

# Role of epithelial–mesenchymal transition involved molecules in the progression of cutaneous melanoma

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**Abstract** Epithelial–mesenchymal transition (EMT) has been suggested to have a driving role in the acquisition of a metastatic potential by melanoma cells. Important hallmarks of EMT include both E-cadherin downregulation and increased expression of N-cadherin. This switch in distinct classes of adhesion molecules leads melanoma cells to lose contact with adjacent keratinocytes and interact instead with stromal fibroblasts and endothelial cells, thus promoting dermal and vascular melanoma invasion. Consequently, tumor cells migrate to distant host tissues and establish metastases. A key regulator in the induction of EMT in melanoma is the Notch1 signaling pathway that, when activated, is prompt to upregulate N-cadherin expression. By means of this strategy, melanoma cells gain enhanced survival, proliferation and invasion properties, driving the tumor toward a more aggressive phenotype. On the basis of these statements, the present study aimed to investigate the possible association between

N-cadherin and Notch1 presence in primary cutaneous melanomas and lymph node metastases. Our results from immunohistochemical analysis confirmed a positive correlation between N-cadherin and Notch1 presence in the same tumor samples. Moreover, this study highlighted that a concomitant high expression of N-cadherin and Notch1, both in primary lesions and in lymph node metastases, predicts an adverse clinical outcome in melanoma patients. Therefore, N-cadherin and Notch1 co-presence can be monitored as a predictive factor in early- and advanced-stage melanomas and open additional therapeutic targets for the restraint of melanoma metastasis.

**Keywords** N-cadherin · Notch1 · Immunohistochemistry · Survival · Cutaneous melanoma

## Introduction

Melanoma represents one of the most challenging diseases to treat, because of the high metastatic potential of even thin lesions with lethal outcome. Identifying within the tumor, at the time of diagnosis, the molecular markers fostering metastasis could help in clear-cut prognosis and allow a focused individual treatment.

Epithelial–mesenchymal transition (EMT), which applies to malignant melanocytes changing from an epithelial to a functional mesenchymal phenotype, has been suggested as a driving event in the acquisition of metastatic potential by melanoma cells (Kim et al. 2013; Alonso et al. 2007; Caramel et al. 2013). A determinant hallmark of EMT is represented by the epithelial (E)- to neural (N)-cadherin switch, by means of a strong reduction of E-cadherin expression versus an increased expression of N-cadherin, a 99.7 kDa transmembrane glycoprotein, linked to the actin molecules

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by catenins and deeply involved in the intercellular adhesion (Gravdal et al. 2007; Kuphal and Bosserhoff 2012). As a specific result of N-cadherin overexpression, malignant melanocytes lose contact with adjacent keratinocytes and acquire new adhesion properties in parallel with the enhancement of cell motility. In particular, these dramatic changes induce strong contact with stromal fibroblasts and vascular endothelial cells and trigger their access to the circulatory and lymphatic system (Bachmann et al. 2005; Yan et al. 2016; Guarino et al. 2007). On the other hand, N-cadherin-mediated cell adhesion activates the antiapoptotic Akt/PKB pathway that, in turn, inactivates the proapoptotic Bcl-2-associated agonist of cell death (BAD) and stabilizes the antiapoptotic protein  $\beta$ -catenin, thus promoting melanoma cell survival (Li et al. 2001).

With regard to melanoma, an important key regulator in the induction of EMT is represented by the Notch1 signaling pathway that, when activated, gives a boost to its oncogenic properties by up-regulating N-cadherin expression. In this way, melanoma cells gain enhanced survival, proliferation and invasion advantages, driving the tumor toward a more aggressive phenotype (Liu et al. 2006). The role of EMT and in particular the involvement of N-cadherin and Notch1 in tumor development and progression has been the object of investigation not only in melanoma (Yan et al. 2016) but also in other malignancies including prostate, pancreatic, breast cancer and rhabdomyosarcoma (Gravdal et al. 2007; Bao et al. 2011; Shao et al. 2015; Masià et al. 2012).

Taking into account the published data, the goal of our study was to investigate the expression of N-cadherin by immunohistochemical analysis in tissue samples from primary cutaneous melanomas and lymph node metastases, its possible association with Notch1 presence in the same tumor samples, and to determine whether the expression profile of the above N-cadherin and Notch1 proteins was associated with the survival outcome in melanoma patients. N-cadherin expression was simultaneously evaluated in a series of pre-neoplastic lesions, including junctional, compound and intradermal dysplastic melanocytic nevi.

## Materials and methods

### Patients and tumor specimens

Archival tissue blocks (104 sporadic primary cutaneous melanomas and 35 lymph node metastases) were obtained from 128 patients: the primary tumors were obtained from 93 patients, the lymph node metastases from 24 patients, and both the primary tumor and the lymph node metastasis from 11 patients. The patients underwent observation at the Businco Oncologic Hospital, Cagliari, Italy, and at the Department of Pathology, Cancer Center of

Solca, Cuenca, Ecuador, from November 1995 through June 2009, and were selected for further study according to the following criteria: melanoma with vertical growth phase and complete clinical data including follow-up until July 2009. Lymph node status and the presence of metastases were verified by a clinical and pathological examination. The clinicopathological characteristics of the 128 Stage I–IV melanoma patients are shown in Table 1 (Greene et al. 2002; Clark et al. 1989). Following surgical resection, each tumor was fixed in 10% buffered formalin and paraffin-embedded. Tumor areas were identified on H&E-stained sections and on adjacent sections immunohistochemically stained for melanoma-associated antigens, including S-100 protein, melan A, and HMB-45. An independent histopathological analysis was performed by two pathologists (L.P. and C.F.).

