EJGO European Journal of Gynaecological Oncology

Expression of estrogen receptors in melanoma and sentinel lymph nodes; a "female" clinical entity or a possible treatment modality?

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Summary

Purpose: The natural history of human malignant melanoma suggests that steroid hormones may affect the biological behavior of this tumor. The purpose of the current study was to investigate the specific immunostaining patterns of estrogen receptors in malignant melanomas and their sentinel lymph nodes (SLNs), as well as to examine any possible association with patients' prognosis and overall survival. *Materials and Methods:* A retrospective analysis of prospectively collected data was conducted during a 12-year period (2001-2012). Sixty patients with mean age of 54.4 ± 14.5 years diagnosed with melanomas of varying depth (Clark) and thickness (Breslow) after excision biopsy of pre-existing melanocytic lesions, were included in the study. All patients underwent wide excision of the primary tumor and SLN identification. Determination of estrogen receptor alpha (ERa) and beta (ERb) status by immunohistochemistry on tumor and nodal paraffin blocks was performed in all feasible cases. *Results:* ERb but not ERa was the predominant estrogen receptor found in all primary tumors and SLNs examined. The most intense ERb immunostaining was seen in negative SLNs associated with thinner, less invading melanomas. ERb expression in the primary tumor seems to correlate with the cellular microenvironment, possibly altering the process of SLN invasion. *Conclusions:* ERb expression is down-regulated in aggressive melanomas with sentinel nodal metastatic disease, suggesting its possible usefulness as a surrogate marker for metastatic potential and prognosis in malignant melanoma.

Key words: Malignant melanoma; Sentinel lymph node; Estrogen receptors; Immunostaining.

Introduction

The role of estrogens in the cause and progression of many cancers is well documented [1]. The effects of estrogens are mediated by two estrogen receptors, estrogen receptor a (ERa) and estrogen receptor b (ERb), representing members of the nuclear steroid receptor superfamily. Estrogen receptors classically mediate their action by ligand-dependent binding to the estrogen responsive element, leading to transcriptional regulation of target genes [1]. Both of these proteins have a high degree of homology in the DNA-binding domain but differ considerably in the N-terminal domain and to a lesser extent in the ligand-binding domain (E domain) [2]. These differences suggest that most likely these proteins represent two different subtypes rather than splice variants [3]. Various isoforms of each subtype of the two receptors have been discovered, suggesting that they could have distinct functions in terms of gene regulations and biologic responses or that they could contribute to the selective actions of 17-β-estradiol and of other estrogenic molecules on target cells [1, 3, 4].

The skin is an important estrogen-responsive tissue and the fundamental role of estrogen in the regulation of hair follicle cycling and self-renewing is well known [5]. However, the relative contribution of estrogens in skin tumorigenesis remains unclear. This is the reason why there is an extensive debate whether skin cancers, especially their most aggressive form, melanoma, express the two ERs. Faint indications are derived through epidemiological data which clearly state a survival benefit for female patients with metastatic melanoma versus male individuals [4, 6]. Studies based on the immunoreactivity failed to demonstrate a role for ERa in the pathophysiology of either melanoma precursor lesions or melanomas [7-9]. After ERb was identified in 1995, no controlled, follow-up studies have examined whether ERb plays a role in the evolution or prognosis of malignant melanomas. Furthermore, no study so far has examined the association between progression of the disease and ER expression in the primary tumor and the sentinel lymph node (SLN) in malignant melanomas; SLN represents the gold-standard procedure in order to estimate the stage of the disease and to determine if further therapeutic actions are required.

