

## Galectin-3 Expression Is Associated with Tumor Progression and Pattern of Sun Exposure in Melanoma

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**Abstract Purpose:** Most studies accept a multistep pathogenic process in melanoma that may include the phases of benign nevi and dysplastic nevi, melanoma, and metastatic melanoma. Dysregulation of cellular proliferation and apoptosis is probably involved in melanoma progression and response to therapy. We have studied the expression of galectin-3, a  $\beta$ -galactoside-binding protein involved in apoptosis, angiogenesis, and cell proliferation, in a large series of melanocytic lesions, and correlated the expression with clinical and histologic features.

**Experimental Design:** Tissue microarray blocks of 94 melanocytic lesions were semiquantitatively evaluated by immunohistochemistry for the cytoplasmic or nuclear expression of galectin-3.

**Results:** Primary and metastatic melanomas expressed galectin-3 at a significantly higher level than nevi in both cytoplasm and nuclei ( $P < 0.0073$ ). There was a significant association between anatomic source (as indirect indication of level of sun-exposure) and cytoplasmic and nuclear expression. Lymph node and visceral metastases had a higher level of expression than s.c. lesions ( $P < 0.004$ ). Interestingly, there was an almost significant finding of worse survival in those patients with lesions showing higher levels of cytoplasmic than nuclear galectin-3 expression (log-rank test,  $P = 0.06$ ).

**Conclusions:** Melanocytes accumulate galectin-3 with tumor progression, particularly in the nucleus. The strong association of cytoplasmic and nuclear expression in lesions of sun-exposed areas suggests an involvement of UV light in activation of galectin-3.

Melanoma is a relatively common and one of most malignant tumors in humans. The incidence of melanoma has increased significantly since 1930 in both men and women and is probably related to changes in sun exposure and environmental levels of UV light. The social impact is significant because many melanoma cases occur in young individuals and there is little effective treatment available once metastatic disease develops. In contrast, >90% of patients with *in situ* or early invasive melanoma are cured with surgical excision (1). The incidence of reported melanoma cases has strikingly increased

over the past several decades. Although part of this increment may be due to increased awareness or changes in diagnostic criteria, there also seems to be a real increase (2).

In addition to sun exposure, other factors contribute to the development of melanoma, including, among others, the presence of a preexisting nevus and hereditary factors (3). Furthermore, several lines of evidence seem to suggest that pathogenesis of malignant melanoma is a multistep process that may include the phases of benign nevi, dysplastic nevi, radial and vertical growth phase melanoma, and metastatic melanoma (4, 5). Melanocytes are normally present in the dermal-epidermal junction and have the capacity to proliferate and form epidermal and dermal aggregates (i.e., melanocytic nevus). Most of these lesions are usually self-limited because their melanocytes modify their phenotype (so-called "maturation"), undergoing a final terminal differentiation resembling that of Schwann cells (6, 7) and not further progressing.

The mechanisms that mediate this proposed melanoma progression still remain mostly unknown. Previous studies have shown that several classes of molecules, including tumor suppressor genes (*p16* and *p53*), transcription factors (cAMP-responsive element binding protein/activating transcription factor-1, activator protein-2), cell adhesion molecules (E-cadherin), and metalloproteinases (matrix metalloproteinase-2), may be involved in the initiation and progression of malignant melanoma (8–18). In addition, it is likely that other pathways are involved.

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**Table 1.** Cases of melanocytic lesions

Melanocytic lesions	Cases
Benign nevi	17
Dysplastic nevi	18
Melanoma	23
Superficial spreading	10
Nodular	2
Acral lentiginous	4
Lentigo maligna	7
Metastatic melanoma	31
Lymph node metastasis	9
S.c. metastasis	12
Visceral metastasis	10

