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Evaluation of Immunostaining for 4-Hydroxy-2-Nonenal Receptors in Cutaneous Malignant Melanoma Immunohistochemical Study of 55 Cases

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Abstract

Background and objectives: Reactive oxygen species have been extensively studied in a wide range of human cancers, but are poorly studied in cutaneous malignant melanomas, particularly with respect to the lipid peroxidation product 4-hydroxy-2-nonenal. This was the reason why we chose to introduce this marker in this study, its use still being a novelty in these neoplasms. In addition, comparative analyzes of 4-hydroxy-2-nonenal expression in benign and malignant (primary or metastatic) human melanocytic lesions have not been performed so far.

Materials and methods: The immunohistochemical study was performed in a total of 55 patients, divided into a control group, which included 5 cases of simple nevi and 5 cases of dysplastic nevi, as well as a study group consisting of 35 cases of primary malignant melanoma and 10 metastases. The immunohistochemical method used to identify epitopes of interest was two-time, polymer-specific, with is characterized by high sensitivity, specificity and affinity.

Results: Immunostaining of the control and study groups with anti-4-HNE antibody included the analysis of the percentage of positive cells (with membranous/cytoplasmic staining and staining intensity for each type of melanocytic lesion analyzed (benign, dysplastic and malignant).

Conclusion: Oxidative stress-induced expression of the lipid peroxidation product 4-hydroxy-2-nonenal is significantly increased in dysplastic nevi versus benign nevi and is maintained at a level comparable to that in dysplastic lesions in cutaneous malignant melanomas. 4-hydroxy-2-nonenal intervenes early in the tumorigenic process of cutaneous malignant melanomas, being highly overexpressed once melanocytes have undergone dysplastic changes. Metastases lose the lipid peroxidation product, aspect correlated with the increased proliferative activity detected in metastases from cutaneous malignant melanoma.

Keywords: Malignant melanoma; Biomarkers; Immunohistochemistry; Reactive oxygen species; 4-hydroxy-2-nonenal

Abbreviations: MM: Malignant Melanoma; 4-HNE: 4-hydroxy-2-nonenal; IHC: Immunohistochemistry/Immunohistochemical; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; SSMM: Superficial Spreading Malignant Melanoma; NMM: Nodular Malignant Melanoma; LMM: Lentigo Malignant Melanoma; ALM: Acral Lentiginous Melanoma; SPSS: Statistical Package for the Social Sciences; L4CL: Tetralinoleoyl Cardiolipin; PUFA: Polyunsaturated Fatty Acids; MDA: Protein-Bound Malondialdehyde

Introduction

Recent advances in molecular biology bring additional information that solves a number of problems related to MM tumorigenesis [1,2]. It appears that, in addition to melanocytes, melanocytic stem cells participate in the initiation and progression of cutaneous MM [3-5].

In general, the clinical progression of cutaneous MM is partly correlated with the expansion of its germ cell compartment. Thus, the proportion of cells employed in the cell proliferation cycle will increase in cutaneous MM [6,7].

ROS have been extensively studied in a wide range of human cancers, but are poorly studied in cutaneous malignant melanomas, particularly with respect to the lipid peroxidation product 4-hydroxy-2-nonenal. This was the reason why we chose to introduce this marker in this study, its use still being a novelty in these neoplasms. In addition, comparative analyzes of 4-hydroxy-2-nonenal expression in benign and malignant (primary or metastatic) human melanocytic lesions have not been performed so far.

ROS are the normal product of a variety of essential biochemical reactions. The normal ROS level has physiological functions, while an increased ROS level is toxic to cells. Overproduction of ROS by endogenous or exogenous aggression is harmful to living organisms and is called oxidative stress [8].

Oxidative stress can cause damage to cellular macromolecules, resulting in DNA and protein modifications and lipid peroxidation [9]. Oxidative stress caused by free radicals has been associated with the development of several diseases, such as cardiovascular diseases, chronic inflammation and cancer [10,11].

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Antibody	Clone	Dilution	Pretreatment	Manufacturer	External control
4-HNE	4-HNE	1:600	EDTA solution pH 8 × 20 min. at microwave	Abcam	Tegument with actinic elastosis

Table 1: The antibody used: clone, dilution, pretreatment, manufacturer and external control.