For all of these reasons, the present authors embarked on a project of evaluating ER expression in tissues of the primary tumor and the SLN of humans diagnosed with malignant melanoma. The primary target was to identify

Revised manuscript accepted for publication October 21, 2013

Table 1. — *Patients' and tumor characteristics.*

Patients	60 (41 males, 19 females)
Age (mean ± SD)	54.4 ± 14.5 years
Tumor location	
Head/Neck	5
Trunk	33
Extremities	22
Histology type	
Superficial spreading (SS)	53
Nodular	5
Borderline	2
Clark level	
I	5
II	9
III	14
IV	30
V	2
Breslow thickness grouping	
Group 1 < 1 mm	15
Group 2 1-4 mm	33
Group 3 > 4 mm	12
Number of SLNs removed	1.7 ± 0.88 (1-4)
Presence of nodal disease	18 patients (36%)
Absence of nodal disease	42 patients (64%)

whether ERa and mainly ERb expression has a role in tumor progression and metastatic potential through the lymph route in malignant melanomas.

Materials and Methods

The protocol of the study was approved by the ethics committee of the present institution. The expression of ERa and ERb in primary tumors and their representative SLNs was investigated in 60 patients (41 men, 19 women) diagnosed with malignant melanoma on varying locations during a 12-year period (2001-2012). The diagnosis was based on excision biopsy of suspicious pre-existing melanocytic nevi. All patients included in the study had never had any kind of hormonal replacement therapy and none of them was obese.

Patients presenting with clinical or laboratory evidence of lymph node invasion or distal metastatic disease were excluded from the study. Since diagnosis was confirmed, all patients underwent SLN biopsy under general anesthesia and simultaneous broad excision of the skin and the subcutaneous tissue on wider, clear margins. If the histopathological report was indicative of SLN invasion, therapeutic lymph node dissection at the specific nodal site was performed in a new scheduled operation (within a time period of 15-20 days). All patients were operated on by a single surgeon (E.T). Patients' and tumors' characteristics are listed in Table 1.

Surgical technique

All patients underwent preoperative diagnostic lymphoscintigraphy at the Department of Nuclear Medicine of the present hospital, seven days prior to the scheduled operation, with the injection of 0.5–1 mCi (18.5–37 MBq) of filtered technetium 99m sulfur colloid (TC) in a volume of 0.5– 1.0 ml injected at four to six intradermal points surrounding the site of the primary melanoma. The anatomic region of the sentinel node(s) was identified and recorded. One hour prior to the operation, a new dose of 0.5 mCi (17.5 MBq) of TC (in a volume of 0.5 ml) was injected intradermally in the skin surrounding the primary melanoma and the location(s) of the lymph node(s) was marked on the nodal sites with a marker pen. At the beginning of the operation, an intradermal injection of one to two ml of methylene blue was additionally given in the surrounding skin of the tumor. Within 15–30 minutes, the radioactive sentinel nodes were identified with a handheld gamma counter. An incision was made through the skin and into the subcutaneous tissue overlying the marked radioactive site. If a blue lymphatic vessel was identified in the subcutaneous tissue, this was followed to its respective draining SLN. In each case, any radioactive SLN was identified by the gamma probe alone and removed until less of 10% of the maximum radioactivity level was recorded at the surgical field. Wider excision of the skin and the underlying subcutaneous tissue was then performed in order to achieve 1.5 cm clear margins around the primary lesion and the operation was terminated. The excised lymph nodes were sent for pathological examination. No cases of any type of allergic reactions to the dye product amongst any of the patients in this study were recorded.

Immunohistochemistry

Tumor and nodal tissues were fixed in 4% buffered formalin, processed, and paraffin embedded according to conventional techniques. Representative sections of four μm were cut and stained with hematoxylin–eosin. Immunostains were conducted using the polymer-based immunohistochemical method.

