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SIRT1 and Ki67 immunohistochemical expression in progression of cutaneous malignant melanoma

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Abstract— *The current study evaluated the proliferation index and immunohistochemical expression of SIRT1 on normal skin and cutaneous melanocytic nevi (CMN) and cutaneous malignant melanoma (CMM). Formalin-fixed paraffin-embedded tissue samples from 43 CMN, 22 CMM and 10 normal skin were obtained and clinical data were abstracted from the electronic medicalrecord.SIRT1 and Ki67 proteins expressions were evaluated regarding to clinic pathological behavior in CMM. The level of significance was set at $\alpha = 5\%$ ($p < 0.05$) .Our findings showed that SIRT1 positivity was significantly higher in benign melanocytic nevi than that in cutaneous malignant melanoma ($p=0.002$). As expected, the proliferation index was significantly higher in samples of cutaneous malignant melanoma as compared to the normal skin and melanocytic nevi ($p=0.001$). However, the expression of Ki67 protein was not also significantly related to the expression of SIRT1 ($p > 0.05$). In conclusion, low expression of SIRT1 and high proliferation index may play an important role in progression of cutaneous melanoma.*

Keywords— ***melanocytic nevi; skin lesions; proliferation; sirtuins.***

I. INTRODUCTION

Skin cancer is the third most common human malignancy and its global incidence is rising at an alarming rate, with basal cell carcinoma, squamous cell carcinoma and melanoma being the most common forms[1].There are an estimated 2–3 million cases of skin cancer across the world each year, and although cutaneous malignant melanoma (CMM) only accounts for about 200,000 of these (World Health Organization), it is the most dangerous form, accounting for most skin cancer deaths[2]. CMM diagnosed in early stage can be cured by surgical resection, and about 80% of cases are dealt with in this way[3]. However, metastatic malignant melanoma is largely refractory to existing therapies and has a very poor prognosis, with a median survival rate of 6 months [4].

Malignant melanoma is a tumor that originates from the melanocytes and manifests mainly on the skin[5]. UVR exposure is a major risk factor, especially in light skin populations[6]. Lightly pigmented skin and a large number of melanocytic nevi are associated with increased risk of developing malignant melanoma[6,7] Cutaneous melanocytic nevi (CMN) are benign proliferations of melanocytes that are postulated to result from sun-induced mutations [8],typically BRAF[9]and genes associated melanocytic system[10]. In the majority of such neoplasms, subsequent melanocyte senescence is induced by tumor suppressor proteins such as p16 and the

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nevus therefore ceases to grow and becomes stable or even involutes[11]. The accumulation of alterations in key genes controlling processes such as proliferation, apoptosis, senescence, and response to DNA damage can favor the formation morphologically atypical melanocytes predisposing the risk of developing melanoma[12,13]. Cutaneous melanocytic nevi can progress to the intra-epidermal lesion that can involve some local microinvasion of the dermis. The next phase, the cells can progress invading the dermis, a more dangerous stage in which the cells have metastatic potential, with nodules or nests of cells. Not all melanomas pass through each of these individual phases but develop directly from isolated melanocytes or nevi, and both can progress directly to metastatic malignant melanoma [14].

Silent mating-type information regulation 2 homologue 1 (SIRT1) is a protomember of the sirtuin family (SIRT1-7) that is involved in a variety of biological processes, including genetic control of aging, regulating transcription, apoptosis, stress resistance and energy efficiency during low-calorie conditions[15,16]. To date, the role of SIRT1 remains controversial as previous data suggest that SIRT1 can act as an oncogene or a tumor suppressor, likely depending on cell type, its distribution and biological targets[17–19].

Recent studies demonstrated that SIRT1 levels are reduced in some types of cancers, and that SIRT1 deficiency results in genetic instability and tumorigenesis[20,21]. SIRT1-deficiency resulted in an increased tumor formation in p53-null mice[22]. SIRT1 inhibits proliferation of pancreatic cancer cells expressing pancreatic adenocarcinoma up-regulated factor[23]. On the other hand, SIRT1 has been considered as a tumor promoter because of its increased expression in some types of cancers and its role in inactivating proteins that are involved in tumor suppression and DNA damage repair [24].

