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Immunohistochemical Markers in the Differential Diagnosis of Melanoma and Nevus in Humans

Marcadores Inmunohistoquímicos en el Diagnóstico Diferencial de Melanoma y Nevos en Humanos

Nelson Quilaqueo[1]; Felipe Navarrete[1]; Cristian Sandoval[2,3]; Ignacio Roa[4]; Mario Pellón[5] & Marco Paredes[1]

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SUMMARY: Immunohistochemistry allows in situ detection of cell and extracellular components through specific antibodies. The objective was to compare the immunohistochemical expression patterns of the S-100, HMB-45 and MART-1 proteins for differential diagnosis of malignant melanoma and melanocytic nevus in human skin biopsies. Thirty-nine biopsies of human tissue were used. They were divided into two groups: 19 in malignant melanoma and 20 in melanocytic nevi. Next, the samples were fixed with paraformaldehyde and processed following the protocol for inclusion. Then, immunohistochemical staining was performed. Finally, the histological and qualitative analysis of the samples was carried out. S-100, HMB-45, and MART-1 markers showed positive immunoreaction in melanoma biopsies. HMB-45 marker was generally present with weaker expression than S-100 and MART-1 in melanocytic nevus biopsies. No expression pattern was observed which specifically associates one or more markers with some types of histopathological diagnosis. Immunohistochemistry is fundamental in differential diagnosis of melanomas and melanocytic nevi. However, there is no antibody or set of antibodies which allows unequivocal diagnosis between melanoma and nevus. It is therefore necessary to analyze with care the expression pattern and location of the lesion using standard morphological characteristics.

KEY WORDS: Antigen antibody complex; Immunohistochemistry; Immunohistochemical markers; Pathology.

INTRODUCTION

Immunohistochemistry (IHC) combines a series of techniques and protocols which allow in situ detection of cell and extracellular components (antigens) through specific antibodies, using enzymatic detection systems (Montuenga *et al*., 2009), based on their high specificity and affinity in recognizing and bonding to molecules. Conjugation with enzymes or fluorescent substances allows detection of minute quantities of molecules present in the tissue studied. Today, IHC is a fundamental diagnostic tool in dermal pathologies (Fuertes *et al*., 2013); it offers advantages over other methods (RIA, Western Blot, ELISA), and ensures that the relations between the different components analyzed are conserved, thus allowing cell identification (Martín-Lacave & García-Caballero, 2014). IHC is used in dermal pathologies to determine the strain or differentiation of a neoplasia, and to define a prognosis; to distinguish benign from malignant neoplastic processes; to establish the molecular architecture of a tissue; and to detect infectious agents in cells or tissues (Fuertes *et al*.). An example of this is the study of melanoma, diagnoses of which have increased strongly in recent decades (Geller *et al*., 2013). One objective of these studies is to identify antibodies which will allow it to be reliably distinguished from other, non-melanocytic pathologies (Ferringer, 2015), such as melanocytic nevus. This search, however, has proved fruitless to date.

The most frequently used marker for diagnosing melanocytic lesions is the S-100 protein, which presents high sensitivity (between 97 % and 100 %), but low specificity (between 75 % and 87 %) (Ohsie *et al*., 2008; Hoang *et al*., 2010). This is because it acts as a marker for

1 Departamento de Ciencias Básicas, Facultad de Medicina, Universidad de La Frontera, Temuco, Chile.
2 Departamento de Ciencias Preclínicas, Facultad de Medicina, Universidad de la Frontera, Temuco, Chile.
3 Escuela de Tecnología Médica, Facultad de Salud, Universidad Santo Tomás, Los Carreras 753, Osorno, Chile.
4 Departamento de Ciencias Básicas Biomédicas, Facultad de Ciencias de la Salud, Universidad de Talca, Chile.
5 Departamento de Especialidades Médicas, Facultad de Medicina, Universidad de La Frontera, Temuco, Chile.
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normal melanocytes, dendritic (Langerhans) cells, histiocytes, chondrocytes, lymphocytes, skeletal and cardiac striated muscle, Schwannocytes, epithelial and myoepithelial cells of the mammary, salivary and sudoriferous glands, and cells of the glia (Morrison & Prayson, 2000). It should be noted that neoplasias derived from these cells also express the S-100 protein, although not uniformly (Patel *et al*., 2002). As a result, it is necessary to use other markers, like HMB-45 and MART-1 (Melan-A), generating a pool of IHC detection techniques which help to obtain more accurate diagnoses (Cho *et al*., 1990).