Four-micrometer-thick paraffin sections were mounted on super-frost/plus microscope slides. The sections were deparaffinized in xylene and rehydrated in ethanol/H_2O. Anti-ERa and anti-ERb required antigen retrieval by microwave pre-treatment for 15 minutes in 0.01 mol/l citric acid, pH 6.0 and in one mM EDTA, pH 8.0, respectively. Endogenous peroxidase activity was blocked by immersion in methanol containing 0.2% hydrogen peroxide for 15 minutes. Tissue sections were incubated with mouse monoclonal anti-ER-alpha (clone 6F11; dilution 1:40) and mouse monoclonal anti-ER-beta (clone EMRO2; dilution 1:100), for one and two hours, respectively, at room temperature. The sections were treated with a specific detection system and the immunoreactions were visualised by the application of 3, 3′-diaminobenzidine. All slides were counterstained with hematoxylin, dehydrated in ascending ethanol concentrations, immersed in xylene, and mounted. Negative controls were obtained by omitting the primary antibody. Breast carcinoma sections were used as positive controls for both antibodies. Cells with brown-coloured nuclear and/or cytoplasmic staining were considered as positive. A semi-quantitative grading scale from 0 to 4 was used to estimate the immunoreactivity of both melanocytic nuclei and cytoplasm, as follows: Score 0: < 5% positive staining; Score 1: 5-25% positive staining; Score 2: 26-50% positive staining; Score 3: 51-75% positive staining; Score 4: ≥ 76% positive staining.

Statistical analysis

Data analysis was performed by means of SPSS software (release 13.0). Clinical and laboratory data were correlated by using the Mann–Whitney test whereas Spearman's r correlation coefficient was used to detect any potential correlation between ER expressions. Survival curves were estimated using the Kaplan–Meier method and statistical significance was determined by the log rank test. The Cox proportional hazards regression model was used to estimate disease-free survival (DFS) and overall survival (OS) according to ER expression levels. When comparison between groups of lesions was needed, a paired Wilcoxon test was used. Any *p*-value less than 0.05 was considered statistically significant.