The study also included 81 dysplastic melanocytic nevi (23 junctional, 38 compound and 20 intradermal) that were formalin-fixed and paraffin-embedded for the

**Table 1** Clinicopathological characteristics of the 128 cutaneous melanoma patients

Characteristics	No. patients (%)
Gender	
Men	60 (47)
Women	68 (53)
Age at diagnosis (range 10–94)	
$\leq 64$ years <sup>a</sup>	65 (51)
$> 64$ years	63 (49)
Stage <sup>b</sup>	
I–II	68 (53)
III–IV	60 (47)
Breslow thickness <sup>c</sup>	
T1–T2	33 (32)
T3–T4	71 (68)
Clark level <sup>c,d</sup>	
II–III	31 (30)
IV–V	73 (70)
Ulceration <sup>c</sup>	
Absent	41 (39)
Present	63 (61)
Anatomic location <sup>c</sup>	
Head and neck	28 (27)
Trunk	25 (24)
Upper extremities	16 (15)
Lower extremities	35 (34)

<sup>a</sup>Median value

<sup>b</sup>American Joint Committee on Cancer (AJCC) staging system (Greene et al. 2002)

<sup>c</sup>Only primary tumors ( $n = 104$ )

<sup>d</sup>Clark's classification (Clark et al. 1989)

immunohistochemical analysis. Normal skin samples, from eyelids and external auditory canal, were obtained from healthy donors.

### Immunohistochemistry

Serial microtome sections (5- $\mu$ m) were treated for the immunohistochemical detection of N-cadherin, activated Notch1 (N<sup>IC</sup>, Notch intracellular domain) and melanoma-associated antigens S-100, melan A and HMB-45, applying the alkaline phosphatase–streptavidin method, as previously described (Murtas et al. 2015). Antigen retrieval was performed by heating at 95 °C for 40 min in 10 mM citrate buffer (pH 6.0), followed by gradual cooling for 20 min, for the demonstration of N-cadherin, activated Notch1 and melan A, and by immersion in 0.1% trypsin in phosphate-buffered saline (PBS, pH 7.4), at 37 °C for 10 min for S-100 protein and HMB-45 antigen. Non-specific binding was blocked with 10% normal goat serum (NGS; Sigma-Aldrich, St. Louis, MO, USA) for 45 min at room temperature (RT). Mouse monoclonal antibodies to human N-cadherin (clone 13A9, 1:100, overnight at 4 °C; Santa Cruz Biotechnology, Heidelberg, Germany), to human melan A (clone A103, 1:100, 1 h at RT; Dako, Glostrup, Denmark), and to human HMB-45 (clone HMB-45, 1:100, 1 h at RT; Dako), rabbit polyclonal antibodies to human activated Notch1 (1:100, overnight at 4 °C; Abcam, Cambridge, UK) and to bovine S-100 protein (1:1000, 1 h at RT; Dako) were used as primary antisera. Table 2 shows the details of the primary antibodies used for the immunohistochemical staining, according to the Resource Identification Initiative (Bandrowski et al. 2015). Evidence for the antibody specificity is given in the technical specifications provided by the respective manufacturers. In particular, regarding the N-cadherin antibody, a knock-down validation was performed by Alaei et al. (2016). Biotinylated goat anti-mouse and anti-rabbit immunoglobulins G (1:200, 30 min at RT; Vector Laboratories, Burlingame, CA, USA) were used as secondary antisera. The sections were further incubated in alkaline phosphatase–streptavidin

(1:1000; Vector Laboratories) for 30 min at RT. The Fast Red substrate–chromogen system (Sigma-Aldrich) was used to develop the alkaline phosphatase reaction product. The sections were counterstained with Carazzi's haematoxylin. Known positive target tissues were used as controls: human lymphoid and myocardial tissues for activated Notch1 and N-cadherin, respectively. To check non-specific binding of secondary antibodies, negative controls were established by replacing the primary antibodies with normal serum.

### Evaluation of immunohistochemical staining

The immunostained tumors were examined and scored by two pathologists (L.P. and C.F.) in a blinded fashion, with no previous knowledge of the clinical status of the patients. For each analyzed section, the two estimates were then averaged to provide the final value of the staining.

Counting of tumor cells resulting positive for N-cadherin or activated Notch1 was performed by the microscopic examination of the whole tumor at 200 $\times$  magnification and the evaluation was then confirmed through adjacent fields at 400 $\times$  magnification. For each case, the average of the single counts of positive tumor cells per field was considered. With regard to N-cadherin, an immunoreactivity scoring system previously used by Lade-Keller et al. (2013) was applied, based on the proportion of positive tumor cells showing at least a discrete staining. The percentage of immunoreactive tumor cells was scored as 0 (<5% positive cells), 1 (5–50% positive cells), or 2 (>50% positive cells). To dichotomize the obtained data, the overall status of cases was designated as either low (<5%, group 0) or high N-cadherin expression ( $\geq$ 5%, group 1 and 2). For activated Notch1, a scoring system based on the proportion and intensity of positive tumor cells, previously used by Chu et al. (2011), was applied. (A) Number of positive stained cells:  $\leq$ 25% (scored 0), 26–50% (1), and >50% (2). (B) Intensity of staining: colorless/weak (0), moderate (1), and strong (2). The staining score was obtained by multiplying (A) and (B) and stratified as absent/weak (0–1 score) and moderate/strong (2–4 score). Tumors

**Table 2** Primary antibodies characterisation

Antibody name	Immunogen	Clonality	Clone	Host	Catalog number	Vendor	RRID <sup>a</sup>
N-cadherin	Recombinant N-cadherin cytoplasmic domain fusion protein of human origin	Monoclonal	13A9	Mouse	sc-59987	Santa Cruz	AB_781744
Melan A	Recombinant protein expressed in E. coli corresponding to melan A	Monoclonal	A103	Mouse	M7196	Dako	AB_2335691
Melanosome	Extract of pigmented melanoma metastases from lymph nodes	Monoclonal	HMB45	Mouse	M0634	Dako	AB_2335682
Activated Notch1	Synthetic peptide corresponding to activated Notch1 aa 1755-1767 (intracellular)	Polyclonal	–	Rabbit	ab8925	Abcam	AB_306863
S100	S100 isolated from cow brain	Polyclonal	–	Rabbit	Z0311	Dako	AB_10013383

<sup>a</sup>The Resource Identification Initiative (Bandrowski et al. 2015); Resource Identification Portal (<http://scicrunch.com/resources>)



with moderate/strong immunostaining were classified as showing high activated Notch1 expression, whereas an absent/weak immunostaining was classified as low activated Notch1 expression.