Lesion type (55)	IHC expression of 4-HNE (%)			
	0	Below 10%	10%-50%	Over 50%
Simple nevus (5)	5	0	0	0
Dysplastic nevus (5)	0	1	1	3
LMM (3)	0	0	1	2
SSMM (15)	1	1	5	8
NMM (10)	6	3	1	0
ALM (3)	0	1	1	1
Amelanotic MM (4)	2	1	1	0
Metastasis (10)	6	2	1	1

Table 2: Distribution of the percentage of positive cells for 4-HNE marker according to the histological type of the analyzed lesions.

Intensiy	IHC expression of 4-HNE (%)			
	0	Below 10%	10%-50%	Over 50%
0	20	0	0	0
+1	0	3	4	2
+2	0	4	3	4
+3	0	2	4	9

Table 3: Distribution of the percentage of positive cells for 4-HNE marker depending on staining intensity.

Material and Methods

The IHC study was performed in a total of 55 patients admitted to the Craiova County Emergency Hospital over a period of five years (2012-2016). The 55 patients were divided into a control group that included 5 cases of simple nevi and 5 cases of dysplastic nevi, as well as a study group consisting of 35 cases of primary MM (15 cases of SSMM, 10 cases of NMM, 3 cases of LMM, 3 cases of ALM and 4 cases of amelanotic MM) and 10 metastases (1 intestinal, 3 cutaneous-one satellite and two distant, and 6 lymph node metastases).

The IHC method used to identify epitopes of interest was two-time, polymer-specific, characterized by high sensitivity, specificity and affinity. The characteristics of the used antibody and the external controls used are shown in Table 1.

From the paraffin blocks, 3-micron thick sections were cut and then inserted into the Leica BOND-MAX automated stainer. The sections were incubated with a primary antibody for 1 hour and a Bond Polymer Refine RED Detection Kit was used to detect the primary antibody, the immune reaction visualization being done with RED. Counterstaining was performed with Meyer's hematoxylin solution.

Appropriate positive and negative external controls were used throughout the testing process (Table 1). Negative external controls were tissue samples collected from the analyzed cases, to which the primary antibody was replaced with non-immune Ig serum from the same species as the used primary antibody. Macrophages were used as internal positive control for 4-HNE. Tumor cells showing cytoplasmic and/or membranous immunoreactivity to 4-HNE were considered positive.

The methods of scoring 4-HNE immunostaining were the determination of the percentage of positive cells and the cases were classified into one of the following categories: 0 (negative cases, no positive cells), cases with less than 10%, between 10-50% and over 50% positive cells. Staining intensity was recorded semi-quantitatively, from 0 (negative), +1 (weak), +2 (medium / moderate positive) to +3 (intense).

Statistical analysis was performed with SPSS v.10. For two categories of comparisons, the Student t-test was used. When comparing more than two categories a one-way ANOVA test with post-hoc test was used, and Tukey test was used to evaluate the differences between pairs of categories.

The statistical results were reported as follows:

- $p<0.05$, significant (S)
- $p<0.01$, highly significant (HS)
- $p<0.001$, very highly significant (VHS)
- $p>0.05$, not significant (NS)

Throughout the study we complied with the ethical principles of the Declaration of Helsinki and University Code of Good Research Practice, along with the Codes of Practice based on the principles established in the Code of Medical Ethics.

Results

Immunostaining with anti-4-HNE antibody of the control and study groups comprised the analysis of the percentage of positive cells (with membranous/cytoplasmic staining) and staining intensity of each type of melanocytic lesion analyzed (benign, dysplastic and malignant) at tumor cell level. Percentage 4-HNE expression and staining intensity for each category of analyzed melanocytic lesion are shown in Tables 2 and 3.

In all 5 cases of simple nevi, 4-HNE immunostaining was absent in the nevic cells (Figure 1), while all 5 cases of dysplastic nevi showed staining for 4-HNE in the dysplastic foci (Figures 2 and 3). Thus, in most cases (3 cases, 60%) over 50% of the dysplastic tumor cells were positive, and in one case each (20%) 10%-50% and less than 10% of tumor cells, respectively exhibited staining for 4-HNE. Staining intensity was +2 and +3 in all positive cases (Tables 2 and 3).