Galectin-3 is a protein member of the group of lectins that bind to  $\beta$ -galactosides. Of the three major groups described thus far, galectin-3 belongs to the chimera type containing a short NH<sub>2</sub>-terminal domain of 12 amino acids (for cellular targeting), a collagen-like long sequence (substrate to matrix metalloproteinases), and a COOH-terminal domain (for binding to the carbohydrates; ref. 19). Galectin-3 plays an important role in tumor cell adhesion, proliferation, differentiation, angiogenesis, and metastasis (19) in multiple tumors (20, 21). Galectin-3 seems to bind to several molecules, which may explain the observation of a range of functions; particularly relevant is an antiapoptotic or proapoptotic function that appears depending on the studied tumor. Galectin-3 was reported to be an antiapoptotic molecule, inhibiting Fas-induced T-cell apoptosis as well as epithelial cell apoptosis induced by staurosporine, cisplatin, genistein, and anoikis (22–26). The antiapoptotic activity of galectin-3 has also been shown in galectin-3 knockout mice, in which macrophages from these mice were more sensitive to apoptosis than macrophages from normal control mice (27). In addition, enforced expression of galectin-3 in epithelial cells rendered them resistant to apoptotic stimuli (22–26). Indeed, galectin-3 contains the anti-death Asp-Trp-Gly-Arg (NWGR) motif that is conserved in the Bcl-2 homology domain 1 of the Bcl-2 family (22, 28). A previous study on a small series of melanocytic lesions (29) revealed that some nevi and melanomas express galectin-3. The goal of this study was the analysis of galectin-3 expression as a potential biomarker and possible therapeutic target. We have used a human melanocytic lesion microarray to examine the possible involvement of galectin-3 in melanoma progression. We report here that melanocytes acquire higher levels of galectin-3 with tumor progression with higher nuclear expression. Because studies in other neoplasms seem to indicate that galectin-3 contributes to chemotherapy and apoptosis resistance, we propose that anti-galectin-3 targeted therapy may represent a novel approach to treat patients with malignant melanoma.

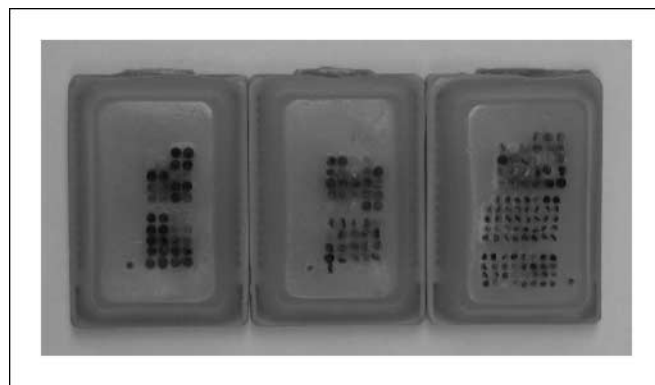
**Materials and Methods**

**Materials and case selection.** A total of 89 cases of melanocytic lesions including benign nevi (17 cases), dysplastic nevi (18 cases), melanoma *in situ* (superficial spreading, nodular, acral, and lentigo

malignant melanoma; melanoma, 23 cases), and metastatic malignant melanoma to s.c. tissue, lymph node, and visceral organs (metastatic melanoma, 31 cases) were obtained from the Department of Pathology of The University of Texas M.D. Anderson Cancer Center (Table 1). The diagnoses had been rendered by dermatopathologists. In particular, the grading of dysplastic nevi was done using previously reported criteria (30). Melanomas were classified according to the histologic subtype as well as to the anatomic location, thus likely reflecting the pattern of sun exposure. Lesions on the head, neck, and arms (excluding hands) were considered to be high level of sun exposure; lesions on the trunk and proximal extremities were considered to be intermittently sun exposed; and lesions on hands and feet, possible intermittent sun exposure and repetitive trauma. The specimens included small biopsies or excisions. The study was approved by the Institutional Review Board.