The role and functional significance of SIRT1 in cancer development and progression is currently an intense area of research investigation. SIRT1 has been shown to be upregulated in several cancers such as prostate cancer, cutaneous T-cell lymphoma, colorectal cancer and pancreatic[16,25–28].SIRT1 is also overexpressed in non-melanoma skin cancers, including squamous and basal cell carcinomas, actinic keratosis, and especially in Bowen's disease[25,29]. However, several studies have shown that both the overexpression and low expression of SIRT1 has been linked to poor disease prognosis and survival depending on the type of cancer[30–34]. In spite of the controversial role of SIRT1 in tumorigenesis[35,36], it is evident that SIRT1 is significantly involved in the process of tumorigenesis, however, its expression status in melanoma is poorly defined and are required further investigation. In this research, we observed the expressions of SIRT1 and index proliferation in skin lesions, and investigated the association between the expressions and clinicopathological characteristics.

II. MATERIAL AND METHODS

Patients

This retrospective and cross-sectional study analyzed samples of human tissues in 17 normal skins (control), 40 benign cutaneous melanocytic nevi (CMN) and 22 cutaneous malignant melanoma (CMM) with confirmed histopathological diagnosis. Clinic data were obtained from medical records of patients attended at public health centers for Oncology treatment at Montes Claros city, Minas Gerais state, Brazil. The normal human skin samples were obtained from patients who experienced aesthetic or corrective surgical procedures in women's breasts.

Ethical approval for this study was obtained from a relevant local ethic committee (Committee on ethic in research – FaculdadesIntegradasPitágoras: protocol no: 714.865/2014).

Clinic and pathological analyses

All CMM cases were classified according to the American Joint Committee on Cancer melanoma staging[37]. CMM patients were categorized as T1/T2 (≤ 2 mm thickness, n = 5 (22.72%) and T3/T4 (> 4 mm thickness, n = 17 (77.27%). Metastatic diseases were diagnosed in 7 (31.8%) CMM patients. Furthermore, cases of CMM presented the following clinical aspects: superficial spreading (n = 11, 50.0%), nodular (n= 3, 13.63%), lentigo malignant (n = 5, 22.72%), and acral melanomas (n = 3, 13.63%).

According to anatomical sites, CMM samples were classified as low-risk (lower trunk, thigh, lower leg, foot, lower arms, hands, and face) and high-risk (back and breast/thorax, upper arm, neck, and scalp) for death caused by CMM [37,38].

Formalin fixed and paraffin embedded samples were submitted to histopathological analysis. Tissue sections were cut at a thickness of 3–5µm and stained with hematoxylin and eosin (H&E). CMM samples were subjected to analysis of tissue invasion of melanoma cells by Breslow's thickness [39]and Clark's level[40]criteria. According to Breslow's thickness grade, CMM samples were categorized as follows: TI (up to 0.75mm, n = 3, 13.6%), TII (from 0.75 to 1.5mm, n = 7, 31.8%), TIII (1.5 to 4mm, n = 11, 50.0%), and TIV (≥ 4mm, n = 1, 4.5%). According to Clark's level (degree of invasion), CMM samples were categorized as follows: level I (limited to

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the epithelium, n = 2, 8.3%), level II (invasion up to the papillary dermis, n = 6, 25.0%), level III (invasion fills the entire reticular dermis, though without invading it, n = 11, 45.8%), level IV (invasion of the reticular dermis, n = 5, 20.8%), and level V (invasion of the hypodermis, n = 0).

Immunohistochemical reactions

The 5-µm tissue sections were deparaffinized, hydrated and the antigen retrieved. The tissue sections were incubated with 3% (v:v) hydrogen peroxide for 30 min at room temperature to quench the endogenous peroxidase.