All LMM cases showed staining for 4-HNE, most of them in over 50% of tumor cells (2 cases, 66.67%) and in 10%-50% in 1 case. Staining

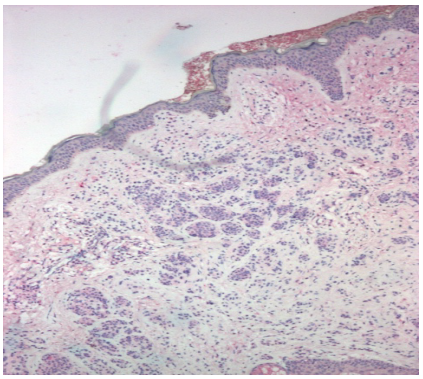


Figure 1: Dermal nevus, negative staining for 4-HNE, 40x.

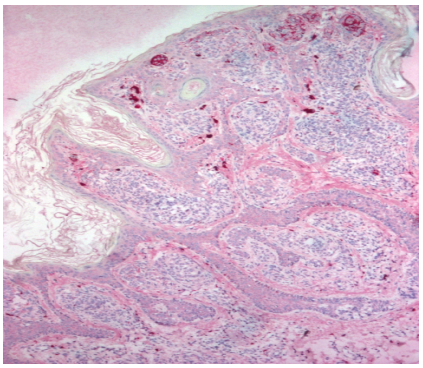


Figure 2: Compound not with dysplastic focus, 4-HNE positive, 40x.

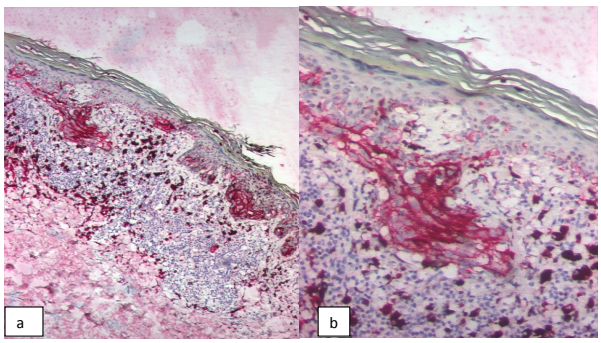


Figure 3: Junctional dysplastic nevus-staining for 4-HNE, a. 40x and b. 100x.

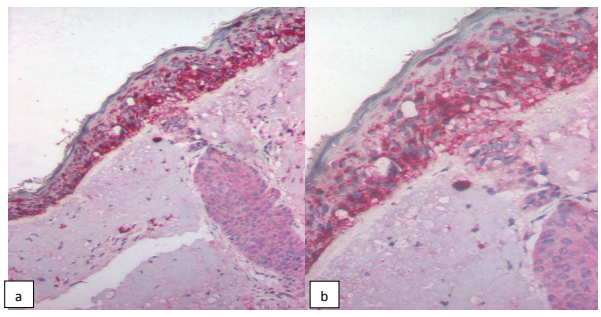


Figure 4: LMM-malignant lentigo area-immunostaining for 4-HNE, a. 100x and b. 200x.

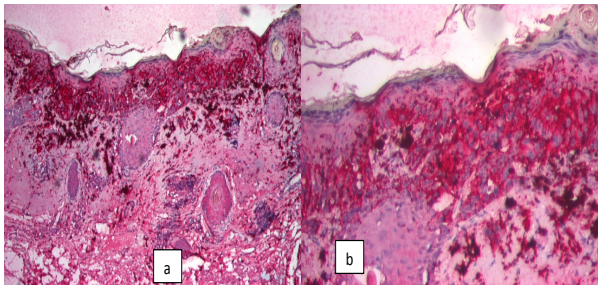


Figure 5: LMM-area of MM-4-HNE immunostaining, a. 40x and b. 100x.

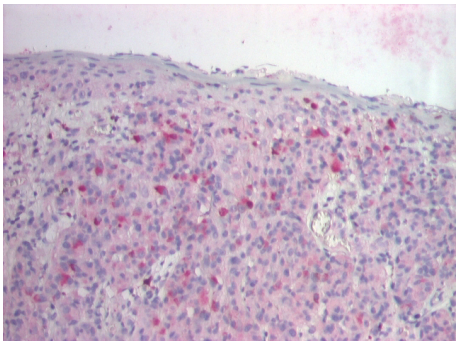


Figure 6: SSMM-positive focal staining for 4-HNE, with moderate intensity, 100x.