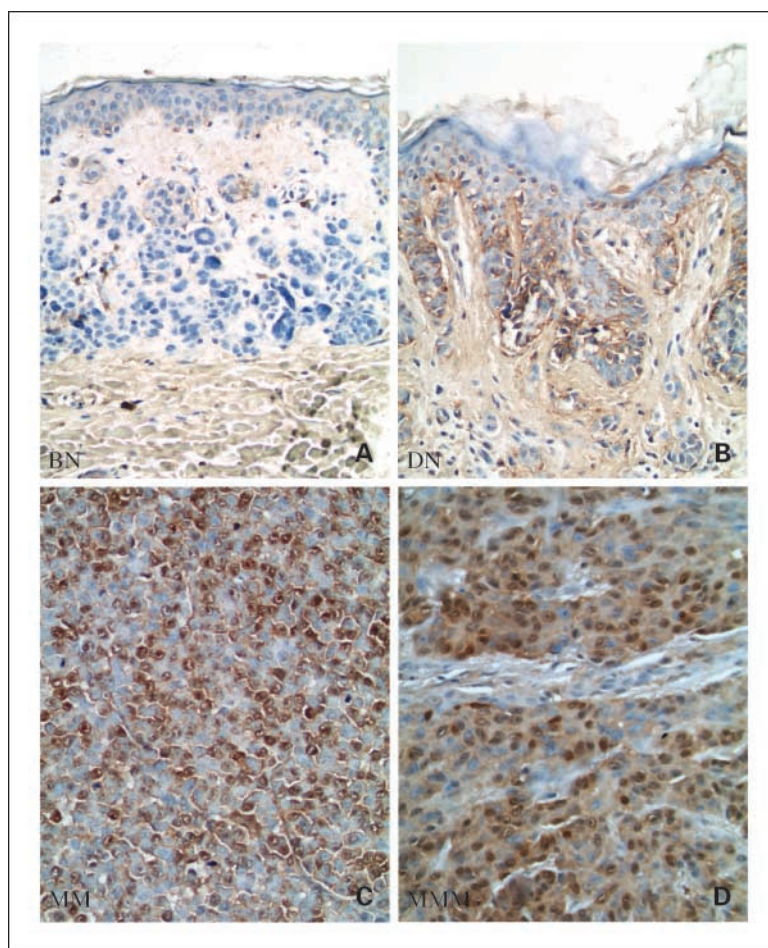
**Tissue microarray construction.** For tissue microarray construction, H&E-stained sections were reviewed from each block to define the selective areas. Either 0.6-mm (punch biopsies of benign and dysplastic nevi cases) or 1.0-mm (excision specimens) cylindrical cores of tissue were punched out from donor blocks to preserve the original tissue block. The selected tissue cores were inserted in a standard 4.5 × 2 × 1 cm recipient block using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD) with an edge-to-edge distance of 0.1 or 0.15 mm. At least two tissue cores were taken for each case for a total of 187 cores in three tissue microarrays (Fig. 1). Two same control cases (one benign nevi and one melanoma) were included in all three blocks. Serial 5- $\mu$ m-thick sections of all three blocks were cut and one standard H&E-stained slide was examined to verify the presence of diagnostic lesional cells (17).

**Antibodies and immunohistochemical analysis.** Routine immunohistochemical labeling protocols were used to detect galectin-3. Anti-galectin-3 monoclonal antibody (TIB 166) was obtained using a hybridoma from the American Type Culture Collection (Manassas, VA). Antigen labeling was optimized using tissue sections before applying to tissue microarray sections. Heat-induced epitope retrieval was done by steaming in boiling antigen retrieval solution (Dako, Carpinteria, CA) consisting of 10 mmol/L citrate buffer (pH 6.0) for 10 to 20 minutes followed by cooling at room temperature for 20 minutes. Next, tissues were washed thrice for 3 minutes with PBS and incubated with protein block (5% normal horse serum in PBS) for 15 minutes at room temperature. Diaminobenzidine was used as the chromogen with light hematoxylin as the counterstain. The percentage of positive cells and the intensity of staining were separately recorded for both nuclear and cytoplasmic regions in a semiquantitative scale following a previously published method (17): 0, 0% to 5% of cells; 1, 6% to 25%; 2, 26% to 75%; 3, >75%. The intensity of expression was categorized into negative, weak, moderate, or strong. For a lesion to be considered positive, it had to have >25% of the cells expressing