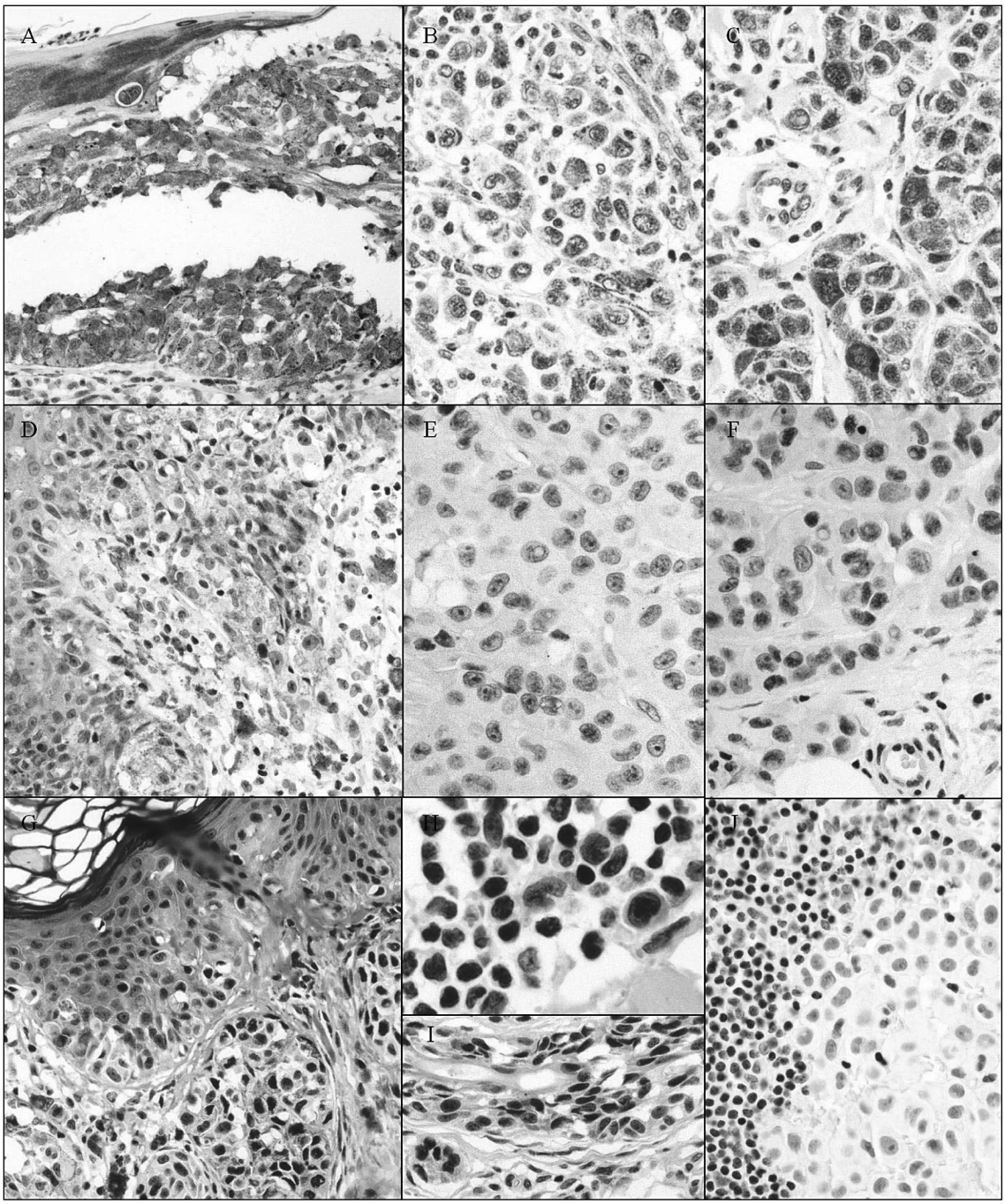

Figure 1. — Immunohistochemical expression of ERb in three different cases of superficial spreading melanoma (SSM) with radial and vertical growth. A-C: ERb in a SSM (three mm deep) from the knee of a 49-year-old female. The great majority of melanoma cells show nuclear and/or cytoplasmic immunoreactivity throughout the lesion. Micrographs A and C highlight the stronger immunoexpression of intraepidermal melanoma cells (KAMs) and of melanoma cells in association with stromal invasion (SAMs), respectively, than the immunoreaction of dermal melanoma cells (MAMs) (B). D-F: ERb in an 8.5 mm-deep melanoma from the elbow of an 48-year-old male. Micrograph D demonstrates cytoplasmic immunostaining in the majority of keratinocyte-associated melanoma cells (KAMs). Micrographs E and F show nuclear immunostaining in tumor cells. Melanoma cells along tumor perimeter (SAMs) (F) show a higher ERb immunopositivity compared to melanoma-associated melanoma cells (MAMs) in the centre of the lesion (E). G-J: ERb in an 1.7 mm-deep melanoma from the trunk of a 62-year-old female and ERb immunostaining of positive SLN. Micrographs G and I show increased nuclear ERb immunoreactivity in melanocytic nests located just below the epidermis (G), as well as in melanoma cells that are surrounded by stroma (I). In contrast, MAMs demonstrate low immunopositivity for ERb (H). Metastatic melanoma in SLN. The minority of tumor cells show a weak nuclear ERb immunostaining. Note the positive immunostaining of several lymphocytes (J). Original magnification A-G, I, J: x200, H: x400.