The antibody against the HMB-45 protein is a pre-melanosome cytoplasm marker which is relatively useful for melanocytic differentiation; however, although it is more specific than the S-100 protein, it is much less sensitive, as studies show that the S-100 protein is present in 78 % to 93 % of cases of melanoma (Gown *et al*., 1986; Palazzo & Duray, 1989; Smoller *et al*., 1989; Sun *et al*., 1990; Skelton 3rd *et al*., 1991; Sundram *et al*., 2003; Ohsie *et al*.). It is known that the expression of the HMB-45 marker is variable, being highest in primary melanoma (77 % to 100 %) and lower in metastasis (58 % to 83 %) (Ordóñez *et al*., 1988; Bishop *et al*., 1993; Zubovits *et al*., 2004).

The melanoma antigen recognized by T cells, also called MART-1 (Melan-A), is a cytoplasmic protein of melanosome differentiation. Today, it is known that this protein detects different types of melanocytic lesions in a very similar pattern to S-100, the difference being that MART-1 less frequently detects desmoplastic melanomas (Busam *et al*., 1998; Prasad *et al*., 2001; Mangini *et al*., 2002; Yu *et al*., 2005). It is, therefore, thought that the MART-1 protein may be a specific antigen of melanocytic differentiation for melanoma cell lines (Chen *et al*., 1996; Jäger *et al*., 1996); and, therefore, more useful in diagnosis of pathologies of this type.

The objective of the present study objective was to compare the immunohistochemical expression patterns of the S-100, HMB-45 and MART-1 proteins for differential diagnosis of malignant melanoma and melanocytic nevus in human skin biopsies.

MATERIAL AND METHOD

Biological material. Biopsies of human skin with histopathological diagnosis were obtained. They were divided into two groups by their diagnosis: 19 malignant melanoma and 20 melanocytic nevi. Then, for histological and qualitative analysis, the samples were fixed with paraformaldehyde at 4 % and processed following the conventional protocol for inclusion in Paraplast Plus (Paraplast® Embedding Media, McCormick Scientific, St. Louis, MO, USA). Three histological sections of 5 mm thickness were taken from each sample.

Immunohistochemical analysis. Paraplast Plus was eliminated with xylol and the samples were hydrated with a battery of ethanol in decreasing concentrations. The activity of the endogenous peroxidase was eliminated by incubation with perhydro™ (H_2O_2) at 0.3 % for 10 minutes, followed by washing three times for 2 minutes each with phosphate buffer saline (PBS). Non-specific sites were blocked by incubation with blocking solution (bovine serum albumin 2 % in PBS) for 20 min. Then, the sections were incubated with the anti-S-100, anti-HMB-45 and anti-MART-1 primary antibodies (Cell Marque®, Sigma-Aldrich Co., St. Louis, MO, USA), diluted at 1:1000 under saturated moisture conditions for 5 hours at 37 ºC, and finally washed three times in PBS for 2 minutes. Detection was carried out with the ABC (avidin-biotin complex) indirect method, using the VECTASTAIN® Kit (Vector Laboratories®, Burlingame, USA), following the manufacturers' instructions. The reaction was analyzed using diaminobenzidine solution (0.5 %) and perhydro™ (1 %) (DAB-H_2O_2) in PBS for 5 to 10 minutes. The samples were washed with distilled water, then contrast staining was carried out with Harris hematoxylin at 3 %, incubated for 2 minutes. Finally, the samples were washed with tap water, dehydrated with a battery of ethanol at increasing concentrations, passed through xylol and mounted with mounting medium (Entellan, Merck & Co., Kenilworth, NJ, USA).

Analysis and capture of digital images. The processed samples were viewed under a Leica® DM 750 microscope and photographed with a Leica® ICC50 HD camera, and processed with the Image Pro Plus 6.0 Software (Media Cybernetics Inc., Silver Spring, MD, USA).

Qualitative analysis of immunohistochemical expression. Qualitative analysis was carried out using score ranges to assess the level of immunohistochemical expression described by Fitzgibbons *et al*. (2014). A value range was established from "negative" (-) to "positive" (+), reported by a different number of "+" signs. The most common spectrum of categories to describe the level of immunohistochemical expression includes: "negative" (-), "weak" (+), "moderate" (++), "strong" (+++) and variations of these (Raica *et al*., 2007; Kapoor & Deshmukh, 2012; Bösmüller *et al*., 2013; Kukreja *et al*., 2013).

