, Córdoba, Argentina) in Dulbecco´s modified Eagle´s medium (DMEM, Sigma), supplemented with sodium pyruvate, glutamine, and antibiotics (all from Sigma).

Expression of cell surface MICA was analyzed by flow cytometry (FC) with the anti-MICA/B mAb D7[14] and PE-labeled anti-mouse IgG (DAKO) in a FACScalibur flow cytometer (BD). Specific fluorescence index (SFI) was calculated as the mean fluorescence produced by the D7 mAb divided by the mean fluorescence produced by the IC mAb. SDS-PAGE of cell lysates and Western blot were performed using anti-MICA polyclonal Ab, as described[14]. Bound Ab were detected using HRP-labeled anti-rabbit IgG (Bio-Rad) and chemiluminescence. No bands were observed in Western blots incubated with normal rabbit IgG.

First, we analyzed cell suspensions of the nevi obtained from patients enrolled in this study for cell surface expression of MICA. We observed that none of the 15 samples of primary nevi (intradermic, junction, mixed, lentigo and congenital samples) as well as in normal skin, benign lesions (seborrheic keratosis), premalignant lesions (actinic keratosis) and benign basocellular cancer derived from 11 different patients showed staining with the anti-MICA/B mAb D7. Representative staining patterns from 2 different samples are shown in Fig. 1A. Conversely, one freshly isolated melanoma and the IIB-MEL-IAN cell line, which is known to express cell surface MICA, (14), showed intense staining, as shown in Fig. 1B.

TABLE 1.- *Characteristics of the samples used in this study*

Patient	Sample
CF	Basocellular nevus
	Seborrhoeic keratosis
	Intradermic cellular nevus
	Actinic keratosis
LM	Verrucous nevus
	Intradermic cellular nevus
	Intradermic cellular nevus
JC	Junction nevus
	Lentigo
TA	Intradermic cellular nevus
HL	Mixed cellular nevus
CG	Intradermic cellular nevus
	Intradermic cellular nevus
	Intradermic cellular nevus
CR	Intradermic cellular nevus
HG	Intradermic cellular nevus
EP	Intradermic cellular nevus
LS	Intradermic cellular nevus
NM	Congenic melanocytic nevuswith sclerodermiform halus
FR	Congenic melanocytic nevuswith nodular melanoma

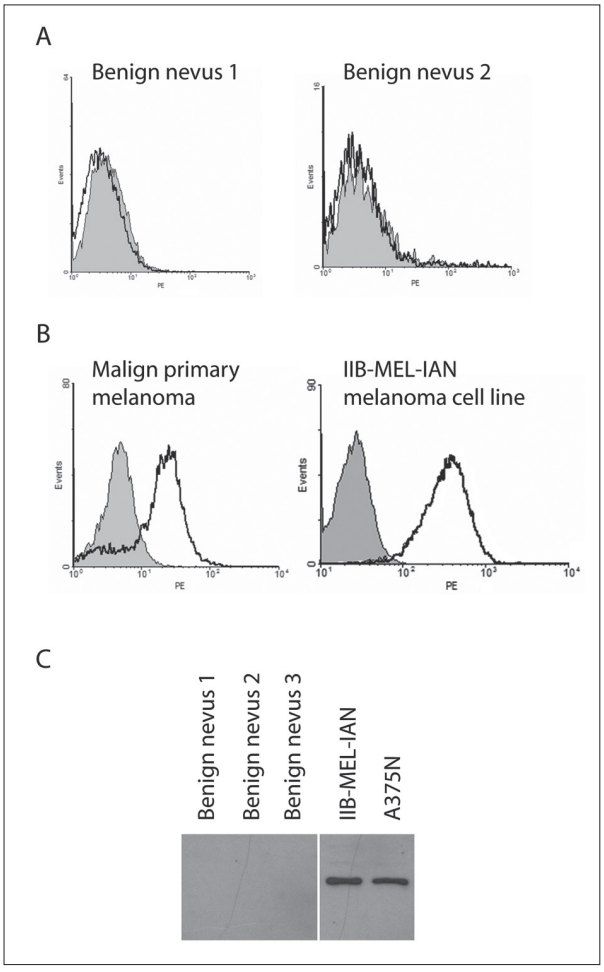

Fig. 1.– Analysis for MICA expression in benign quiescent nevi and melanomas Expression of MICA was analyzed in 2 benign human quiescent nevi (A) or a primary melanoma (at the time of diagnosis) and the melanoma cell line IIB-MEL-IAN (B) by flow cytometry. Open histograms: anti-MICA/B mAb. Filled histograms: isotype negative control mAb. Also, expression of MICA was analyzed by Western blot in cell lysates of 3 benign quiescent nevi and 2 melanoma cell lines. Western blots were normalized against β-actin (not shown).