**Fig. 1.** Tissue microarray blocks (total of three) of melanocytic lesions.

**Fig. 2.** Expression of galectin-3 in benign nevi (BN; A), dysplastic nevi (DN; B), melanoma (MM; C), and metastatic melanoma (MMM; D). Note the minimal expression in benign nevi and focal cytoplasmic expression in the intraepidermal component of a dysplastic nevus. Cytoplasmic and nuclear expression in primary and metastatic melanoma (anti-galectin-3; diaminobenzidine and light hematoxylin).



galectin-3 (levels 2 or 3 percentage) with mild, moderate, and marked intensity of labeling. Because nuclear expression was detected in the cells of a number of melanoma cases, we also calculated a variable of nuclear to cytoplasmic ratio (numeric value of cytoplasmic expression divided by numeric value of nuclear expression, both for intensity and percentage of positive cells).

**Statistical analysis.** The statistical analysis was supervised by two of the authors (M.M.J. and A.L.). McNemar and Stuart-Maxwell tests were used to assess the intragroup association. For the McNemar or Stuart-Maxwell test, the frequency table is required to be square (same number of rows and columns). Thus, in some of the evaluations done, a frequency of 0.00001 was added in the corresponding cell of the row (level) or column that was not observed (i.e., zero count). Furthermore, because some of the levels

had few samples within that level, consecutive levels (i.e., 2 and 3) of the variable were collapsed.

Fisher's exact test was used to test the intergroup association between all the groups and pairs of groups. Due to the various two-group comparisons done, we used the Bonferroni correction to adjust the significance level for an individual test to maintain an overall significance level of 5%. For example, because seven two-group comparisons were done, an individual significance level of 0.0071 ( $0.05/7 = 0.0071$ ) was used. Statistical analyses were carried out using SAS 8.02 and JMP 5.1 (SAS Institute, Cary, NC). Kaplan-Meier method was used to estimate survival distributions and the log-rank test was used to compare the survival distributions between the groups of tumor characteristics. Cox proportional hazard models were used to determine the relationship of tumor characteristics with survival outcomes.

**Table 2.** Comparison of cytoplasmic intensity among the four subgroups

Cytoplasmic intensity levels	Benign nevi	Dysplastic nevi	Melanoma	Metastatic melanoma	Total	P
0	1 (5.9)	0 (0.0)	3 (11.5)	3 (10.3)	7	0.0073
1	11 (64.7)	22 (100.0)	17 (65.4)	15 (51.7)	65	
2	5 (29.4)	0 (0.0)	6 (23.1)	10 (34.5)	21	
3	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.5)	1	

NOTE: P value from the Fisher's exact test. All values expressed as n (%).

**Table 3.** Comparison of cytoplasmic percentage among the four subgroups

Cytoplasmic percentage levels	Benign nevi n (%)	Dysplastic nevi n (%)	Melanoma n (%)	Metastatic melanoma n (%)	Total	P
0	2 (11.8)	0 (0.0)	4 (15.4)	3 (10.3)	9	<0.0001
1	6 (35.3)	20 (90.9)	8 (30.8)	4 (13.8)	38	
2	7 (41.2)	2 (9.1)	12 (46.2)	17 (58.6)	38	
3	2 (11.8)	0 (0.0)	2 (7.7)	5 (17.2)	9	

NOTE: P value from the Fisher's exact test.