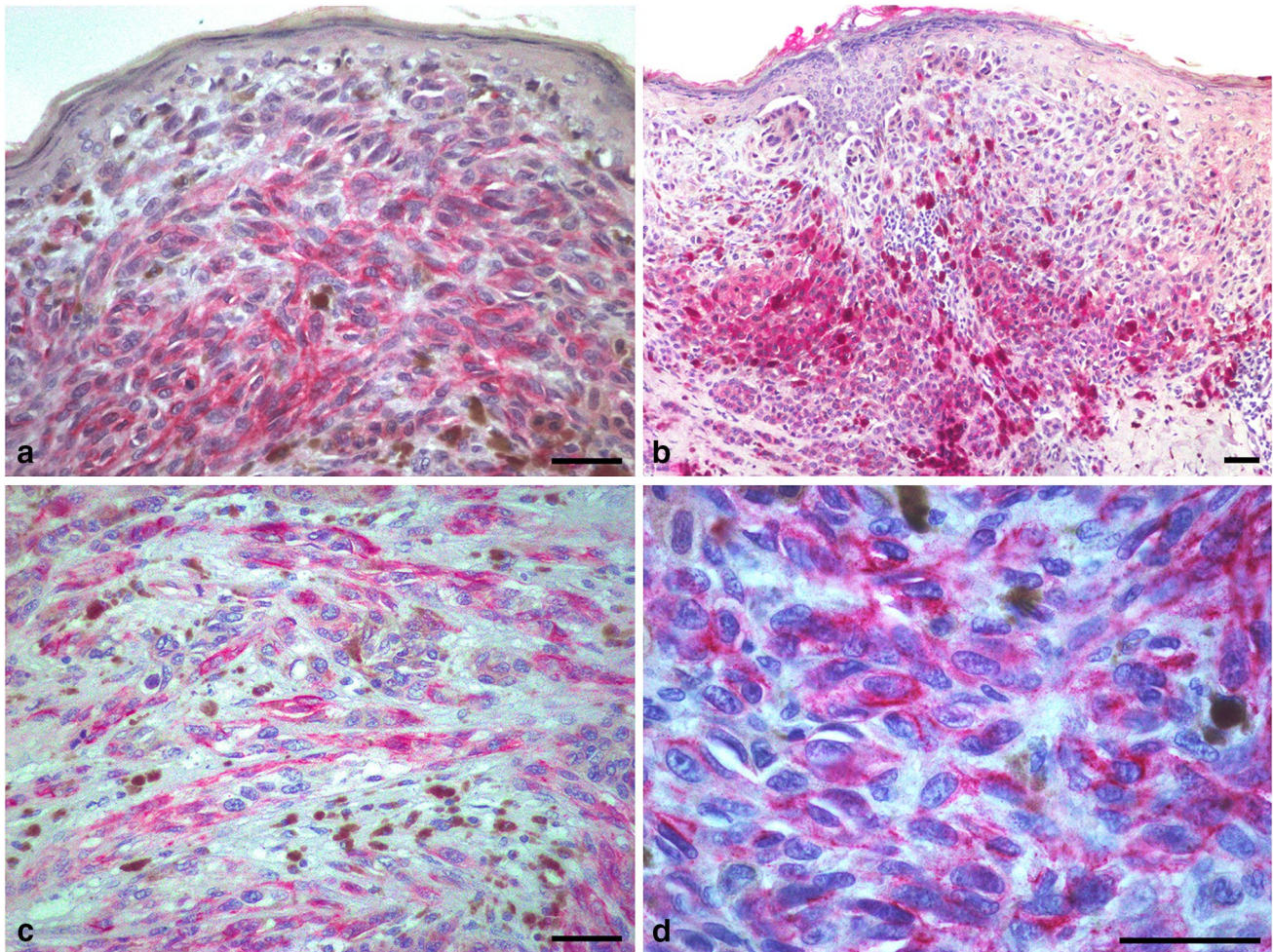
### Double immunofluorescence

A simultaneous procedure was used for the staining of N-cadherin and activated Notch1. Following deparaffinization, rehydration, antigen retrieval, and blocking of non-specific binding, sections were incubated overnight at 4 °C with a mixture of mouse anti-human N-cadherin (clone 13A9, 1:100; Santa Cruz Biotechnology) and rabbit anti-human activated Notch1 (1:25; Abcam) primary Abs. Donkey Alexa Fluor 594 anti-mouse IgG (H+L) and Alexa Fluor 488 anti-rabbit IgG (H+L) (1:200 and 1:500, respectively;

Invitrogen Life Technologies, Paisley, UK) secondary Abs were used for immunofluorescence detection. The sections were mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclear detail (Vector Laboratories). A Zeiss Axioplan 2 (HBO 100 illuminator; mercury vapor, short arc lamp) microscope was used for sample analysis and image processing.