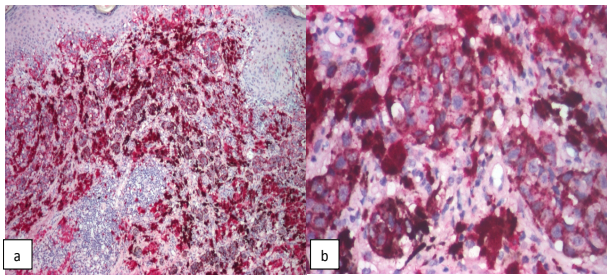


Figure 7: SSMM-intensely positive staining for 4-HNE, a. 40x and b. 200x.

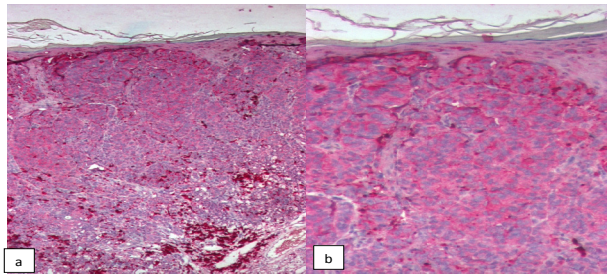


Figure 8: SSMM-positive staining for 4-HNE, positive of moderate intensity and decreasing to depth a. 40x and b. 100x.

intensity was high and moderate, with scores +3 and +2 in all cases. The described aspects were seen both in lentigo maligna areas and in invasive MM areas of LMM lesions (Figure 4 and 5) (Tables 2 and 3).

As to SSMM, only one case (6.67%) with negative 4-HNE staining and also one case (6.67%) with less than 10% tumor cells exhibiting weak intensity (+1) staining for 4-HNE were identified. Five (33.33%) cases exhibited 10%-50% immunostained tumor cells and the majority,

8 cases (53, 33%), over 50%. There was a slight decrease in staining towards tumor depth compared to its superficial area (Figures 6-8) (Tables 2 and 3).

NMMs were generally negative for 4-HNE (60% of cases), the remainder demonstrating positivity in less than 50% of malignant tumor cells (3 cases/30% - less than 10% of the cells and only 1 case/10% between 10% and 50% of the cells). Staining intensity in all 4 positive cases was weak, score +1 (Figure 9) (Tables 2 and 3).

All ALM cases were positive for 4-HNE, with one case in each category of percentage of stained cells, and intensity was moderate and high (Tables 2 and 3).

Amelanotic MMs were similar to NMMs, with 50% of the cases negative for 4-HNE, and 25% cases each positive in less than 10% and 10-50% of tumor cells, respectively. However, staining intensity was weak and moderate (Tables 2 and 3).

The analyzed MM metastases did not express 4-HNE in 60% of cases (Figure 10). Less than 10% of tumor cells stained positive in 2 (20%) cases and over 10% in the remaining 2 (20%) cases (10%-50% and over 50% of metastatic cells, respectively) (Tables 2 and 3). Staining intensity was variable: weak (Figure 11), weal moderate and intense (Figure 12).

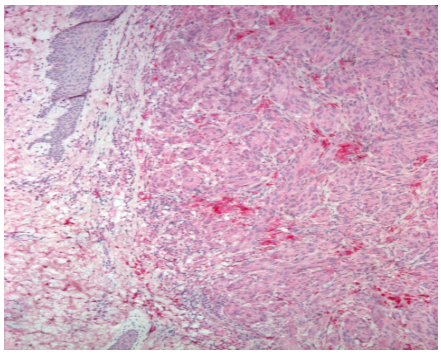


Figure 9: NMM-low staining for 4-HNE, 40x.

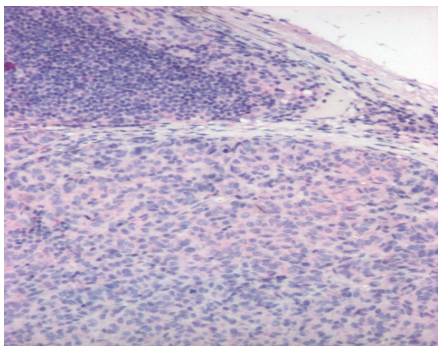
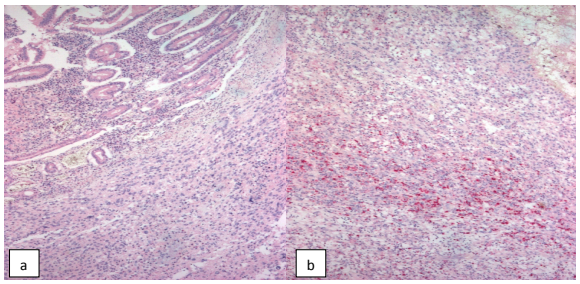


Figure 10: Nodal metastasis of MM-negative staining for 4-HNE, 40x.



