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

Human Cell

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--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
Corresponding Author:	Eleonora Salvolini Università Politecnica delle Marche: Universita Politecnica delle Marche ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Università Politecnica delle Marche: Universita Politecnica delle Marche
Corresponding Author's Secondary Institution:	
First Author:	Davide Sartini
First Author Secondary Information:	
Order of Authors:	Davide Sartini
	Elisa Molinelli
	Valentina Pozzi
	Roberto Campagna
	Eleonora Salvolini
	Corrado Rubini
	Gaia Goteri
	Oriana Simonetti
	Anna Campanati
	Annamaria Offidani
	Monica Emanuelli
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/221441304430O__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

Eleonora Salvolini
Department of Clinical Sciences
Università Politecnica delle Marche
Ancona, ITALY
e.salvolini@univpm.it

Replies to reviewers

- **Reviewer #1**
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- **Reviewer #2**
  N/A

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

Davide Sartini[1], Elisa Molinelli[2], Valentina Pozzi[1], Roberto Campagna[1], Eleonora Salvolini[1], Corrado Rubini[3], Gaia Goteri[3], Oriana Simonetti[2], Anna Campanati[2], Annamaria Offidani[2], Monica Emanuelli[1,4].

[1] Department of Clinical Sciences, Marche Polytechnic University, Ancona, Italy

[2] Department of Clinical and Molecular Sciences, Marche Polytechnic University, Ancona, Italy

[3] Department of Biomedical Sciences and Public Health, Marche Polytechnic University, Ancona, Italy

[4]New York-Marche Structural Biology Center (NY-MaSBiC), Marche Polytechnic University, Ancona, Italy

Corresponding Author:
Eleonora Salvolini
e-mail address: e.salvolini@univpm.it

D. Sartini and E. Molinelli contributed equally to this work

https://orcid.org/0000-0003-3879-8647 (D. Sartini)
https://orcid.org/0000-0002-7939-0026 (E. Molinelli)
https://orcid.org/0000-0003-3941-0341 (V. Pozzi)
https://orcid.org/0000-0003-2607-2734 (R. Campagna)
https://orcid.org/0000-0003-2405-8947 (E. Salvolini)
https://orcid.org/0000-0003-0632-8632 (C. Rubini)
https://orcid.org/0000-0001-5198-8387 (G. Goteri)
https://orcid.org/0000-0001-7941-5089 (O. Simonetti)
https://orcid.org/0000-0002-3740-0839 (A. Campanati)
https://orcid.org/0000-0001-5445-1200 (A. Offidani)
https://orcid.org/0000-0002-8520-519X (M. Emanuelli)

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 Polytecnhic 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.

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

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

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

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

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

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

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

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

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

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

Figure Legends

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

Funding

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

Conflicts of interest

The authors have no conflicts of interest to declare.

Ethics approval

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

Informed consent

All participants gave their informed consent.

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Figure

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