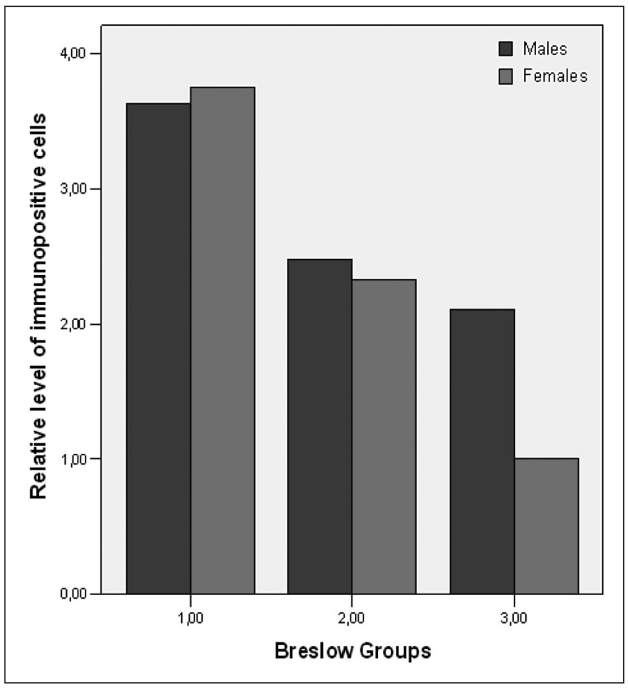

Figure 2. — ERb immunoreactivity of primary melanomas in male and female patients, divided into three Breslow groups (Group 1: depth < 1 mm, Group 2: depth 1-4 mm, Group 3: depth > 4 mm).

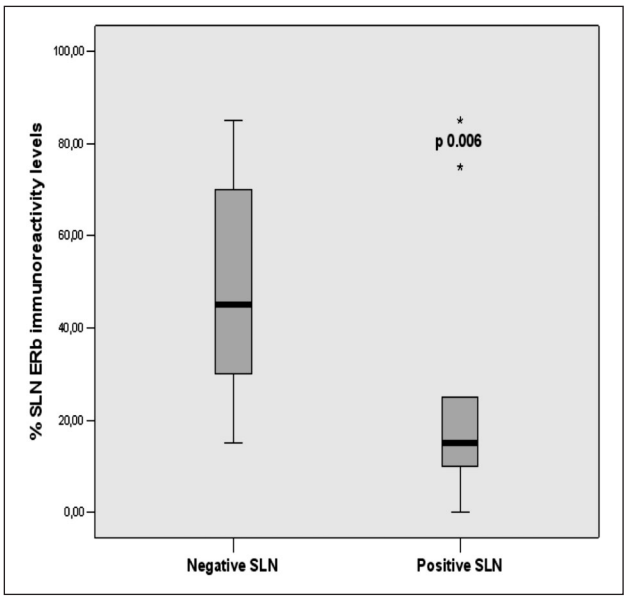

Figure 3. — ERb immunoreactivity levels in positive and negative SLNs.

Results

Although the original goal of the study was to determine the distribution and levels both of ERa and ERb receptors in malignant melanomas and their SLNs, immunodetection of ERa was only noted in seven out of 60 patients (11.9%). Therefore, ERb distribution formed the basic focus of our research.

Sixty cases of primary melanomas were studied. Sentinel node detection was successful in all patients (100%) while the mean number of nodes removed was 1.7 ± 0.88 (range 1-4). Pathological examination revealed nodal metastatic disease in 18 patients (36%) and completion lymph node dissection was performed in these cases with null morbidity and mortality.

The melanomas were divided into three groups, according to the Breslow thickness: group one included thin melanomas (≤ one mm), group two included melanomas of depth one – four mm and group three any deeper lesions (> four mm) (Table 1). A constant observation was that ERb immunoreactivity was significantly decreasing when Breslow depth was increasing ($p = 0.024$ and $p = 0.001$ when group one was compared to group two and three, respectively) (Figure 1). Deep melanomas represented the least ERb immunoreactive lesions in the present series (Figure 2). ERb expression was significantly more intense in males compared to females, only in deep melanomas ($p = 0.04$).

When ERb immunoreactivity was examined in SLN tissues, a constant decrease of ERb levels was recorded, analogously to increasing Breslow thickness of the primary tumor ($p = 0.017$). A concomitant decrease in the levels of ERb expression in the primary tumors and their SLNs was noted when Breslow thickness increased, however a statistically significant difference in ERb immunoreactivity between primary tumors and SLNs was noted only in Breslow group two ($p = 0.001$). Nevertheless, a strong association of ERb immunoreactivity levels and negative SLN status was steadily documented in the present study ($p = 0.006$, Figures 1J, 3).