Figurea 11: Small metastatic intestine MM -a, b. weak staining for 4-HNE, 40x.

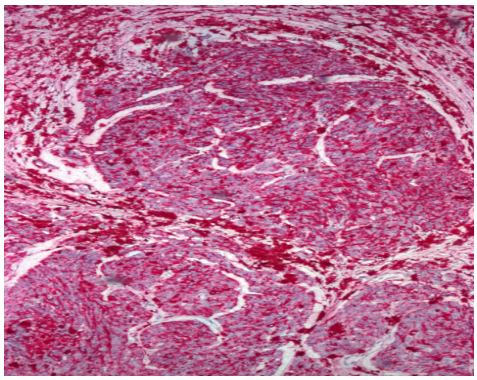


Figure 12: Satellite skin metastasis-positive staining for a 4-HNE, 40x.

Lesion type (55)	IHC Expression of 4-HNE (%)		
	Below 10%	10%-50%	Over 50%
Nevus (10)	6 (60%)	4 (40%)	0.338 (NS)
Melanoma (35)	15 (42.86%)	20 (57.14%)	-
Metastasis (10)	8 (80%)	2 (20%)	0.0382 (S)

Table 4: Distribution of cases with less than 10% and ≥ 10% cells stained for 4-HNE according to lesion type.

Lesion type (55)	Expresia IHC a 4-HNE (%)		
	Below 10%	10%-50%	Over 50%
Simple nevus (5)	5 (100%)	0 (0%)	0.00982 (S)
Dysplastic nevus (5)	1 (20%)	4 (80%)	-
Melanoma (35)	15 (42.86%)	20 (57.14%)	0.329 (NS)

Table 5: Distribution of cases with less than 10% and ≥ 10% cells stained for 4-HNE according to the type of primary lesions.

Comparative analysis of 4-HNE expression in nevi (simple/dysplastic nevi) and primary MM, choosing a threshold of 10% positive cells, showed that although generally nevi express 4-HNE in less than 10% of tumor cells and MMs express 4-HNE in over 10% of malignant cells, the differences are not significant (p=0.388) (Table 4). In contrast, MM metastases express this marker in most cases in just under 10% of the cells, the differences being significant compared to their primary tumors (p=0.0382) (Table 4). This latter observation shows that metastases lose the lipid peroxidation product.

When 4-HNE expression in simple and dysplastic nevi was comparatively analyzed, the differences were highly statistically significant (p=0.00982), showing a much higher 4-HNE expression with the occurrence of dysplastic lesions but these differences later disappear during progression to MM (p=0.329), the level of expression of this lipid peroxidation product being similar to that recorded in dysplastic lesions. Also, 4-HNE expression was significantly higher in the analyzed MM cases compared with simple benign nevi (p=0.0168) (Table 5).

In all 4-HNE immunostained cases, staining intensity in cases with less than 10% and ≥ 10% positive cells was comparatively analyzed, and it was found that absence of staining or weak staining is much more frequent when few cells are stained (less than 10%), whereas moderate or intense staining is characteristic (p=0.00003) in cases with

Intensity	Expresia IHC a 4-HNE (%)		
	Below 10%	10%-50%	Over 50%
0/+1 (29)	23 (79,31%)	6 (20.69%)	0.00003 (S)
+2/+3 (26)	6 (23.08%)	20 (7.92%)	-

Table 6: Distribution of cases with less than 10% and ≥ 10% cells stained for 4-HNE according to staining intensity.

a significant number of stained cells (≥ 10%) (Table 6). Thus, moderate and high 4-HNE expression intensity is characteristic to cases with a significant number of positive cells.

Discussion

The IHC study was focused on analysis of 4-HNE, marker involved in tumor progression and prognosis, and which may be a therapeutic target in patients with cutaneous MM. This study was conducted in a number of 55 cases, represented by a control group of 10 cases and a study group consisting of 35 cases of primary MM and 10 metastases.

Free radicals are very reactive, short-lived chemical entities that contain one or more unpaired electrons and which induce cell damage through the non-participating electron, which leads to the oxidation of cellular components and molecules. They can also be considered as negatively influencing the signaling involved in the normal process of differentiation and migration [12,13].