After blocking in normal goat serum, the tissue sections were incubated with the primary antibodies anti-Ki67 (Mouse monoclonal, Dako, 1:200 dilution, clone MIB-1, Glostrup, Denmark) and anti-SIRT1 (Clone sc-15404, Santa Cruz Biotecnology, Dallas - TX) overnight at 4°C. The slides were then washed in PBS and incubated with LSABTM-Kit Plus Peroxidase® for 1h. Tissues were stained with a chromogen amino-etil-carbazol, counterstained with Mayer's hematoxylin, cover slipped, and visualized under an optical microscope Olympus® BH2 microscope (model: CX31; RTSF, Miami, USA).Positive and negative controls were applied according to the manufacturer's instructions (DakoCytomation, Glostrup, Denmark).All control, CMN, and CMM samples were examined by two independent investigators who were blind to the clinical data.

Counting of immunostained cells with Ki67and SIRT1

The immunohistochemical staining for Ki67 and SIRT1 were measured manually using custom software ImageJ®, version 1:44, for Windows® to assist in performing the cells counts. Ten images were acquired per case at a total magnification of ×400 using an optical inverted Olympus® FSX100 microscope (model: CX31; RTSF, Miami, USA). Selected fields were those with highest density of Ki67 or SIRT1 positive cells. Ki67 labeling index was performed as follows: % marking = (positive nuclei/[positive nuclei + negative nuclei]) [41,42].

The immune reactivity of SIRT1 was evaluated in the normal epithelial /nevi/neoplastic cells considering the cytoplasmatic and/or nuclear staining, or even absent. It was estimated the proportion of cells labeled with both cytoplasmic and nuclear expression in each one of the photomicrographs. Next, the average of the ratios was calculated for each case, considering individual nuclear and cytoplasmic expression for statistical analysis. It aimed to further determine the best cutoff point to define the expression of the protein as positive or negative in samples to the lesions types and location of this protein, using the receiver operating characteristic curve (ROC curve). In cytoplasmic expression, it was found that any ratio of higher than 36.1% represented a good cut-off for positive cytoplasmatic staining ($p < 0.021$). In the evaluation of nuclear expressions identified that positivity would be better represented in values above 1.8150% markup ($p = 0.198$). In the final evaluation of the positive immunohistochemical expression of SIRT1, cases were further ranked as cytoplasmic or nuclear staining to study statistical inferences.

Statistical analysis

Statistical analyses were performed using SPSS® 18.0 (SPSS Inc., Illinois, USA) and Graph Pad Prism®5.0 Softwares. Results were expressed as mean ± SE or as percentages. P values ≤0.05 were considered statistically significant.

Comparisons of immunohistochemical expressions of studied proteins between the lesions were evaluated using Mann-Whitney and Fisher's exact test. Kruskal-Wallis was used to evaluate the differences between SIRT1 and Ki67 expressions and clinicopathological characteristics of melanoma.The analysis immunohistochemical of the expression of SIRT1 and variables clinicopathological lesion and comparing of the expression of SIRT1 between the types of study samples was performed by Chi-square test and Fisher's exact test with application of ROC ("Receiver-operator curves") curve. The curve ROC was used to assess the sensitivity and specificity as the cutoff point for analysis of SIRT1 expression.

III. RESULTS

SIRT1immunohistochemical expression is reduced in human cutaneous malignant melanoma

SIRT1 immunohistochemical staining was localized in tumor and normal cells (lymphocytes and fibroblasts) with varying intensities.The immunohistochemical staining of SIRT1 in CMM, CMN and normal skin is shown in Figure 1.

Based on the ROC curve, it was simulated a cutoff point to distinguish samples with low and high staining of SIRT1, according to the diagnosis of the sample. Applying the values on a ROC curve, the area under the curve[43,44]was 0.684 (95% CI) with best estimates occurring in the amount of 36.1, which was a sensitivity of 59.1% and specificity of 23.5% ($p = 0.021$).

