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Immunohistochemical expression of nicotinamide N-methyltransferase in lymph node metastases from cutaneous malignant melanoma

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Original

Immunohistochemical expression of nicotinamide N-methyltransferase in lymph node metastases from cutaneous malignant melanoma / Sartini, Davide; Molinelli, Elisa; Pozzi, Valentina; Campagna, Roberto; Salvolini, Eleonora; Rubini, Corrado; Goteri, Gaia; Simonetti, Oriana; Campanati, Anna; Offidani, Annamaria; Emanuelli, Monica. - In: HUMAN CELL. - ISSN 0914-7470. - STAMPA. - 36:1(2023), pp. 480-482. [10.1007/s13577-022-00793-3]

Availability:

This version is available at: 11566/306463 since: 2024-04-13T14:39:54Z

Publisher:

Published

DOI:10.1007/s13577-022-00793-3

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note finali coverpage

(Article begins on next page)

Human Cell

Immunohistochemical expression of Nicotinamide N-methyltransferase in lymph node metastases from cutaneous malignant melanoma --Manuscript Draft--

Manuscript Number:	HUCE-D-22-00517R1
Full Title:	Immunohistochemical expression of Nicotinamide N-methyltransferase in lymph node metastases from cutaneous malignant melanoma
Article Type:	Letter to the Editor
Keywords:	Cutaneous melanoma; Immunohistochemistry; Lymph node metastases; Nevi; Nicotinamide N-methyltransferase
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Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Abstract is not required since it is a Letter to the Editor
Response to Reviewers:	Replies to reviewers •Reviewer #1 1.Size bars have been provided on each histology image, as suggested. 2.In agreement with the reviewer's observation, Figure 1b has been replaced with a more appropriate picture in which the percentage of positive cells is clearly lower with respect to metastatic melanoma. 3.As concerns the ethics statement, please note that, according to the Ethics Committee of the Marche region [https://www.ospedaliriuniti.marche.it/portale/index.php?id_sezione=146&id_doc=446 , https://www.ospedaliriuniti.marche.it/portale/moduli/downloadFile.php?file=oggetto_modulistica/2214413044300__OProcedure+per+richiesta+di+parere+versione+1.7+del+2

5+maggio+2022+%284%29.doc,] ethical approval for retrospective studies is not required; it is sufficient to send a notification.

•Reviewer #2
N/A

Ancona, September 8, 2022

To the Editor-in-Chief of
Human Cell
Hiroaki Kataoka

Dear Editor,

here enclosed you'll find the manuscript HUCE-D-22-00517, entitled "**Immunohistochemical expression of Nicotinamide N-methyltransferase in lymph node metastases from cutaneous malignant melanoma**", revised according to the reviewer's suggestions. We are grateful to the reviewers for their comments, which we feel have considerably improved our manuscript. In the paper the revised parts are pointed out with red characters.

Hoping that our paper will be now suitable for the publication in your journal, I remain sincerely yours,

Eleonora Salvolini

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Replies to reviewers

- **Reviewer #1**

1. Size bars have been provided on each histology image, as suggested.
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[\[https://www.ospedaliriuniti.marche.it/portale/index.php?id_sezione=146&id_doc=446,https://www.ospedaliriuniti.marche.it/portale/moduli/downloadFile.php?file=oggetto_modulistica/2214413044300_OProcedure+per+richiesta+di+parere+versione+1.7+del+25+maggio+2022+%284%29.doc,_\]](https://www.ospedaliriuniti.marche.it/portale/index.php?id_sezione=146&id_doc=446,https://www.ospedaliriuniti.marche.it/portale/moduli/downloadFile.php?file=oggetto_modulistica/2214413044300_OProcedure+per+richiesta+di+parere+versione+1.7+del+25+maggio+2022+%284%29.doc,_) ethical approval for retrospective studies is not required; it is sufficient to send a notification.

- **Reviewer #2**

N/A

Immunohistochemical expression of Nicotinamide N-methyltransferase in lymph node metastases from cutaneous malignant melanoma

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Keywords Cutaneous melanoma; Immunohistochemistry; Lymph node metastases; Nevi; Nicotinamide N-methyltransferase.

Acknowledgements

The authors would like to thank Dr. Donatella Diotallevi (Unit of Pathologic Anatomy and Histopathology, Department of Biomedical Sciences and Public Health, Marche Polytechnic University, United Ancona Hospitals, Ancona, Italy) for her support.

Author contributions

D.S. and E.M.: writing-original draft preparation, methodology; V.P.: data curation; R.C.: software; E.S.: writing-review and editing, project administration; C.R.: visualization, formal analysis; G.G.: resources; O.S.: funding acquisition; A.C.: validation; A.O.: conceptualization, supervision; M.E.: conceptualization, supervision; writing-review and editing. All authors read and approved the final version of the manuscript.