Free radicals can be produced from the non-enzymatic reactions of oxygen-containing organic compounds as well as from those initiated by ionizing radiation, but this process can also occur in mitochondria by oxidative phosphorylation [14,15].

In order to escape the harmful effects of these free radicals, the body has different mechanisms to endogenous or exogenous produce antioxidants which will neutralize the increased amount of free radicals, protect the cells against their toxic effects and contribute to disease prevention. Oxidative stress can be defined as any disturbance in the antioxidant-pro-oxidant-balance in favor of the latter due to different factors such as aging, drug actions and toxicity, inflammation and/or addiction [14].

Generally, oxidative stress is an excess formation and/or insufficient removal of highly reactive molecules such as reactive nitrogen species (RNS) and reactive oxygen species (ROS). Until now, free radicals have been involved in the pathogenesis of over 50 diseases, and when the antioxidant level is limited, this damage can become debilitating and cumulative. Oxidative damage to DNA, proteins and other molecules has been implicated in the pathogenesis of a wide variety of diseases, the most important being cancer and cardiac disease [16,17].

The increased level of oxygen and nitrogen free radicals (ROS/RNS) has been linked to lipid peroxidation, non-enzymatic glycation of proteins and oxidation of glucose [13]. Thus, ROS overproduction, called oxidative stress, can cause polyunsaturated fatty acids (PUFA) oxidation in cellular and mitochondrial membranes through free radical chain reactions and the formation of lipid hydroperoxides as primary products [18]. Some of these primary oxidation products may decompose and lead to the formation of reactive lipid electrophiles. Of these lipid peroxidation products, 4-HNE is one of the most bioactive and well-studied lipid alkenes [19].

4-HNE can modulate a series of signaling processes, mainly by forming covalent bonds with nucleophilic functional groups in proteins, nucleic acids (DNA) and membrane lipids. These properties have been extensively discussed in some recent reviews [20-23].

Mitochondrion is vital for cellular bioenergy and is considered the most important cell site for ROS production and 4-HNE formation [24,25]. Moreover, it has been well documented that oxidative stress and ROS generation are intimately associated with cancer [26,27]. Accumulated data suggest that mitochondrial macromolecules derived from 4-HNE are involved in the initiation and progression of cancer [28,29].

At low levels 4-HNE can protect cancer cells against damage, while at high levels cells undergo apoptosis or even necrosis, the capacity of this protective mechanisms being exceeded. Thus, strategies focused on manipulating the generation of mitochondrial ROS, lipid peroxidation, and 4-HNE may have therapeutic value in the prevention or treatment of cancer [30-32].

Thus, mitochondria are one of the most important cellular sites of 4-HNE production, probably from the oxidation of PUFA-rich lipids, such as L4CL. Evidence suggests that this process plays a critical role in apoptosis. Secondly, in response to 4-HNE induced toxicity, the mitochondria developed a number of defense mechanisms to turn 4-HNE into less reactive chemical species and to minimize its toxic effects. In addition, 4-HNE macromolecules in mitochondria are involved in the initiation and progression of cancer by modulating the mitochondrial function and metabolic reprogramming. Manipulation of mitochondrial ROS generation, lipid peroxidation, and lipid electrophile production may be a viable approach to cancer prevention and treatment [25].

Lipid peroxidation is initiated by the attack of free radicals on membrane lipids, generating large amounts of reactive products that have been involved in initiating and promoting cancer development [28].

Gradual progression from benign to malignant in B16 murine MM after *in vitro* exposure to hypoxia had already been described, and Zarkovic N. et al. tested whether exposure of MM B16-F10 cells to 4-HNE aldehyde could have opposite effects. The obtained results suggest that while hypoxia can increase malignancy of murine MM cells, exposing these cells to one of the major "second toxic messengers" of oxygen free radicals, 4-HNE, has almost opposite effects and also suggests the possible use of this aldehyde *in vivo* [33].

The antiproliferative and pro-apoptotic effects of HNE have been extensively demonstrated in a wide variety of tumor cell types *in vitro*. Thus, it could represent a new promising molecule in anticancer therapy strategies [34].

Solar UV radiation is a major etiological factor for MM and non-melanocytic skin cancers, and numerous studies indicate that oxidative stress is implicated in photocarcinogenesis. However, there are still no *in vivo* studies in human skin on oxidative stress and MM, although ROS participates in a number of pathophysiological processes, including DNA alteration and lipid peroxidation, being considered a key factor in tumor progression. Moreover, comparative studies of the expression of lipid peroxidation products in human benign and malignant melanocytic lesions and lesions are almost nonexistent, at this time a single research on human study material being identified [35].