During evaluation of the immunostaining patterns of ERb in the present series, there was a constant finding that the cellular levels of ERb immunoreactivity depended most upon their microenvironment within the lesion. Based on the methodology by Schmidt *et al.* [10], the present authors found that the data on ERb expression varied according to specific cellular spatial associations within the primary tumor; three spatial groups presented reproducible patterns of ER immunostaining: keratinocyte – associated melanoma cells (KAMs), stroma – associated melanoma cells (SAMs) and melanoma – associated melanoma cells (MAMs). KAMs are melanoma cells very close to the epidermal surface and adjacent to keratinocytes. SAMs represent melanoma cells in proximity to the stroma, mostly at the periphery of downwardly progressing nodules. Finally, MAMs are melanoma cells which are not in association with keratinocytes or stroma and they are only surrounded by other melanoma cells.

Estrogen receptor b immunoreactivity of KAMs was reversely related to the Breslow thickness of the primary lesion, varying from 60-80% (score 3-4) in thin melanomas (group one) to less than 40% (score 1-2) in thicker melanomas (group three) ($p < 0.004$) (Figure 4). There was no statistically difference between male and female patients. Interestingly, cytoplasmic ERb immunoreactivity was more intense than nuclear immunoreactivity in all KAMs studied, however no

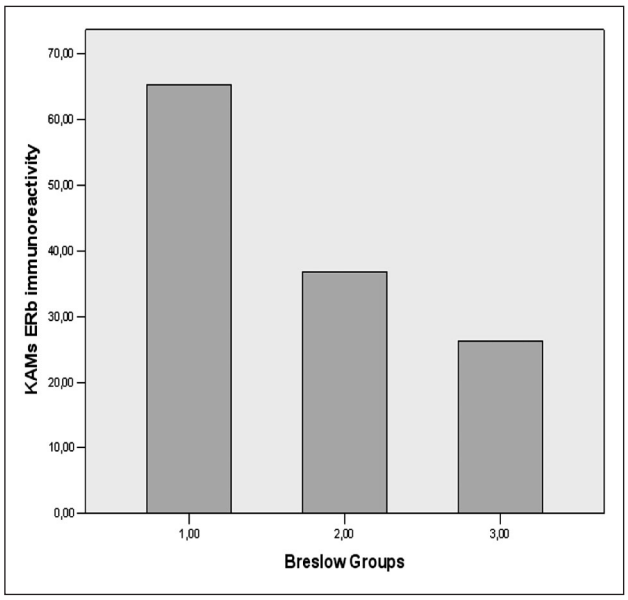

Figure 4. — ERb immunoreactivity levels in keratinocyte – associated melanoma cells (KAMs) among the three Breslow groups (Group 1: depth < 1mm, Group 2: depth 1-4 mm, Group 3: depth > 4mm).

statistically significant difference was recorded ($p = 0.101$) (Figures 1A, D, G).

Within the SAMs population there was no statistically significant difference in ERb immunostaining patterns between the three groups of melanomas in both males and females. The SAMs averaged 35-65% immunoreactivity (score 2-3) in both the nuclei and cytoplasm (Figures 1C, F, I).

Melanoma cells located adjacent to one another (MAMs) were also identified in all Breslow groups. Within the MAMs population ERb immunoreactivity levels were low, presenting though a slight gradual increase in melanoma tissues of depth > four mm (Figures 1B, E, H). However, immunopositivity levels never exceeded 40% (score 2). No difference was recorded in nuclear and cytoplasmic patterns. ERb immunostaining was more intense in male patients with melanomas > four mm deep compared to female patients of this group ($p < 0.001$).

Among all subpopulations examined, KAMs exhibited the most intense ERb immunopositivity levels in thin melanomas, compared to SAMs and MAMs of all the Breslow groups ($p < 0.001$). However, in thicker melanomas, immunoreactivity levels presented gradual decrease in all subpopulations examined. Although KAMs and SAMs immunostaining was again more intense compared to MAMs among melanomas of depth > one mm, no statistically significant difference was recorded between these cellular compounds in Breslow groups 2 and 3. Overall immunostaining score among these spatial cellular compounds is summarized in Table 2.