### Statistical analysis

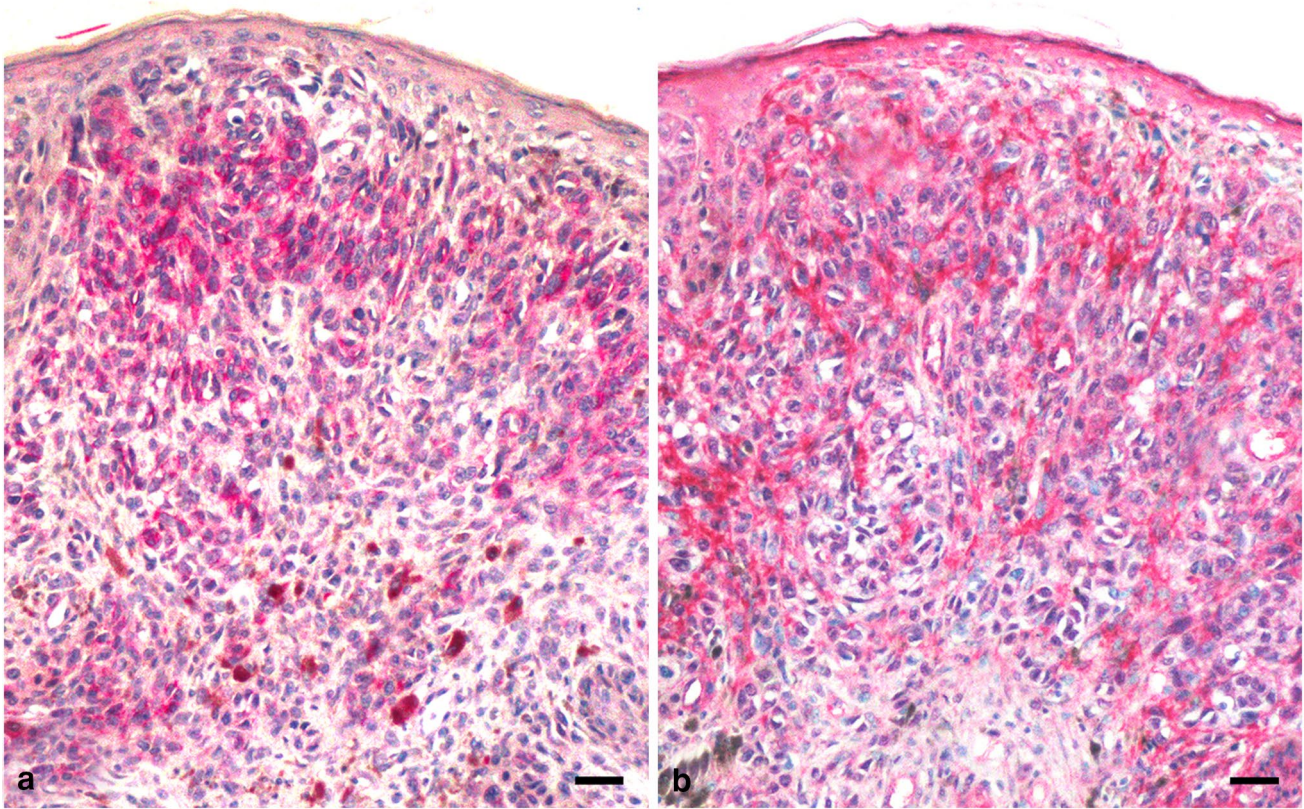
Data were computed with the IBM SPSS Statistics 21 software. The correlation between N-cadherin and activated Notch1 expression and between N-cadherin expression and clinicopathological characteristics of Stage I–IV melanoma patients was assessed by Fisher's exact or Pearson's  $\chi^2$  test. The tests used were two-tailed. Overall and 5-year survival



**Fig. 1** Immunohistochemistry for N-cadherin in primary cutaneous melanoma. Positive tumor cells were distributed in the whole tumor (**a**) as well as at the invasive tumor front (**b**). N-cadherin was expressed in the cytoplasm and lining the cellular border of tumor

cells, with a moderate/strong staining intensity (**c**, **d**). **d** The figure represents a higher magnification better showing the staining intensity pattern ranging from moderate to strong. Scale bars **a–d** = 50  $\mu$ m





**Fig. 2** Immunohistochemistry for N-cadherin (**a**) and activated Notch1 (**b**) in primary cutaneous melanoma. Immunoreactivity for N-cadherin (**a**) and activated Notch1 (**b**) was observed in the same tumor area on adjacent histological sections. Scale bars **a**, **b** = 50  $\mu$ m

of patients with primary melanoma or lymph node metastasis was calculated according to N-cadherin, activated Notch1 expression, and the histopathological parameter Breslow thickness, from the date of histological diagnosis to the date of fatal outcome due to melanoma or to the last follow-up, until July 2009. Primary tumors were stratified into two categories (T1–T2; T3–T4) according to thickness. Survival curves were obtained using the Kaplan–Meier method and comparisons were made using the log-rank test. Differences were considered statistically significant at  $p \leq 0.05$ .

## Results

### N-cadherin and activated Notch1 expression by immunohistochemistry

N-cadherin-positive cells were diffusely distributed throughout the tumor (Fig. 1a) as well as at the invasive tumor front (Fig. 1b) and at the periphery of tumor nests. N-cadherin immunoreactivity was identified in the cytoplasm and lining the cellular border of tumor cells, with a staining intensity pattern from weak to moderate/strong (Fig. 1c, d).

Among primary tumors, 38% of cases showed a high immunoreactivity for N-cadherin both throughout the tumor and in the advancing edge; a strong immunostaining for activated Notch1 in the entire tumor was observed in 78% of cases. Regarding lymph node metastases, a high immunostaining for N-cadherin was found in 29% of samples and for activated Notch1 in 77% of cases.

It is remarkable that, in both primary and metastatic lesions, N-cadherin-positive cells were mainly found in the tumor areas concurrently with activated Notch1-presenting cells (Fig. 2a, b). Activated Notch1 immunostaining was strongly detectable within the cytoplasm and in fewer nuclei of tumor cells, regardless to the whole tumor and the invading front, as shown in our previous study (Murtas et al. 2015). The frequencies of N-cadherin and activated Notch1 expression in tumor cells are shown in Table 3.

Interestingly, at the dysplastic melanocytic nevi sites, the nests of nevus cells confined to the epidermis did not display any N-cadherin immunostaining in junctional nevi (Fig. 3a). The junctional component, as well as the epidermis, was negative also in all compound and intradermal nevi. In 24% of compound and in 35% of intradermal nevi, a moderate/strong staining for N-cadherin was exclusively visible in the nests of nevus cells within the dermis (Fig. 3b,

**Table 3** N-cadherin and Notch1 expression in primary melanomas and lymph node metastases

	No. positive cases (%)
Stage I–II <sup>a</sup> primary tumors	
N-cadherin	
Whole tumor	20 (29)
Tumor front	20 (29)
Notch1	
Whole tumor	49 (72)
Tumor front	49 (72)
Stage III–IV <sup>a</sup> primary tumors	
N-cadherin	
Whole tumor	20 (56)
Tumor front	20 (56)
Notch1	
Whole tumor	32 (89)
Tumor front	30 (83)
Lymph node metastases	
N-cadherin	
Whole tumor	10 (29)
Notch1	
Whole tumor	27 (77)

<sup>a</sup>American Joint Committee on Cancer (AJCC) staging system (Greene et al. 2002)

c). N-cadherin was not expressed in normal skin since both epidermal keratinocytes and melanocytes were totally lacking in the same protein (Fig. 3d).

### N-cadherin and activated Notch1 expression by double immunofluorescence

With the assumption that co-localisation may mean interaction, as we previously investigated for the transcription factors NF- $\kappa$ B and IRF-1 (Murtas et al. 2013), we tested whether N-cadherin and Notch1 co-localized. Tumor cells mostly showed a co-localisation of N-cadherin and activated Notch1 in the cytoplasm (Fig. 4a–d), although a co-distribution of cytoplasmic N-cadherin and nuclear-activated Notch1 was sometimes evident.