### Results

**Expression of galectin-3.** The pattern of expression was predominantly cytoplasmic (Fig. 2). Among the groups, there were marked differences in the pattern of cytoplasmic and nuclear expression of galectin-3, with higher levels of expression from benign nevi to dysplastic nevi to melanoma to metastatic melanoma. With the exception of nuclear intensity (that showed the trend but only to a level of  $P = 0.09$ ), the differences were highly significant among the subgroups, ranging from  $P < 0.0001$  (percentages of melanocytic cells with cytoplasmic galectin-3) to  $P = 0.0073$  (intensity of cytoplasmic expression). In general, the highest levels were seen in melanoma and metastatic melanoma (Tables 2–5). Particularly marked were the differences in nuclear expression: although there was some overlap among the groups, no nevi (whether benign nevi or dysplastic nevi) expressed galectin in the nucleus above the selected cutoff of >25% of the cells.

Regarding the histologic type and anatomic location of melanoma, there was significant correlation of galectin-3 expression and histologic subtype/pattern of sun exposure. More than 50% (5 of 9) of sun-exposed lesions (from head and neck and arms) showed cytoplasmic expression; three of these cases had also nuclear expression. Sixty-six percent (6 of 9) of lesions with intermittent sun exposure had cytoplasmic expression of galectin-3; however, none of these lesions showed nuclear expression ( $P = 0.048$  and  $P = 0.038$ , nuclear and cytoplasmic expression, respectively; Figs. 3 and 4).

With respect to a possible relationship with Breslow thickness, there was a trend toward increased cytoplasmic expression of galectin-3 in thicker melanomas but this finding did not reach statistical significance in our series ( $P = 0.15$ ). There was no significant correlation of galectin-3 expression

and presence or absence of ulceration in the primary melanomas (data not shown).

The differences in the pattern of expression of galectin-3 in the different types of metastases were statistically significant for both nuclear and cytoplasmic expression ( $P = 0.038$  and  $P = 0.001$ , respectively). S.c. lesions consistently expressed galectin-3 in their cytoplasm while only rarely showing nuclear expression. Lymph node metastases showed a higher nuclear expression. Metastases to the viscera showed higher nuclear than cytoplasmic expression (Fig. 5).

The analysis of the cytoplasmic/nuclear ratio showed a strong, statistically significant difference among the three types of metastases (data not shown;  $P = 0.01$  and  $P = 0.008$  for intensity and percentage ratios, respectively). More interestingly, there was an almost significant (log-rank test,  $P = 0.06$ ) association between disease-free survival and nuclear to cytoplasmic ratio of galectin-3 expression: patients with either primary or metastatic lesions in which there was a nuclear percentage of expression equal or higher than the cytoplasmic expression had a shorter disease-free survival (Fig. 6).

### Discussion

In this study, we used a tissue microarray platform to analyze simultaneously the expression of galectin-3 in a spectrum of melanocytic lesions, benign nevi, dysplastic nevi, melanoma *in situ*, and metastatic melanoma. Although the overlap among the groups seems to prevent an analysis of only galectin-3 expression to differentiate between benign and malignant lesions, our data show that there is a statistically significant acquisition of nuclear and cytoplasmic expression of galectin-3 with melanoma progression. Therefore, it is possible that galectin-3 plays a role in the acquisition of

**Table 4.** Comparison of nuclear intensity among the four subgroups

Nuclear intensity levels	Benign nevi n (%)	Dysplastic nevi n (%)	Melanoma n (%)	Metastatic melanoma n (%)	Total	P
0	10 (58.8)	14 (63.6)	12 (46.2)	14 (48.3)	50	0.0930
1	6 (35.3)	8 (36.4)	13 (50.0)	8 (27.6)	35	
2	1 (5.9)	0 (0.0)	1 (3.8)	7 (24.1)	9	
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	

NOTE: P value from the Fisher's exact test.

**Table 5.** Comparison of nuclear percentage among the four subgroups

Nuclear percentage levels	Benign nevi n (%)	Dysplastic nevi n (%)	Melanoma n (%)	Metastatic melanoma n (%)	Total	P
0	11 (64.7)	19 (86.4)	13 (50.0)	14 (48.3)	57	0.0052
1	6 (35.3)	3 (13.6)	11 (42.3)	7 (24.1)	27	
2	0 (0.0)	0 (0.0)	2 (7.7)	8 (27.6)	10	
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0	

NOTE: P value from the Fisher's exact test.