Similarly, in the present study, the comparative analysis of the expression of the lipid peroxidation product 4-HNE in simple and dysplastic nevi showed statistically significant differences (p=0.00982), revealing a much higher expression of 4-HNE with the occurrence of dysplastic lesions, difference that will disappear during progression to MM (p=0.329), the expression of this lipid peroxidation product being maintained at a level comparable to that of dysplastic lesions.

These results indicate that 4-HNE is involved early in the process of MM tumorigenesis, being highly overexpressed once melanocytes have undergone dysplastic changes. Also, 4-HNE expression was significantly increased in the MM compared with simple benign nevi (p=0.0168).

In addition, this study included a series of 10 MM metastases in which the IHC expression of 4-HNE was present in most cases in less than 10% of cells, the differences compared to their primary tumors being significant (p=0.0382). This latter finding suggests that metastases lose the lipid peroxidation product, which correlates with the increased proliferative activity found in the analyzed metastases and therefore with the absence of pro-apoptotic and antiproliferative actions of HNE that have been extensively demonstrated in a wide variety of tumor cell types *in vitro* [34].

This study also brings another element of originality as in all 4-HBE immunostained cases staining intensity in the cases with less than 10% and ≥ 10% positive cells was comparatively analyzed. It was found that absence of staining or weak staining is much more frequent when few cells are stained (less than 10%), whereas moderate or intense staining is characteristic (p=0.00003) of cases with a significant number of stained cells (≥ 10%).

Conclusion

In conclusion, oxidative stress plays an important role in the pathogenesis of human cutaneous melanocytic lesions, the lipid peroxidation products (such as 4-HNE) is overexpressed since the stage of dysplastic melanocytic lesion and an elevated level is maintained in melanocytic malignant cells. Thus, oxidative stress is intense in cutaneous MM and thus it may cause alteration of the surrounding tissues and metastatic spread. After the development of metastasis, the 4-HNE level decreases as compared to the level in their primary tumors, aspect that correlates with the increased proliferative activity detected in the analyzed metastases and thus with the absence of pro-apoptotic and antiproliferative actions of HNE.

The number of cancer patients has increased significantly across the world, reason why it is called the "disease of the century" and represents a true challenge for both researchers and clinicians.

Of the malignant skin tumors, the most common are epitheliomas, accounting for 90% to 95% of skin cancers, but also the least severe. MM, although much more rare (5%), are very severe, being responsible for most deaths from skin cancer.

The aim of this study was to highlight the importance of IHC marker 4-HNE, with tumorigenic, prognostic and therapeutic implications in cutaneous MM.

ROS have been extensively studied in a wide range of human cancers, but little analyzed in cutaneous MM, especially in terms of 4-HNE, product of lipid peroxidation, which is why we chose to introduce this marker in this paper, as an element of novelty in these neoplasias, as well as in our study. In addition, comparative analyzes of 4-HNE expression in human benign and malignant (primary or metastatic) melanocytic lesions have not been conducted until the present research.

Following our study, we came to the following conclusions:

1. As a result of oxidative stress the expression of lipid peroxidation product 4-HNE is significantly increased in dysplastic compared to common (benign) nevi ($p<0.05$) and remains at a level comparable to that in dysplastic lesions in cutaneous MMs ($p>0.05$).

2. 4-HNE occurs early in the process of tumorigenesis of cutaneous MM, highly overexpressed once melanocytes have undergone dysplastic changes. Metastases lose the lipid peroxidation product 4-HNE, aspect correlated with the increased proliferative activity detected in cutaneous MM metastases.

3. Oxidative stress is involved in the initiation and progression of cutaneous MM through lipid peroxidation products of bioactive alkenes type, such 4-HNE generated in mitochondria, which induces the metabolic reprogramming of cells.

4. Manipulation of ROS generation can be a viable approach to preventing and treating MM in the future.

5. 4-HNE is a strong marker involved in tumorigenesis, diagnosis and prognosis of cutaneous MM, and can also represent a major new generation of therapeutic agents.

Due to the biological complexity of MM, the main problem remains how to translate this highly relevant IHC marker into a benefit for MM patients. The solution lies in identifying the true biological "heel of Achilles" for each MM subtype. Most likely, approaches using the 21st century genetic profile technology will yield interesting results.

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