### Statistical analysis

The number of tumor cells positive for N-cadherin was increased in the tumors with high activated Notch1 expression. When analyzed by Fisher's exact test, N-cadherin immunoreactivity was significantly associated with activated Notch1 expression in melanoma cells of both primary tumors and lymph node metastases ( $p = 0.034$ ). Table 4 shows the expression of N-cadherin in relation to activated Notch1 immunoreactivity.

No significant difference in N-cadherin expression was found between primary tumors and lymph node metastases ( $p > 0.05$ ). At the same time, no association was demonstrated between N-cadherin expression and patients' clinicopathological characteristics ( $p > 0.05$ , Table 5).

Survival analysis showed that patients with primary tumors  $\leq 2$  mm thick had a significantly longer overall ( $p = 0.023$ ) and 5-year ( $p = 0.014$ ) survival when compared with patients with thicker primary lesions.

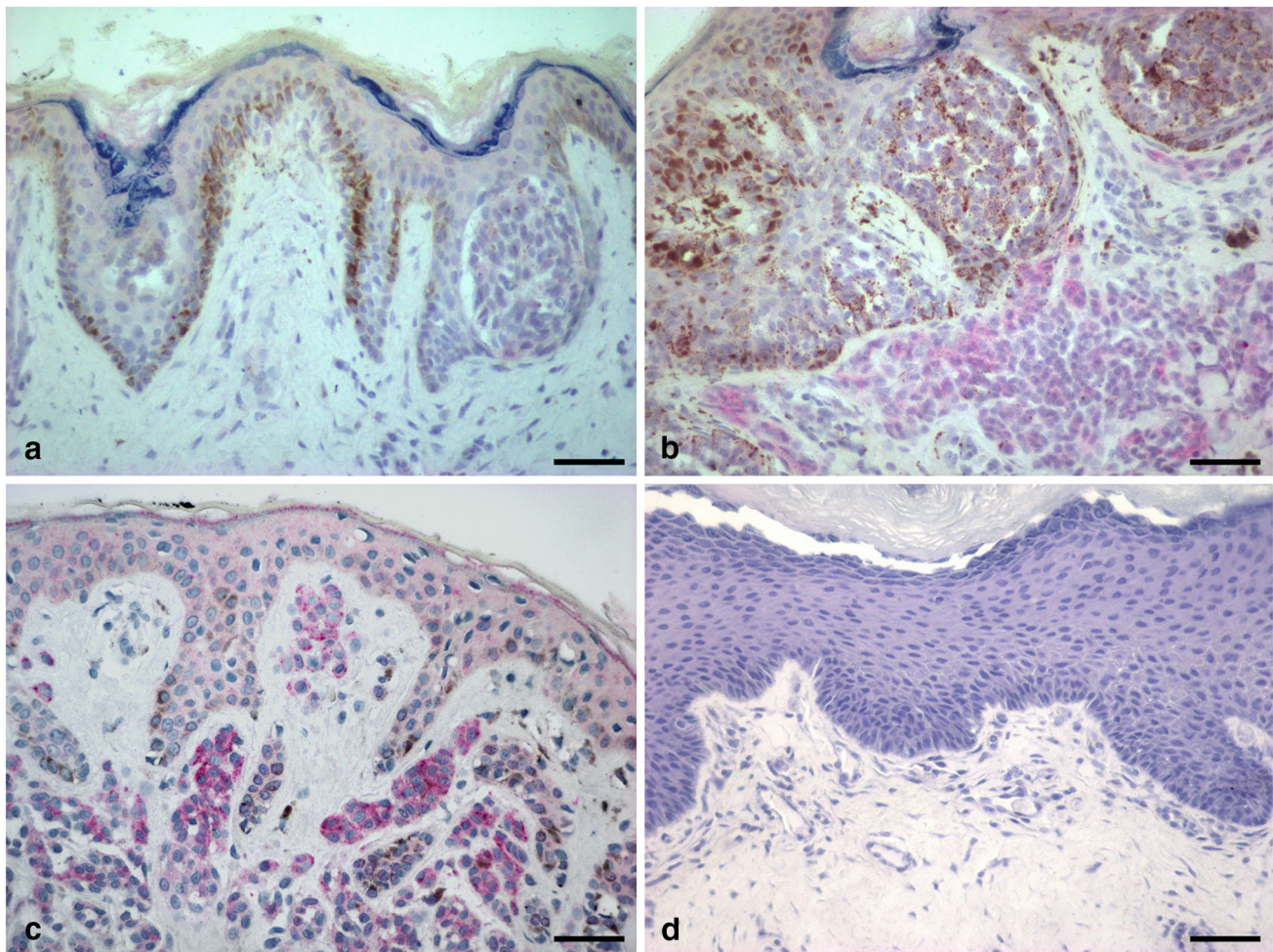
On the basis of the evidence that a high expression of N-cadherin alone, as well as a high activated Notch1 expression, did not show a prognostic value, they were analyzed in combination: patients were categorized based on showing a high N-cadherin/Notch1 expression (high immunoreactivity of N-cadherin and/or activated Notch1) or a low N-cadherin/Notch1 expression (both N-cadherin and activated Notch1 low immunoreactivity). Kaplan–Meier analysis revealed that the group of patients with high N-cadherin/Notch1 expression had a statistically significant poorer outcome in comparison with the group of patients with low N-cadherin/Notch1 expression in the primary tumor cells as well as in the lymph node metastases. Global survival analysis, adjusted for the primary tumor/lymph node metastasis status, showed that a high expression of N-cadherin and/or activated Notch1 significantly predicted poor overall ( $p = 0.039$ ; Fig. 5) and 5-year ( $p = 0.042$ ; Fig. 6) survival of patients.

Regarding N-cadherin and activated Notch1 staining in the primary tumor, Kaplan–Meier estimates of overall survival probabilities were 0.54 for patients with high N-cadherin/Notch1 immunoreactivity and 0.73 for those with low expression; estimates of 5-year survival probabilities were 0.58 for patients with high and 0.80 for those with low expression. Moreover, concerning N-cadherin and activated Notch1 staining in the lymph node metastasis, Kaplan–Meier estimates of overall survival probabilities were 0.12 for patients with high and 0.33 for those with low N-cadherin/Notch1 immunoreactivity; estimates of 5-year survival probabilities were 0.17 for patients with high and 0.33 for those with low expression.