RESULTS

The immunohistochemical expression analysis of the S-100, HMB-45, and MART-1 markers in histological preparations from biopsies with diagnosis of melanoma, in 19 cases analyzed, shows that these three markers present positive immunoreaction (Figs. 1 and 2). In qualitative terms, no cleardifference was observed in the intensity of expression of these markers according to the type of histopathological

Table I. Qualitative analysis of immunohistochemical expression patterns of proteins S-100, HMB-45 and MART-1 in skin biopsies with histopathological diagnosis of malignant melanoma.

Nº	Histopathological diagnosis	S-100	HMB-45	MART-1
1	Ulcerated polypoid malignant melanoma	+++	+++	++
2	Histological finding concordant with stage III malignant melanoma	++	+	+
3	Nodular malignant ulcerated melanoma	-	+	+
4	Histological finding concordant with malignant melanoma *in situ* with chronic inflammation in dermis	+	++	+
5	Malignant epithelioid neoplasm with melanin pigment (melanoma)	++	++	++ melanin
6	Stage II malignant melanoma	+	+	-
7	Metastases of malignant melanoma	++	++	++
8	Metastases of malignant melanoma	+	++	+
9	Histological finding with metastases of malignant melanoma	+	++	+
10	Histological finding concordant with malignant melanoma	++	+	+++
11	Malignant epithelioid neoplasm with melanin pigment (melanoma)	++	++	+
12	Nodular malignant ulcerated melanoma	-	++	+
13	Histological finding concordant with stage V malignant melanoma	+++	++	+++
14	Histological finding concordant with metastases of malignant melanoma	+++	++	+++
15	Nodular malignant melanoma	+	+++	+++
16	Melanoma *in situ*	+	+	+
17	Melanoma *in situ*	+	+	+
18	Histological finding concordant with stage V polypoid malignant melanoma	+	++	+
19	Histological finding concordant with melanoma *in situ* (lentigo maligna type)	+	+	++

-: negative; +: weak; ++: moderate; +++: strong.