1 Skin cancers (SC) are the most common malignancies in White-skinned populations. Although
2 melanoma accounts for only about 1 % of all SC, it causes the majority of SC-related deaths, as a
3 result of its high metastatic potential, and its incidence is rising worldwide, mostly due to the
4 increasing exposure to ultraviolet (UV) radiation. Moreover, a central role in melanoma development
5 is played by host risk factors, such as the number and features of melanocytic nevi, genetic
6 susceptibility and a family history. At the time of diagnosis most patients have early-stage disease,
7 which can be effectively treated by surgical resection. Nevertheless, some of them can experience a
8 relapse. In addition, about 10 % of melanomas are diagnosed at an advanced stage and they are
9 difficult to treat, because of the presence of metastases, the resistance to the majority of currently
10 available therapies, and the genetic heterogeneity [1,2]. The 5-year survival rate drops indeed from
11 99 %, in case of early detection, to 27 % if the diagnosis occurs when the melanoma is already
12 metastatic [3].

21 A deeper understanding of the molecules involved in melanoma evolution is therefore crucial, in
22 order to ameliorate the surveillance strategies and to develop effective targeted therapies.

25 In particular, we focused on nicotinamide N-methyltransferase (NNMT), a human cytosolic enzyme
26 catalyzing the N-methylation of nicotinamide, pyridines, and other structural analogues. NNMT
27 upregulation has been observed in a number of malignancies, including skin cancers [4], thus
28 suggesting a key role for the enzyme in promoting cancer phenotype by shifting the cells toward a
29 less differentiated state [5]. In addition, increased enzyme levels have been associated with tumour
30 progression and worse clinical outcomes, also due to NNMT contribution to chemotherapy drug
31 resistance [6,7].

38 Our aim was therefore to analyze the immunohistochemical expression of NNMT in primary
39 cutaneous melanomas and in regional lymph nodes, which represent the most common site of
40 metastatic disease, in order to evaluate its potential suitability for the improvement of the prognostic
41 assessment and care of melanoma patients.

45 A retrospective study was conducted in accordance with the principles of the Declaration of Helsinki
46 on a total of sixty-seven formalin-fixed and paraffin-embedded tissue samples collected between
47 2019 and 2021 and obtained from the archives of the Unit of Pathologic Anatomy and
48 Histopathology, Marche Polytechnic University. The specimens included 42 primary cutaneous
49 melanomas (27 males and 15 females; age range: 28-96 years; mean age: 62 years), 10 age- and
50 gender-matched dermal melanocytic nevi as the control group and 15 lymph node metastases from
51 cutaneous melanoma (3 males and 12 females; age range: 52-86 years; mean age: 76 years).

58 NNMT expression was evaluated by means of immunohistochemistry (IHC) as previously described
59 [8]. A rabbit polyclonal antibody against human NNMT (1:1500 dilution, Sigma-Aldrich, St. Louis,
60

1 Missouri, USA) was used. Clear cell renal cell carcinoma was used as a positive control, whereas
2 negative control slides were obtained by replacing primary antibody with rabbit IgG isotype.
3 Analyses were independently performed by two investigators blinded to the patient group using a
4 Nikon Eclipse E600 light microscope equipped with a Nikon DS-Vi1 digital camera. Stained cells
5 were counted in at least ten fields per sample (field area: 0.07 mm², magnification: x400) and
6 quantified as a percentage of the total counted cells. Agreement between observers was always >
7 95%. Discrepancies were resolved by simultaneous reexamination of the slides, using a double-
8 headed microscope.
9

10 Differences between groups were assessed by means of Kruskal-Wallis test. A p-value less than 0.05
11 was considered statistically significant.
12

13 Our results evidenced a significantly higher NNMT cytoplasmic immunoexpression in tumour cells
14 with respect to controls (being 48.87 ± 29.84 % of positive cells in primary melanomas and $4.00 \pm$
15 3.94 % in benign nevi; Fig. 1 a,b,d $p < 0.001$) thus confirming previous findings by Ganzetti et al.,
16 who were the first authors to observe an upregulation of NNMT in malignant melanoma [9].
17 Moreover, our observations are consistent with other studies which have reported increased NNMT
18 levels in various tumours [4] and corroborate the hypothesis of an involvement of the enzyme in the
19 carcinogenesis. In addition, in the present work we have analyzed for the first time the
20 immunohistochemical expression of NNMT in lymph node metastases from cutaneous melanoma.
21 Interestingly, the percentage of positive cells was significantly higher in metastatic lymph nodes
22 (94.50 ± 1.12 %) compared to both primary melanomas and nevi (Fig. 1 a-d; $p < 0.01$ and $p < 0.0001$,
23 respectively). These data reinforce the assumption that the enzyme contributes to cell invasion and
24 metastasis, probably through the activation of matrix metalloproteinases-2 expression [10] and are in
25 line with the reduction of melanoma cell proliferation and migration recently observed by our
26 research group following NNMT knockdown [7].
27

28 Taken together, the results obtained confirm the noteworthy potential of the enzyme as a diagnostic
29 and prognostic biomarker in a variety of malignancies. In particular, despite the marked
30 improvements achieved in melanoma treatment over the past decade, its lethality remains high,
31 especially when diagnosed at an advanced stage, due to its elevated metastatic power. The
32 identification of lesions having a high risk of progression is therefore critical. Moreover, our
33 observations support the use of NNMT as a new target for molecular-based cancer treatments, the
34 development of which represent a central goal of the research in this field.
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Figure Legends