### Discussion

Epithelial–mesenchymal transition is a key step in melanoma invasion and metastasis that leads to recurrence and cancer-related death. The expression of the cell adhesion molecule N-cadherin has been shown to be responsible for the acquisition of a metastatic phenotype and is thus considered a melanoma progression marker (Kuphal and Bosserhoff 2012). Emerging evidence suggests that the activation of Notch1 signaling promotes EMT through the up-regulation of mesenchymal cell markers, such as N-cadherin (Zhou et al. 2014). Interestingly, we observed



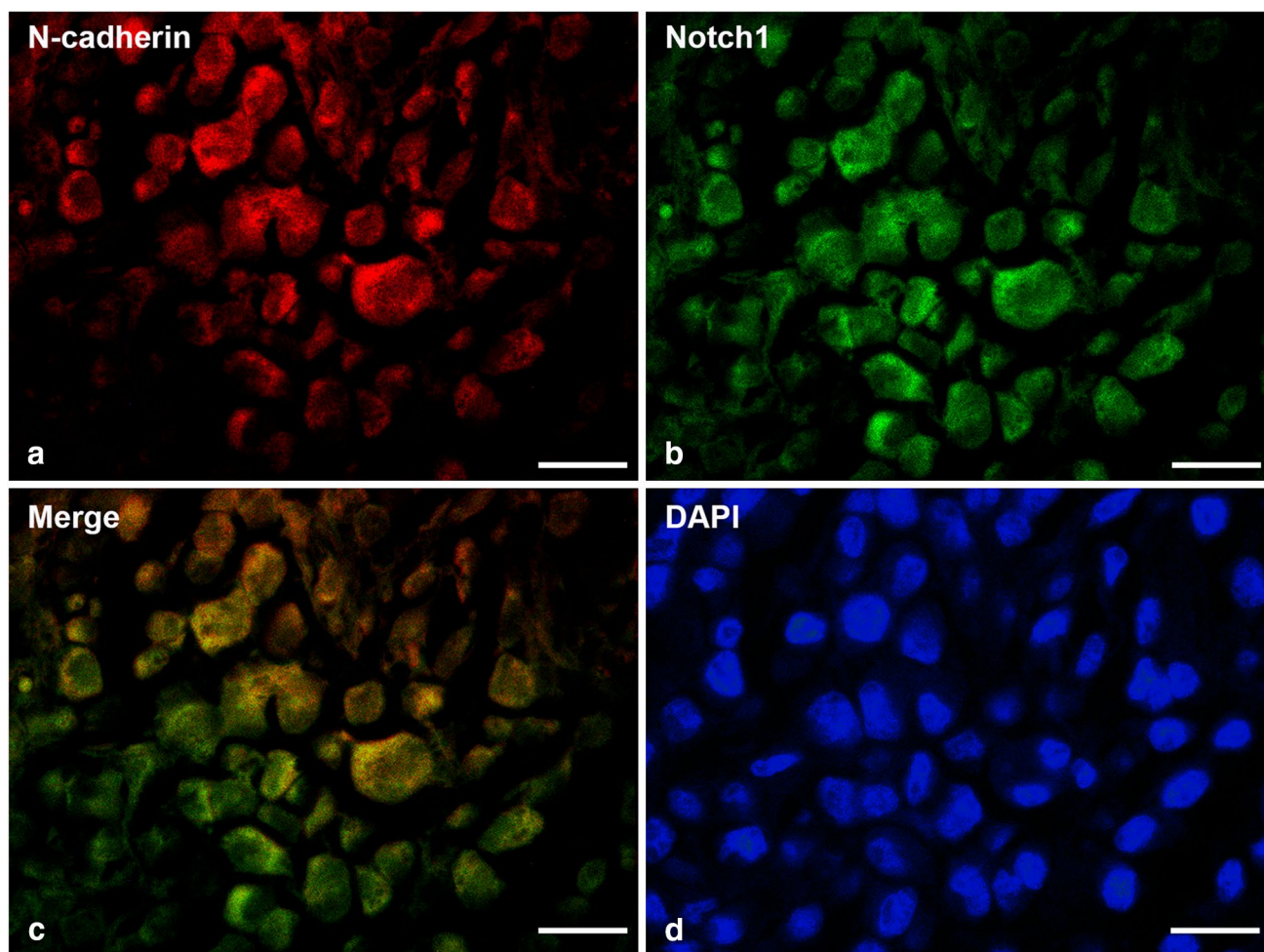


**Fig. 3** Immunohistochemistry for N-cadherin in dysplastic melanocytic nevi. In junctional nevi (**a**), both epidermis and nevus cells were negative for N-cadherin. Positive nevus cells were exclusively observable in the nests within the dermis of compound (**b**) and intradermal

(**c**) nevi, with a moderate/strong staining intensity. N-cadherin expression was not detected in epidermal keratinocytes and melanocytes of normal skin (**d**). Scale bars **a–d** = 50  $\mu$ m

that the expression levels of N-cadherin in melanoma cells of both primary melanoma sites and lymph node metastases were significantly related to the activation of Notch1, assessed by the immunohistochemical detection of the Notch intracellular domain  $N^{IC}$ , after its release upon Notch1 receptor activation and cleavage. The co-presence of N-cadherin and activated Notch1 in the cytoplasm of tumor cells was further confirmed by the immunofluorescence analysis. All these findings corroborate the evidence that the activation of Notch1 in malignant melanocytes switches on N-cadherin expression, which confers a mesenchymal, more aggressive, phenotype to the tumor, as suggested by Liu et al. (2006). Moreover, it has been shown that N-cadherin-mediated adhesion between tumor cells and vascular endothelial cells facilitates transmigration of tumor cells through the vascular endothelium, thus promoting the tumor spread (Li et al. 2001).