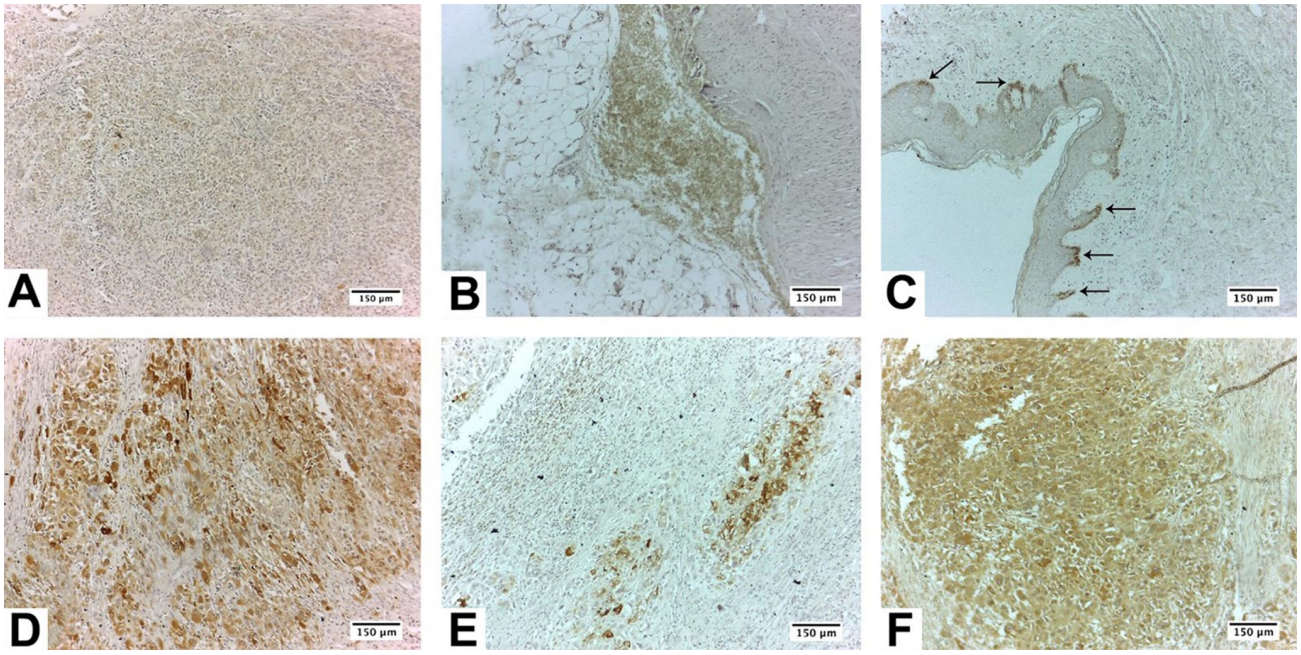

Fig. 1. Comparison of immunohistochemical expression patterns of proteins S-100, HMB-45 and MART-1 in skin biopsies with histopathological diagnosis of ulcerated nodular malignant melanoma (A, B, C) and stage V malignant melanoma (D, E, F), respectively. Small areas of immunoreaction (arrows) are observed.

diagnosis of the biopsies assessed: a variable range of intensity of expression was found, not associated with any particular type of melanoma (Table I). The expression of S-100 was observed to be low in some tumor outbreaks (Fig. 1A) and very intense in other cases (Fig. 1D). In the biopsies diagnosed as melanocytic nevus, in general, the expression

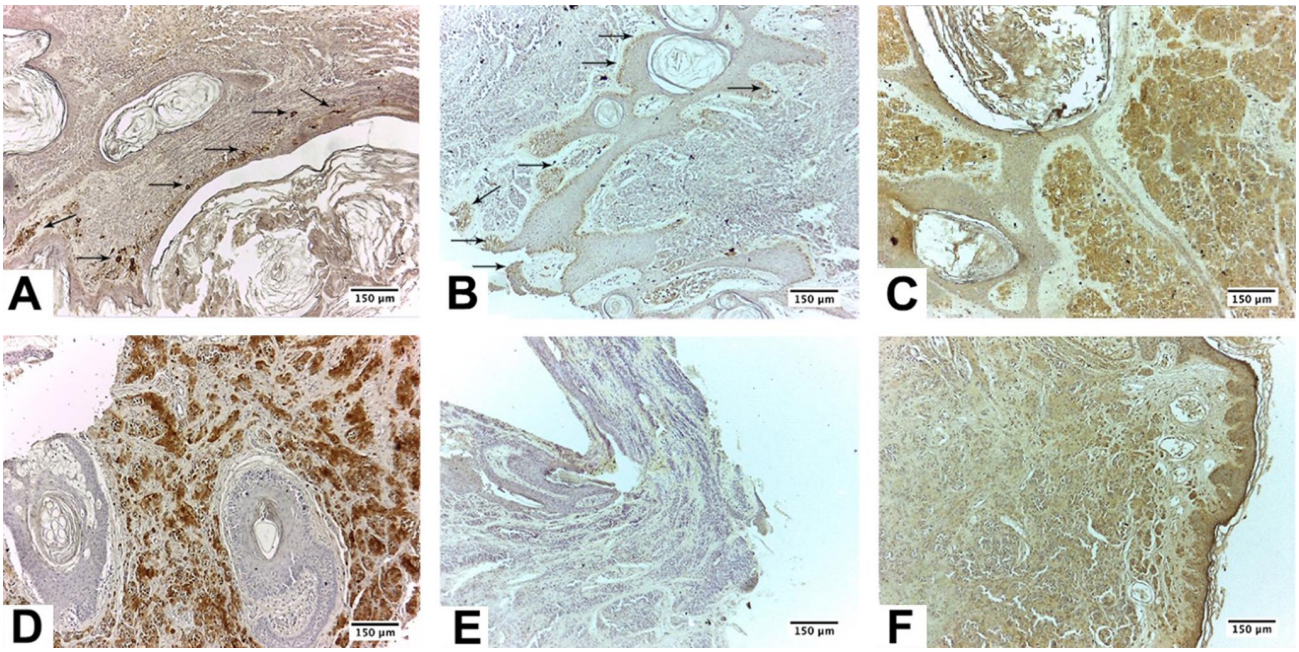

Fig. 2. Comparison of immunohistochemical expression patterns of proteins S-100, HMB-45 and MART-1 in skin biopsies with histopathological diagnosis of melanocytic dermal nevus (A, B, C) and intradermal melanocytic nevus (D, E, F), respectively. Small areas of immunoreaction (arrows) are observed.

Table II. Qualitative analysis of immunohistochemical expression of S-100, HMB-45 and MART-1 proteins in skin biopsies with histopathological diagnosis of melanocytic nevus.