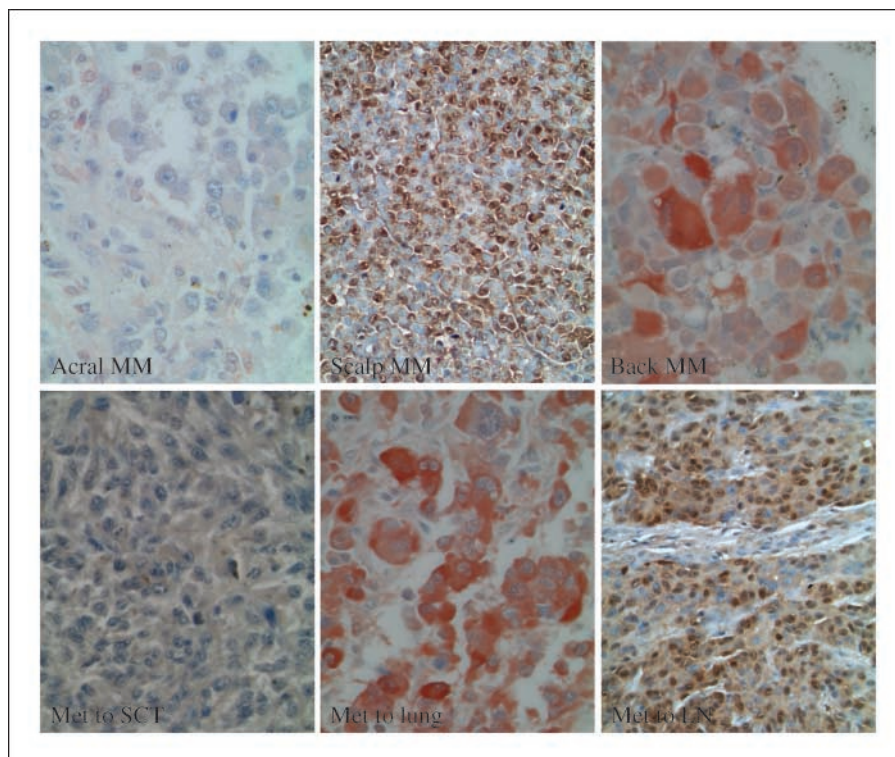
malignant phenotype of melanoma cells. Furthermore, our data suggest a relationship between galectin-3 expression and prognosis, in particular translocation of galectin-3 to the nucleus: lesions metastatic to viscera and lymph nodes had higher nuclear to cytoplasmic ratio expression of galectin-3 than s.c. lesions, with a trend toward worse disease overall survival and disease-free survival. Along these lines, a very recent study indicates that patients with metastatic melanoma have high serum levels of galectin-3, probably produced by the tumor cells (31).

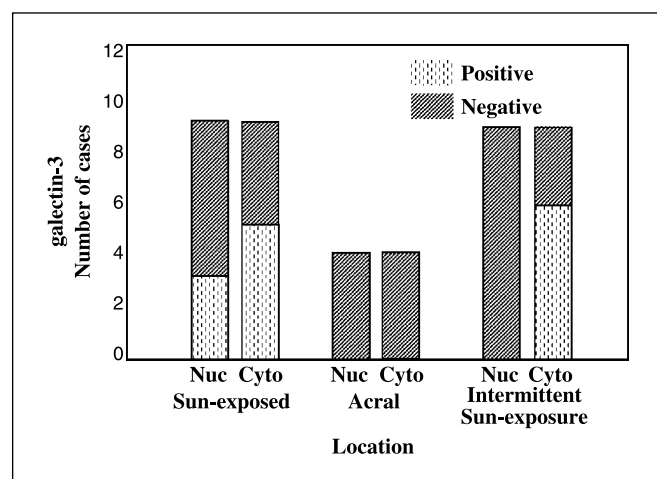
Galectin-3 is a member of the galectin gene family that is expressed at elevated levels in a variety of neoplastic cell types and has been associated with cell growth, cellular adhesion processes, cell proliferation, transformation, metastasis, and apoptosis. Furthermore, galectin-3 is up-regulated in various types of cancer and may be involved in carcinogenesis (32, 33). One possible explanation for these activities is the antiapoptotic activity of galectin-3. Several reports have proposed mechanisms by which galectin-3 protects cells from