The same factors that drive epithelial cells toward a mesenchymal phenotype may also commit endothelial cells to a pro-angiogenic phenotype, meant to be essential for melanoma progression and metastasis (Ribatti 2017). In particular, Notch1 overexpression in melanoma cells can promote tumor angiogenesis in a paracrine manner (Murtas et al. 2015) and meanwhile N-cadherin stabilizes nascent blood vessels by promoting endothelial–mural cell interactions (Luo and Radice 2005). EMT, by its own, orchestrates a pro-angiogenic context through a crosstalk between tumor cells, cells of neoplastic stroma (mainly cancer-associated fibroblasts, CAFs), and the extracellular matrix. Among the signaling molecules involved in this interplay, transforming growth factor  $\beta$  (TGF- $\beta$ ) supports tumor–stroma interaction and EMT in advanced melanoma, resulting in the stimulation of angiogenesis (Ribatti 2016). Indeed, the presence of EMT markers has been associated with proliferative and



**Fig. 4** Double immunofluorescence staining for N-cadherin (a) and activated Notch1 (b) in primary cutaneous melanoma, showing an overlapping pattern for N-cadherin and activated Notch1 expression

in the cytoplasm of tumor cells (c). Nuclei were counterstained with DAPI (d). Scale bars a–d = 20  $\mu$ m

**Table 4** N-cadherin expression in relation to Notch1 immunoreactivity

	Notch1		<i>p</i> value*
	No. cases (%)		
	Negative	Positive	
N-cadherin			0.034
No. cases (%)			
Negative	25 (18)	64 (46)	
Positive	6 (4)	44 (32)	

\* Fisher's exact test

pro-angiogenic proteins expression and with prognosis in cervical cancer (Ribatti 2017).

Currently, the most useful prognostic factor in clinical practice for melanoma is Breslow thickness (Piepkorn and Barnhill 2014), as confirmed by our findings. Since in the

near future it is expected that the identification of newer molecular factors will help in more precisely predicting the outcome of melanoma patients, we undertook investigations to assess whether N-cadherin is a potential candidate that fulfills this role. The prognostic importance of N-cadherin remains unclear in the literature and the findings reported by previous studies are contradictory in some aspects. Bachmann et al. (2005) did not find any correlation between N-cadherin expression and the clinical outcome of melanoma patients. Kreizenbeck et al. (2008) showed that increased levels of N-cadherin expression are associated with better overall survival. Pieniazek et al. (2016) found that increased immunoreactivity for N-cadherin is associated with shorter cancer-specific overall survival. Lade-Keller et al. (2013) reported that high N-cadherin expression alone does not significantly predict survival, but when a “switch profile” (low E-cadherin/high N-cadherin expression) is triggered, it significantly predicts poor melanoma-specific



**Table 5** N-cadherin expression in relation to patients' clinicopathological characteristics

	N-cadherin	
	No. cases (%)	
	Negative	Positive
<b>Gender</b>		
Men	37 (29)	24 (19)
Women	45 (35)	22 (17)
<b>Age at diagnosis</b>		
≤64 years <sup>a</sup>	40 (31)	25 (20)
>64 years	42 (33)	21 (16)
<b>Stage<sup>b</sup></b>		
I–II	48 (37)	20 (16)
III–IV	34 (27)	26 (20)
<b>Breslow thickness<sup>c</sup></b>		
T1–T2	21 (20)	12 (12)
T3–T4	43 (41)	28 (27)
<b>Clark level<sup>c,d</sup></b>		
II–III	21 (20)	10 (10)
IV–V	43 (41)	30 (29)
<b>Ulceration<sup>c</sup></b>		
Absent	25 (24)	16 (15)
Present	39 (37)	24 (23)
<b>Anatomic location<sup>c</sup></b>		
Head and neck	17 (16)	11 (10)
Trunk	18 (17)	7 (7)
Upper extremities	10 (10)	6 (6)
Lower extremities	19 (18)	16 (15)

<sup>a</sup>Median value

<sup>b</sup>American Joint Committee on Cancer (AJCC) staging system (Greene et al. 2002)

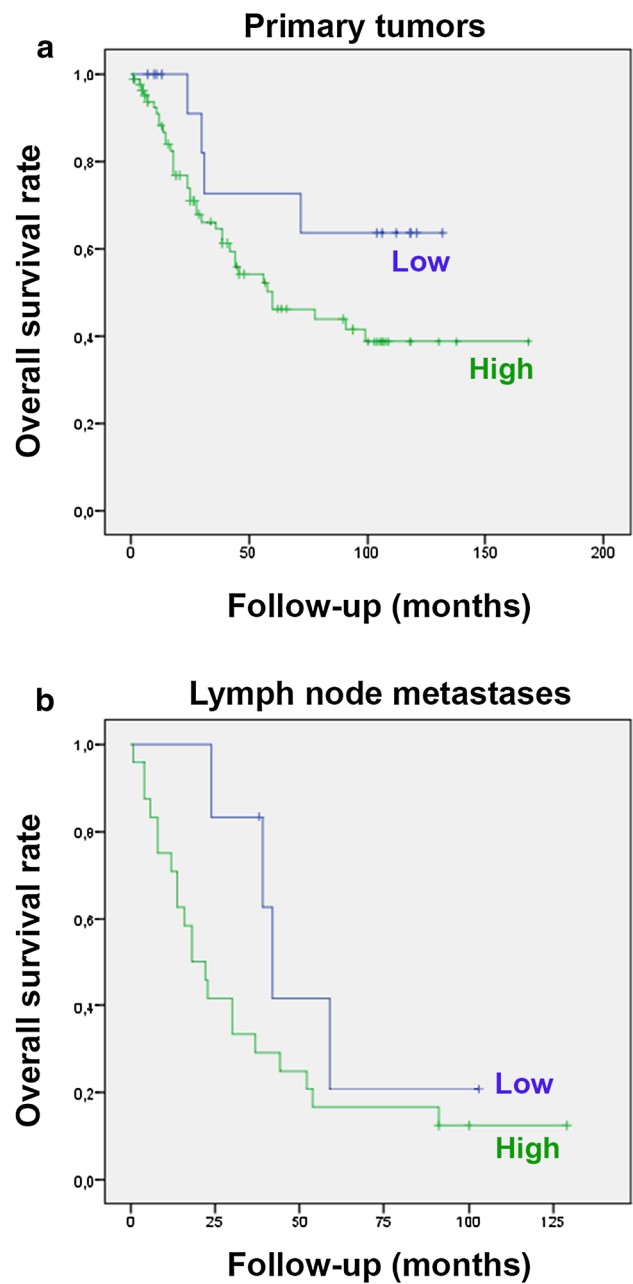
<sup>c</sup>Only primary tumors (*n* = 104)

<sup>d</sup>Clark's classification (Clark et al. 1989)

survival and poor distant metastasis-free survival. These discrepancies might be largely attributable to different methods of patients grouping, immunostaining scoring systems and statistical analyses.