Nº	Histopathological diagnosis	S-100	HMB-45	MART-1
1	Histological finding concordant with compound melanocytic nevus focally pigmented	+++	+	++
2	Concordant finding with focally pigmented intradermal melanocytic nevus with associated focal bone metaplasia	++	+	++
3	Melanocytic dermal nevus of the congenital type	+++	+	+
4	Dermal melanocytic nevus	+	+	+
5	Melanocytic dermal nevus with congenital type	+	+	+
6	Histological finding concordant with intradermal melanocytic nevus pigmented	-	++	+++
7	Histological finding concordant with intradermal melanocytic nevus focally pigmented	-	+	+
8	Dermal melanocytic nevus	-	+	+
9	Dermal melanocytic nevus	+	+	+
10	Intradermal melanocytic nevus	+++	+	++
11	Intradermal melanocytic nevus	+	++	++
12	Intradermal melanocytic nevus	++	+	+
13	Dermal melanocytic nevus with congenital type	+++	+	+++
14	Atypical melanocytic nevus with halo phenomenon	-	++	++
15	Pigmented intradermal melanocytic nevus with congenital type	++	++	+++
16	Intradermal melanocytic nevus	+++	-	+++
17	Pigmented dermal melanocytic nevus	-	+	+++
18	Intradermal melanocytic nevus	+++	++	++
19	Pigmented intradermal melanocytic nevus	+++	+	+
20	Pigmented intradermal melanocytic nevus with associated neuroid involution	++	+	+

-: negative; +: weak; ++: moderate; +++: strong.

of these markers varied between the cases analyzed. This is reflected in Figure 2A, in which a dermal outbreak was observed with scarce expression of S-100; and conversely in the melanocytic nevus in Figure 2D, where intense S-100 marking was observed.

It can also be seen that HMB-45 expression was slight in a tumor outbreak described in Figure 1B, and conversely, there was intense expression in tumor tissue foci in biopsies of other origin (Fig. 1E). The MART-1 marker in tumor tissue is more intense that S-100 and HMB-45, located in some cases in the dermal-epidermal junction and in most in the dermis (Figs. 1C,F).

In biopsies with a diagnosis of melanocytic nevus, the HMB-45 marker was generally present with a weaker expression than that of S-100 and MART-1 (Figs. 2B,E; Table II). MART-1 was present in cases of melanocytic nevus with stronger expression levels than those of HMB-45. In the cases analyzed, it was observed that there was evident variation in the expression of these markers in tumor tissue with diagnosis of melanoma (Table I). In qualitative terms, no expression pattern was observed to specifically associate any of these markers with some types of histopathological diagnosis. Likewise, the samples with a diagnosis of melanocytic nevus of different types showed qualitative variation in the expression of these markers; in general, the expression of HMB-45 was lower in all the samples (Table II).

DISCUSSION

Malignant melanoma is one of the greatest mimickers in pathology, due to the variation inherent in its histological patterns (Hall *et al*., 2013). Correct identification, differentiating it from other benign and malignant entities, is of primordial importance; histological examination of surgical biopsies plays an important role in the diagnosis of primary cutaneous melanomas. IHC permits broad typification of tumors like carcinoma, melanoma, lymphoma or sarcoma, particularly in cases of barely differentiated malignant neoplasias in which the lineage of the cancerous cells is hard to establish by morphology alone (Biernacka *et al*., 2016).

According to previous results, more than 95 % of primary cutaneous melanomas express S-100 (Prieto & Shea, 2011). Our results are consistent with this, since the S-100 antibody against the S-100 cytoplasm protein reacted with 17 of the 19 biopsies with diagnosis of melanoma. Our findings also agree with those of Barrionuevo Cornejo *et al.* (1999) and Biernacka *et al*. who reported high sensitivity of S-100, with immunoreactivity of approximately 96 to 99 %; S-100 is used as a first line melanocyte marker to evaluate poorly differentiated malignant tumors (Argenyi *et al*., 1994). Likewise, positive immunoreaction was found in 15 of the 20 biopsies with histopathological diagnosis of melanocytic nevus. Thus, although it allows us to identify nevi and melanomas, it does not allow differential diagnosis between them (Takahashi *et al*., 1984; Vanstapel *et al*., 1986). Although it is not specific, S100 immunomarkers almost all melanomas, usually with strong staining – in general with diffuse nuclear and cytoplasm staining (Gaynor *et al*., 1981).