Tumors with scores above the 36.1 cut-off values were considered positive for the cytoplasmic expression of SIRT1 protein. According to ROC curve analysis, expression percentage for nuclear SIRT1 above the critical value 1.8150% was defined as positivity. Applying the values on a ROC curve, the area under the

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curve [43]was 0.602 (95% CI) with a sensitivity of 27.4% and specificity of 5.9% (p= 0.198).

Normal skin samples (control) showed weak or negative cytoplasmic staining to SIRT1. In normal skin, cytoplasmic SIRT1 staining was weakly and diffusely expressed in suprabasal epidermal keratinocytes, with only faint and focal staining in the granular layer and stratum corneum. The SIRT1 protein was detected in the normal epithelial tissues in 35.29% of normal skin (Table 1).

The SIRT1 protein was present in both cytoplasmic and nuclear compartments of the cutaneous melanocytic nevi. Therefore, SIRT1-positive cases were classified into two categories (nuclear or cytoplasmic SIRT1). SIRT1 was positive in 76.74% of benign melanocytic nevi cases. Among the SIRT1-positive cases, 63.63% were cytoplasmic positive and 36.36% were nuclear positive. In particular, the junctional component of benign melanocytic nevi was positive in the most cases. SIRT1 positivity is observed in the majority melanocytes, especially those arranged in bridging nests at the dermoepidermal junction and the intradermal component.

The SIRT1 protein was present in cytoplasmic compartment of the malignant melanocytic cells. SIRT1 was positive in twelve of 23 MMC (52, 17%), and all SIRT1-positive MMC cases showed cytoplasmic positivity. SIRT1 positivity was significantly higher in benign CMN than that in MMC (p= 0.002). The invasive component of melanoma shows a weak and diffuse SIRT1 cytoplasmic staining. Most MMC displayed high rate of melanocytes expressing SIRT1 in intradermal component. SIRT1 expression was not significantly related to any of the clinicopathological parameters (Table 2).

Ki67 immunostaining was nuclear and nucleolar (Fig.1). The average number of Ki67-positive cells was significantly higher in samples of cutaneous malignant melanoma as compared to the normal skin and melanocytic nevi (p <0.001) (Table1). The normal skin and benign CMN displayed positive Ki67 immunostaining in basal keratinocytes whereas benign CMN was absent in the majority of nevi cells. The expression of Ki67 it found in intradermal component of MMC.

The associations between the Ki67 expression and clinicopathological factors did not have statistical significance (Table 2). Furthermore, the expression of Ki67 was not also significantly related to the expression of SIRT1 (p>0.05).

IV. DISCUSSION

This study investigated the immunohistochemical expression of Ki67 and SIRT1 in normal skin, CMN and CMM samples. In this study, we noted a significant decrease of staining of SIRT1 from CMN to CMM samples. Also, normal epithelial cells showed weak or negative staining to SIRT1 while CMN samples exhibited a higher SIRT1 expression as compared to the CMM samples. Comparatively, the SIRT1 expression was gradually decreased during carcinogenesis and tumour progression of colorectal adenocarcinoma[33]. This suggests that loss of SIRT1 expression in tumoral lesions may be associated with a more aggressive phenotype. However, the role of SIRT1 in human malignant tumors is controversial. Some previous studies have reported that SIRT1 overexpression was associated with shorter overall survival or poor prognostic indicators in breast and gastric carcinoma [35,45,46].

The expression of SIRT1 is relatively higher in hepatocellular carcinoma, breast cancer, and thyroid cancer but lower in colon and lung cancer[35,47–50]compared with their corresponding normal tissues. In cancer, SIRT1 has been reported as either an oncogenic or a tumor suppressive role, depending on the type of cancer and the context of the analysis [17,21,51]. These results suggest that SIRT1 may acts differently depending on the specific organ or type of tumor involved.

We demonstrated that SIRT1 is predominantly localized in the cytoplasm of CMM. SIRT1 cytoplasmic localization is not commonly identified in cancercells and it is unclear if SIRT1 localization has any changes during carcinogenesis. Similar results have also identified aberrant cytoplasmic localization in human cancer cells [52–54].This finding may suggest a new mechanism for SIRT1 function as a cancer-specific survival factor by targeting cytoplasmic proteins.