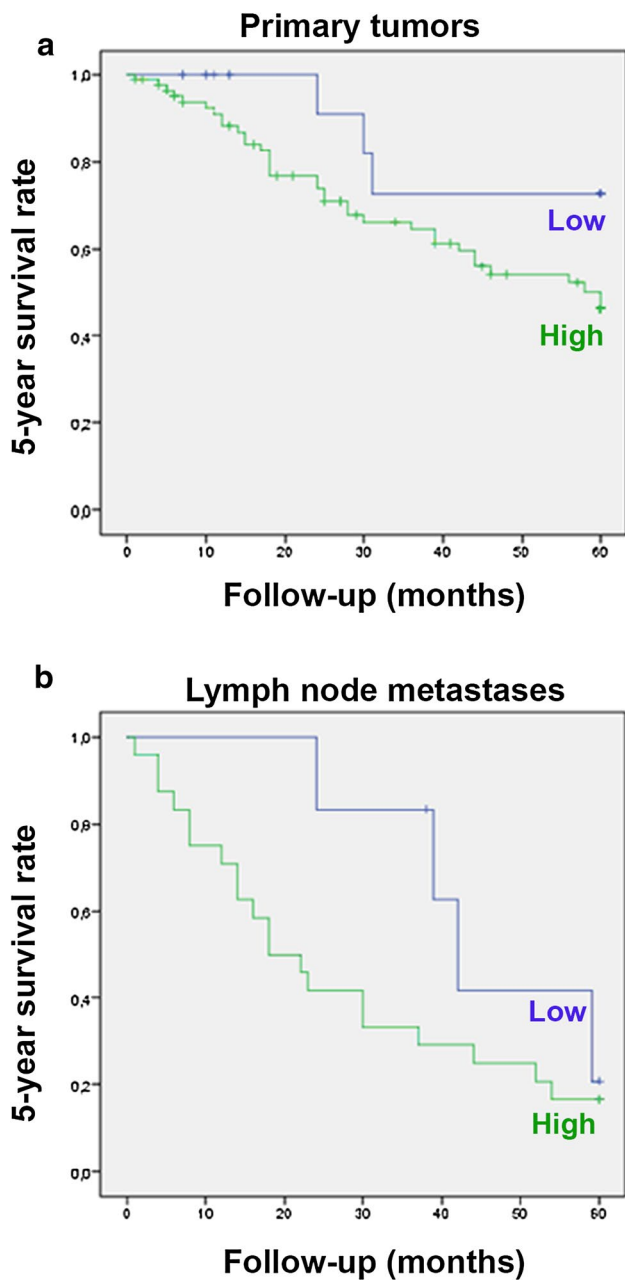
According to our results, while high N-cadherin expression as single marker does not seem to be a prognostic element of survival, as well as high Notch1 expression by itself, when combined they seem to predict an adverse clinical outcome, both considering overall and 5-year survival of melanoma patients. High N-cadherin and Notch1 expression retains their prognostic impact on survival in melanoma patients with both primary lesions or lymph node metastases, thus indicating the utility of the N-cadherin and Notch1 co-presence as a predictive factor both in early- and advanced-stage melanomas.

Our study is part of the growing body of literature regarding the effort to better understand the role of N-cadherin



**Fig. 5** Kaplan–Meier curves comparing the probability of overall survival between patients with a primary tumor (a) or a lymph node metastasis (b) showing a high or a low N-cadherin/Notch1 expression

and Notch1 in EMT and subsequently in the acquisition of a metastatic phenotype by cells of several tumors, including breast, pancreatic, nasopharyngeal carcinoma, and melanoma (Shao et al. 2015; Wang et al. 2010; Nakajima et al. 2004; Luo et al. 2012; Lade-Keller et al. 2013). These studies, taken together with our findings, lead us to draw the conclusion that simply targeting selected molecules involved in EMT may stabilize melanoma by means of preventing the cells from progressing to metastatic spreading at local and/or



**Fig. 6** Kaplan–Meier curves comparing the probability of 5-year survival between patients with a primary tumor (a) or a lymph node metastasis (b) showing a low or a high N-cadherin/Notch1 expression

distant sites, as also suggested by Watson-Hurst and Becker (2006). Finally, this indicates that N-cadherin and Notch1 can be eligible as targets in cancer therapies that may better work by synergic fashion, providing a new promising avenue for the restraint of melanoma metastasis.

Regarding dysplastic melanocytic nevi, N-cadherin immunostaining was only observed in nevus cells present in the dermal compartment of a minority of compound and intradermal nevi, while the epidermis, as well as the

junctional nevus nests, was always negative. These findings better explain the N-cadherin expression pattern previously observed in the context of immunohistochemical analyses of melanocytic nevi (Krengel et al. 2004). The different N-cadherin expression arrangement in the epidermal, junctional and dermal compartments of melanocytic nevi may recapitulate the sequence of changes in the epidermis/dermis architecture, triggered by the E- to N-cadherin switch. In this way, melanocytic cells easily gain the capability to escape the control by neighboring keratinocytes and acquire, in the dermis, the expression of N-cadherin. This latter, in turn, allows invasion of this compartment, leading to nevus cell dissemination and functional contact with dermal fibroblasts. Since acquisition of cell migration properties is potentially linked to a malignant transformation, monitoring the expression of N-cadherin in premalignant lesions, such as dysplastic melanocytic nevi, could suggest which lesions are more likely at risk of progress toward a neoplastic disease giving a hint about those patients to monitor more carefully.

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#### Compliance with ethical standards

**Ethical approval** Due to the retrospective nature of the study and since the analyzed clinical data of the patients were anonymous, formal consent of the patients was not required.

**Conflict of interest** The authors declare that they have no conflict of interest.

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