The HMB-45 antibody is quite specific for melanocyte differentiation; expression of the HMB-45 protein was found in all of the samples diagnosed as melanoma, mainly because it detects a cytoplasm glycoprotein, GP100, present in immature melanosomes. It, therefore, indicates the formation of active melanosomes and melanocyte differentiation (Hall *et al*.); 19 of the 20 biopsies with a diagnosis of melanocytic nevus reacted with the same antibody. Although this antibody is much more specific than S-100, it is also much less sensitive (Gown *et al*.; Palazzo & Duray; Smoller *et al*.; Sun *et al*.; Skelton 3rd *et al*.), similar to Melan-A/MART-1 HMB-45 (Ordóñez, 2014); it is particularly useful for detecting the development pattern of nevi. In contrast, primary cutaneous melanomas generally express HMB-45 with an irregular pattern, presenting isolated cells or small groups throughout the dermis; the same pattern is observed in nevoid melanoma (McNutt *et al*., 1995). As with the intraepidermal component, HMB-45 is notable for a pagetoid growth pattern or ascendant intraepidermal migration (Hancock *et al*., 1991).

One of the most important melanocyte markers is the melanoma antigen recognized by T cells 1 (MART-1) (Chen *et al*.). This protein is detected by two different antibodies and is expressed in the majority of benign and malignant melanocytic lesions. It is thus very useful for detecting melanocytic differentiation (Jungbluth *et al*., 1998). However, other cells may also express this marker (Fetsch *et al*., 1998), and occasionally macrophages (particularly pigmented) are marked with Melan-A antibody (Trejo *et al*., 2002). The Melan-A antibody used in our investigation reacted with both 18 of the 19 biopsies with diagnosis of malignant melanoma and with all the samples diagnosed as melanocytic nevus, so it may be an antigen for melanocytic identification but not for cell differentiation.

CONCLUSION

We believe that immunohistochemistry plays a fundamental role in the differential diagnosis of melanomas and

melanocytic nevi. However, there is no antibody or set of antibodies which allows unequivocal diagnosis between melanoma and nevus. It is therefore necessary to analyze with care the expression pattern and location of the lesion using standard morphological characteristics.

QUILAQUEO, N.; NAVARRETE, F.; SANDOVAL, C.; ROA, I.; PELLÓN, M. & PAREDES, M. Marcadores inmunohistoquímicos en el diagnóstico diferencial de melanoma y nevos en humanos. *Int. J. Morphol., 39(5)*:1509-1515, 2021.

RESUMEN: La inmunohistoquímica permite la detección in situ de componentes celulares y extracelulares a través de anticuerpos específicos. El objetivo de nuestro estudio fue comparar los patrones de expresión inmunohistoquímica de las proteínas S-100, HMB-45 y MART-1 para el diagnóstico diferencial de melanoma maligno y nevo melanocítico en biopsias de piel humana. Se utilizaron treinta y nueve biopsias de tejido humano, las que fueron divididas en dos grupos: 19 en melanoma maligno y 20 en nevos melanocíticos. A continuación, las muestras se fijaron con paraformaldehído y se procesaron siguiendo el protocolo convencional para su inclusión. Luego, se realizó la tinción inmunohistoquímica. Finalmente, se realizó el análisis histológico y cualitativo de las muestras. Los marcadores S-100, HMB-45 y MART-1 mostraron inmunorreacción positiva en biopsias de melanoma. El marcador HMB-45 estuvo generalmente presente con una expresión más débil que S-100 y MART-1 en biopsias de nevo melanocítico. No se observó ningún patrón de expresión que asocie específicamente uno o más marcadores con algunos tipos de diagnóstico histopatológico. La inmunohistoquímica es fundamental en el diagnóstico diferencial de melanomas y nevos melanocíticos. Sin embargo, no existe ningún anticuerpo o panel de anticuerpos que permita un diagnóstico inequívoco entre el melanoma y el nevo. Por tanto, es necesario analizar con cuidado el patrón de expresión y la localización de la lesión utilizando características morfológicas estándar.

PALABRAS CLAVE: Complejo antígeno anticuerpo; Inmunohistoquímica; Marcadores inmunohistoquímicos; Patología.

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Corresponding author:
Dr. Marco Paredes
Departamento de Ciencias Básicas
Facultad de Medicina
Universidad de La Frontera
Avenida Francisco Salazar 01145
Casilla 54-D
Temuco
CHILE

E-mail: marco.paredes@ufrontera.cl

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