Previously, in our laboratory we described a novel tumor immune escape mechanism based on an intracellular retention of immature forms of the MICA polypeptide which conferred immune privilege to some human melanoma cells[14]. To investigate whether similar intracellular pool of MICA may exist in benign nevi, in cases in which we had sufficient number of cells in order to run flow cytometry experiments and prepare cell lysates, MICA expression was also analyzed by Western blot (Fig. 1C). Again, we were unable to detect MICA in benign quiescent nevi. In contrast, cell lysates of IIB-MEL-IAN and A375N melanoma cell lines exhibited easily detectable amounts of MICA. These results indicate that benign quiescent nevi, in opposition to malign melanomas, do not express cell surface MICA.

Although one of the most important differences between benign nevi and melanomas is the ability of melanomas to proliferate without control and eventually metastasize, it is sometimes difficult to distinguish between benign and malign melanocytes[15]. Also, the presence of infiltrating $CD8^+$ and NK cells in benign nevi is not strictly and systematically associated with a benign or malign diagnosis. Thus, correct diagnosis remains uncertain in some cases. In our study, we established that expression of MICA was restricted to malign neotransformed melanocytes (melanomas). Since benign quiescent melanocytic nevi did not express MICA on their cell surface or as intracellular deposits, we believe that neo-expression of MICA is an event that takes place after the cell achieved malign transformation into a melanoma, a characteristic that would put it under the scrutiny of NKG2D-expressing cells. Such critical point would take place once the melanocyte turned on a transcriptional program and a DNA damage response that would be one of the first steps contributing to the acquisition of the malignant phenotype. Assessment of MICA expression may therefore contribute to the differential diagnosis of benign nevi *vs.* malign melanomas.

In summary, the results here described represent the first analysis of expression of MICA in benign melanocytic nevi and lead to the conclusion that the onset of MICA expression is a tightly regulated process that occurs after melanocytes have trespassed the stage of malignant transformation. Therefore, analysis of MICA expression in tissue sections of skin samples may constitute a marker to differentiate between benign and malignant nevi.

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Conflict of interest statement: Authors declare that they have no conflicts of interest.

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Salgo de Estados-Unidos, mi estimado amigo, en aquel estado de excitación que causa el espectáculo de un drama nuevo, lleno de peripecias, sin plan, sin unidad, erizado de crímenes que alumbran con su luz siniestra actos de heroísmo i abnegación, en medio de los esplendores fabulosos de decoraciones que remedan bosques seculares, praderas floridas, montañas sañudas, o habitaciones humanas en cuyo pacífico recinto, reinan la virtud i la inocencia. Quiero decirle que salgo triste, pensativo, complacido i abismado; la mitad de mis ilusiones rotas o ajadas, miéntras que otras luchan con el raciocinio para decorar de nuevo aquel panorama imaginario en que encerramos siempre las ideas cuando se refieren a objetos que no hemos visto, como damos una fisonomía i un metal de voz al amigo que solo por carta conocemos. Los Estados-Unidos son una cosa sin modelo anterior, una especie de disparate que choca a la primera vista, i frustra la expectación, pugnando con las ideas recibidas, i no obstante este disparate inconcebible es grande i noble, sublime a veces, regular siempre; i con tales muestras de permanencia i de fuerza orgánica se presenta, que el ridículo se deslizaría sobre su superficie como la impotente bala sobre las duras escamas del caiman.[…]

Domingo Faustino Sarmiento (1811-1888)

Viajes por Europa, Africa y América 1845-1847 y Diario de gastos. Edición crítica de Javier Fernández (Coordinador). Buenos Aires: ALLCA XX-FCE Argentina, 1993.
Carta al Sr. Don Valentin Alsina. Noviembre 12 de 1847. p 290. Grafía original conservada.