apoptosis. When galectin-3 is transfected into lymphoma T cells, such cells become more resistant to apoptosis induced by anti-Fas antibodies; similarly, galectin-3-expressing breast carcinoma cells are resistant to multiple proapoptotic stimuli (e.g., chemotherapy, nitric oxide, radiotherapy; refs. 23, 34, 35). Particularly interesting is our observation of expression of galectin-3 in the nuclei of some melanoma cells and not in nevus cells.

A history of severe sunburns together with intermittent UV exposure is implicated in the etiology of human melanoma (36). However, the mechanism by which UV radiation participates in melanoma formation is widely debated due to the lack of direct evidence for the UV-induced genetic alterations in a large proportion of primary human melanomas. Early studies revealed mechanistic and topological associations between UV and activating *N-RAS* mutations, which often occur at or near dipyrimidine sequences (sites of UVB-type mutations) and are detected on tumors localized on sun-exposed body sites (37). A hallmark of UVB-induced

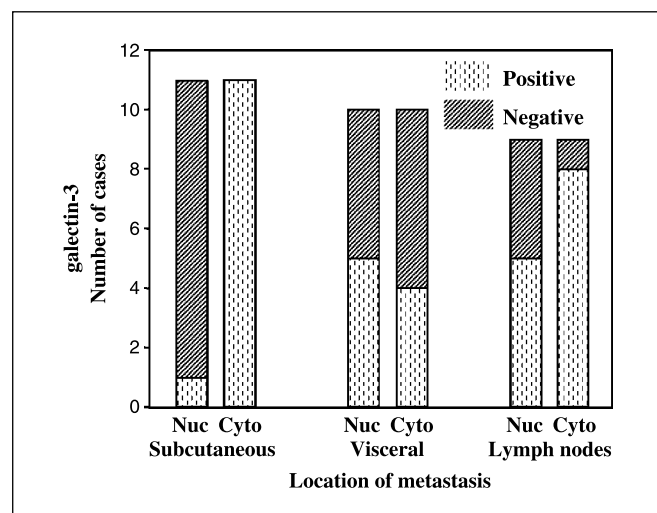
**Fig. 3.** Pattern of expression of galectin-3 in primary (*top row*) and metastatic (*bottom row*) melanoma. Acral melanoma lacking galectin-3 expression. Strong cytoplasmic and nuclear expression in a scalp lesion. Cytoplasmic expression in a lesion from the back. S.c. metastasis with cytoplasmic but no nuclear expression. Predominantly cytoplasmic expression in a metastasis to the lung. Strong nuclear and cytoplasmic expression in a lymph node metastasis [anti-galectin-3; diaminobenzidine (*brown*) or aminoethylcarbazol (*red*) with light hematoxylin].