In contrast to its well-described role in the nucleus, the deacetylation function of cytoplasmic proteins caused by SIRT1 provides important insights into the function of cytoplasmic SIRT1. Zhang, 2007 showed that SIRT1 enhanced IGF-1 signaling by deacetylating the ISH-2 cytoplasmic protein [55]. SIRT1 also deacetylates cytoplasmic cortactin and promotes cancer cell [35,52] In addition, SIRT1 was found to promote the activation of cytoplasmic kinases, including AMPK,.Ras-MAPK, Erk and S6K1 [52].

In the case of certain cancers, including prostate cancer, lung cancer, breast cancer, and melanoma, SIRT1 has been shown to localize to the cytoplasm, while being located predominantly in the nucleus in the corresponding normal tissues[52,53]. This change in localization could theoretically minimize the deacetylation of TP53 in the

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nucleus by SIRT1 while still allowing TP53 to regulate its transcription. Thus, in cancers under these conditions, the oncogenic role of SIRT1 overexpression through TP53 might be minimized, allowing for other targets of SIRT1 to play a more significant role, especially those that are localized to the cytoplasm[17]. Cellular localization of SIRT1 also has been shown to differ among different tissue types in mice [56], which could explain why SIRT1 sometimes exhibits tumor suppressor properties in certain types of cancer but not in others.

In current study, there was no association significant between SIRT1 and Ki67 immunostaining, however, further functional study will be needed to investigate the relationship between SIRT1 and cellular proliferation. Melanoma is known to exhibit aberrant expression of proliferation markers, and these abnormalities are considered important steps in the genesis and progression of melanoma [57].An increasing literature describes the role of proliferation markers in the evaluation of melanocytic tumors[58]. Ki67 staining has been shown positive in multiple lesions, 5% of positivity on melanocytic cells in most benign nevi, although there have been reports of up to 15% positivity in Spitz and dysplastic nevi [59–62]. Conversely, Ki67 staining is reported as positive in 13–30% of the cells in a malignant melanoma, although individual cases can show almost 100% nuclear positivity[62,63]. In our study, we found a lower average than 5% staining in benign melanocytic nevi while in melanoma was greater than 15%. Therefore, Ki67 index was reported to be higher in malignant melanomas than in benign nevi. Correspondingly, no associations between Ki67 and measures of tumor size (thickness and diameter) and invasion (Clark's level) were found. Other studies on cutaneous melanoma have suggested that increased Ki67 expression might be associated with tumor thickness and tumor cell proliferation [62].

Our experiments have some limitations, such as small sample size. The tumor heterogeneity and staining scoring ethod also may to interfere the results. In summary, we need further study on the roles of SIRT1 and Ki67 on clinical and pathological behavior of melanoma.

V. CONCLUSION

Low expression of SIRT1 and high proliferation index may play an important role in progression of cutaneous melanoma.

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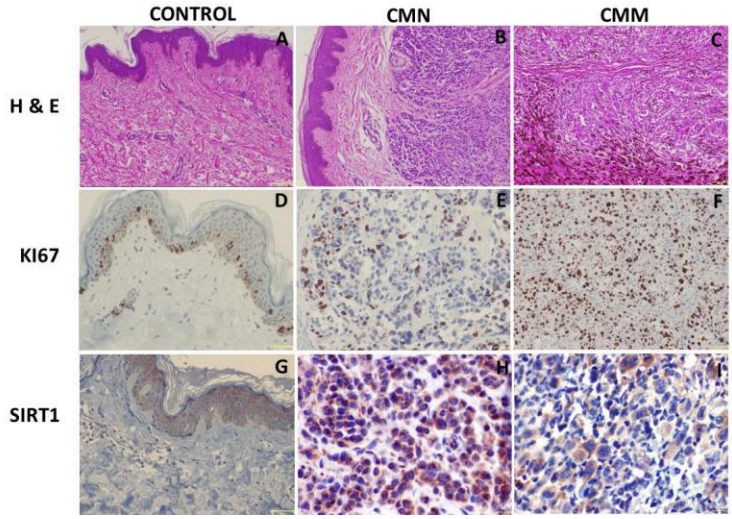