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2 **Fig. 1** Immunohistochemical expression of NNMT in dermal melanocytic nevi (a; the scale bar
3 corresponds to 100 μm), primary cutaneous melanomas (b; the scale bar corresponds to 50 μm) and
4 lymph node metastases from cutaneous melanoma (c; the scale bar corresponds to 30 μm). Arrows
5 indicate a lymph node follicle. d) Comparison between enzyme levels in benign nevi, primary
6 melanomas and metastatic lymph nodes. Values represent the mean percentage of positive cells \pm
7 standard deviation; * $p < 0.001$, ** $p < 0.01$, *** $p < 0.0001$.
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Funding

The authors did not receive support from any organization for this study.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Ethics approval

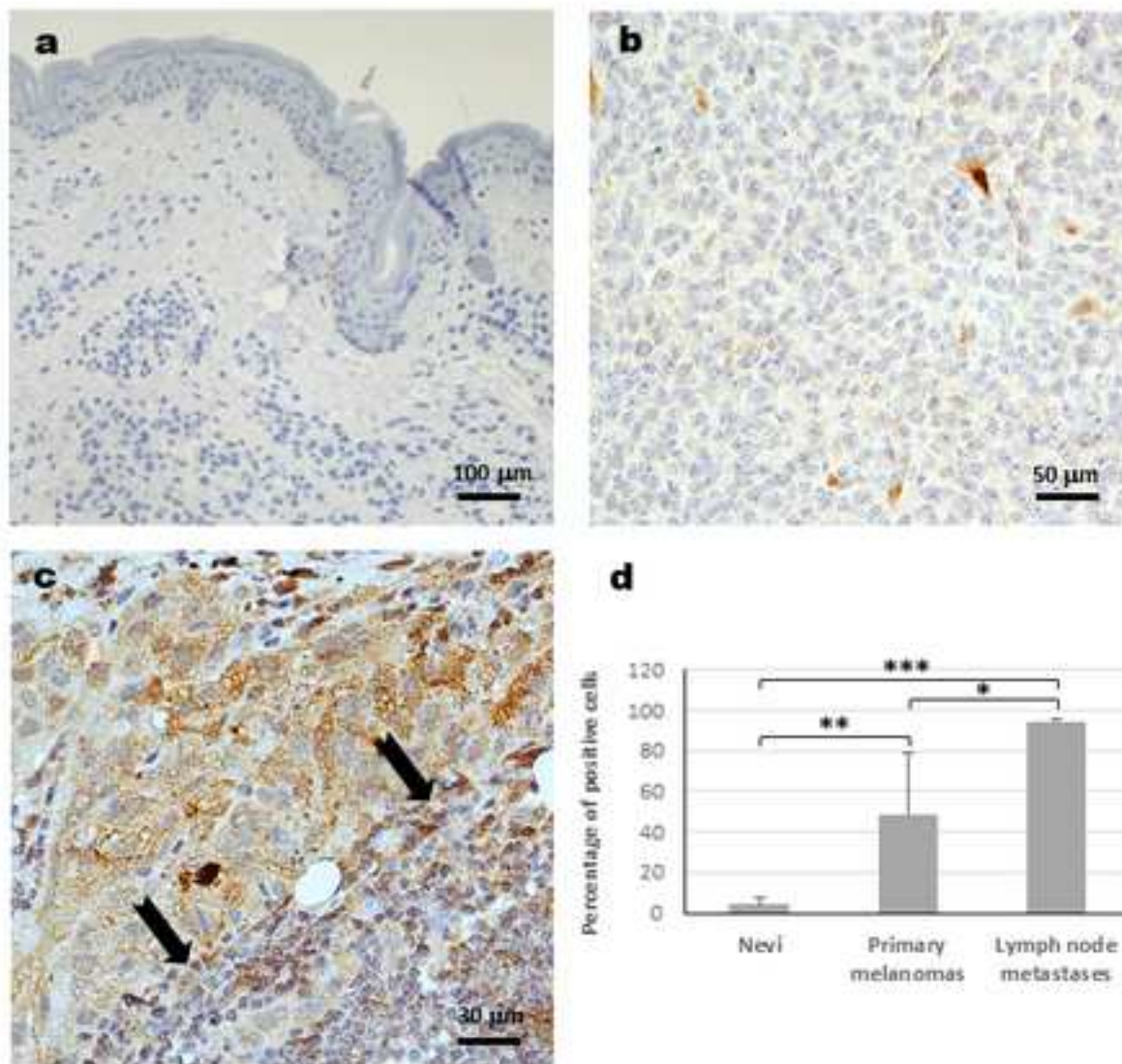
13 This retrospective study was performed on formalin-fixed and paraffin-embedded tissue specimens,
14 previously collected for diagnostic purposes. **According to the Ethics Committee of the Marche**
15 **region ethical approval for retrospective studies is not required; it is sufficient to send a notification.**
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Informed consent

23 All participants gave their informed consent.
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