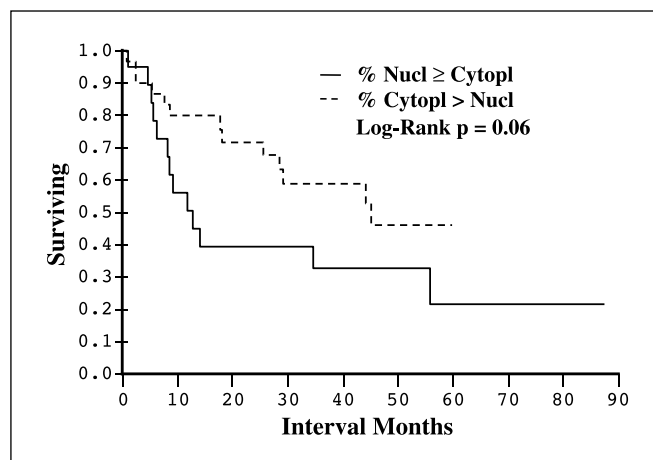


**Fig. 4.** Pattern of galectin-3 expression in primary melanomas subclassified according to the likely pattern of sun exposure: high sun exposure (head and neck, arms), intermittent (trunk and legs), and acral (hands and feet). Note that acral lesions lack galectin-3; intermittently sun-exposed lesions show cytoplasmic but not nuclear expression; the only lesions with nuclear expression are those on sun-exposed areas ( $P = 0.048$  and  $P = 0.038$  for nuclear and cytoplasmic expression, respectively).

damage, UV-specific CC→TT mutation, was identified in the *CDKN2 (p16INK4a)* gene, which was implicated in the development of melanoma (38). Recently, a more comprehensive analysis of primary melanomas revealed that melanoma subgroups develop by distinct mechanistic routes depending on the patterns of sun-exposure (39). Notably, melanomas on skin with intermittent sun-induced damage had frequent mutations in *BRAF* or *N-RAS* oncogenes and frequent loss of phosphatase and tensin homologue deleted on chromosome 10, *PTEN*, whereas melanomas on skin with



**Fig. 5.** Expression of galectin-3 in the different types of metastatic lesions. S.c. lesions consistently express galectin-3 in their cytoplasm while only rarely showing nuclear expression. Lymph node metastases show a higher nuclear expression. Metastases to the viscera show higher nuclear than cytoplasmic expression. These differences are statistically significant ( $P = 0.038$  and  $P = 0.001$ , nuclear and cytoplasmic, respectively).



**Fig. 6.** Analysis of disease-free survival stratified by nuclear to cytoplasmic ratio of galectin-3 expression. Lesions with percentages of nuclear expression equal or higher than cytoplasmic expression showed a trend to worse prognosis than those with nuclear percentages lower than the cytoplasmic percentages. These differences were almost significant (log-rank test,  $P = 0.06$ ).

chronic sun exposure had infrequent mutations in *BRAF* and frequent increases in the number of copies of the *cyclin D1* gene. Distinctively, acral melanomas, which are likely not associated with sun exposure, were found to have a uniquely high degree of focal chromosomal amplifications and losses (39). In our study, we found that melanomas on chronically and intermittently sun-exposed areas were characterized by an intense cytoplasmic expression of galectin-3, as compared with acral melanomas, which did not express the marker. Furthermore, nuclear expression of galectin-3 was restricted to chronically sun-exposed melanomas, suggesting that this nuclear translocation is associated with chronic UV-induced damage. The latter conclusion remains to be further validated in a larger group of samples. Furthermore, our data show a trend for shorter disease-free survival for those patients with lesions in which there is a percentage of nuclear expression equal or higher than the cytoplasmic expression, therefore supporting the hypothesis that the translocation of galectin-3 to the nucleus of melanoma cells results in a more aggressive behavior. Recently, it has been reported that galectin-3 translocates to the nucleus after treatment with chemotherapeutic agents (35) and that in a perinuclear location, it inhibits the release of mitochondrial cytochrome *c* (40). Therefore, overexpression of galectin-3 in melanoma cells may act as a survival factor rendering melanoma cells resistant to chemotherapy and apoptosis.

In summary, this study reveals a shift of galectin-3 immunophenotype from benign nevi to metastatic melanoma, further supporting the theory of a progression model between nevus cells and metastatic melanoma cells. This study also shows a different pattern of galectin-3 expression in different histologic types of primary melanomas, possibly related to the pattern of sun exposure, and in different types of metastatic lesions, possibly associated with survival. Finally, because galectin-3 likely provides cancer cells with antiapoptotic functions, use of small molecules targeting galectin-3 may prove helpful for treatment in melanoma patients.

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