Figure 1. Morphological aspects of normal skin (control), cutaneous benign melanocytic nevi (CMN), and malignant melanoma (CMM) samples (Figures A, B, and C, respectively. H&E; higher magnification of 100x).Immunohistochemical expression of Ki67 (Figures D, E, F) higher magnification of 200x in samples of control, CMN, and CMM.SIRT1 (Figures G, H, and I) higher magnification of 200x in samples of control, CMN, and CMM. Immunostaining: AEC; counterstaining: Mayer's hematoxylin.

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Table 1: Expression of Ki67 and SIRT1 in normal skin (control), benign cutaneous melanocytic nevi and cutaneous malignant melanoma.

Variables	Ki67 Mean (SD)	P	SIRT1+ n (%)	P	SIRT1+ Cytoplasmic n (%)	SIRT1+ Nuclear n (%)	p
Control [a] (17)	0.93 (1.82)	p^{ab}= 0.155	6 (35.3%)	p^{ab}= 0.001 **	6 (100.0%)	0	p^{ab}= 0.151
Melanocytic Nevi [b] (43)	2.04 (4.28)	p^{ac} = <0.001*	33 (76.7%)	p^{ac}= 0.334	21 (63.6%)	12 (36.4%)	p^{ac}= 0.529
Melanoma [c] (22)	16.96 (14.54)	p^{bc} = <0.001*	12 (54.6%)	P^{bc}= 0.035	10 (83.3%)	2 (16.7%)	p^{bc}= 0.287

*Values bearing asterisks show significant association using Mann-Whitney. **Values bearing asterisks show significant association using Chi-square test and Fisher's exact. Level of significance was set at α = 5% (P< 0.05).

Table2: Evaluations Ki67 and SIRT1 immunohistochemical expressions in function of clinic and pathological parameters on melanoma malignant cutaneous.

Variables	KI67 Mean (SD)	p	SIRT1+ n (%)	P	SIRT1 Cytoplasmic n (%)	SIRT1 Nuclear n (%)	p
Anatomical Site							
Lowrisk (18)	13.82 (15.01)	0.631	9 (50.0%)	0.478	8 (88.9%)	1 (11.1%)	0.522
High risk (4)	17.22 (14.09)		4 (100.0%)		3 (75.0%)	1 (25.0%)	
TNM							
I/II (5)	16.89 (11.60)	0.990	2 (40.0%)	0.457	2 (100.0%)	0	0.488
III/IV (17)	16.98 (15.62)		10 (58.8%)		8 (80.0%)	2 (20.0%)	
Clinical Size							
Small (5)	16.89 (11.60)	0.990	2 (40.0%)	0.457	2 (100.0%)	0	0.488
Large (17)	16.98 (15.62)		10 (58.8%)		8 (80.0%)	2 (20.0%)	
Regional Metastasis							
Absent (15)	16.69 (15.74)	0.902	8 (50.0%)	0.867	6 (75.0%)	4 (50.0%)	0.273
Present (7)	17.54 (12.70)		4 (57.1%)		4 (100.0%)	0	
Recurrence							
Absent (18)	17.04 (14.61)	0.646	9 (56.2%)	0.422	7 (77.8%)	2 (22.2%)	0.371
Present (4)	20.86 (15.03)		3 (60.0%)		3 (100.0%)	0	
Level of Invasion							
I-III (17)	14.80 (13.57)	0.206	9 (50.0%)	0.781	7 (77.8%)	2 (22.2%)	0.371
IV-V (5)	24.31 (16.91)		3 (60.0%)		3 (100.0%)	0	
Tumor Thickness							
≤ 2 mm (12)	15.60 (12.38)	0.642	8 (72.7%)	0.211	6 (75.0%)	2 (25.0%)	0.273
≥2 mm (10)	18.59 (17.34)		4 (33.3%)		4 (100%)	0	

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