ERb immunoreactivity was mostly identified in thinner melanomas which generally are associated with better prognosis. Although KAMs were the most intense subpopulation documented in this Breslow group, no statistically significant correlation was found between ERb immunostaining of KAMs lesions and SLN infiltration, both in male and female patients ($p = 0.082$). Interestingly, although MAMs ERb immunostaining was more intense in deeper melanomas (even in lower levels), no significant association between ERb expression in this subpopulation and SLN invasion was also documented ($p = 0.121$). On the other hand, SAMs ERb immunoreactivity levels in melanomas one to four mm deep was strongly associated with SLN status, indicating a possible crucial step in the metastatic process of the disease ($p = 0.03$) (Figure 5).

Table 2. — *Immunostaining score among cellular compounds studied in primary melanomas (KAMs: keratinocyte-associated melanoma cells, SAMs: stroma-associated melanoma cells, MAMs: melanoma-associated melanoma cells).*

Immunoreactivity	KAMs	SAMs	MAMs
Score 0 (< 5%)	7	6	8
Score 1 (5 - 25%)	8	14	28
Score 2 (26 - 50%)	12	21	23
Score 3 (51 - 75%)	25	15	1
Score 4 (≥ 76%)	8	4	0

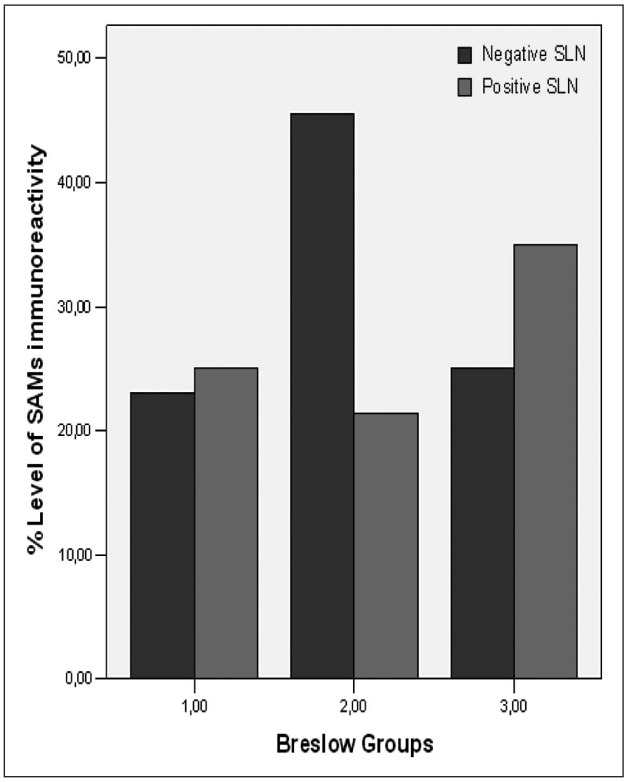

Figure 5. — Association of ERb immunoreactivity levels in stroma – associated melanoma cells (SAMs) and sentinel nodal status (* p 0.03) (Breslow group 1: depth < 1mm, Breslow group 2: depth 1-4 mm, Breslow group 3: depth > 4mm).

Table 3. — *Multivariate analysis for the impact of primary tumor characteristics in overall survival.*

Covariates	*p*	Hazard ratio	(95% Confidence interval)
Age (≤ 40 vs > 40 years)	0.172	1.031	0.987 - 1.076
Sex (male vs female)	0.920	0.920	0.181 - 4.677
Location (trunk vs extremities)	0.086	2.380	0.884 - 6.408
Histology (superficial spreading vs nodular)	0.213	0.206	0.017 - 2.473
Growth phase (radial vs vertical)	0.856	1.075	0.495 - 2.332
Mitotic index (≤ 4/mm^2 vs > 4 mm/^{2})	0.029	1.087	0.976 - 1.210
Cell type (epithelioid vs spindle)	0.065	1.926	0.959 - 3.869
Lymphatic infiltration (present vs absent)	0.870	1.123	0.278 - 4.546

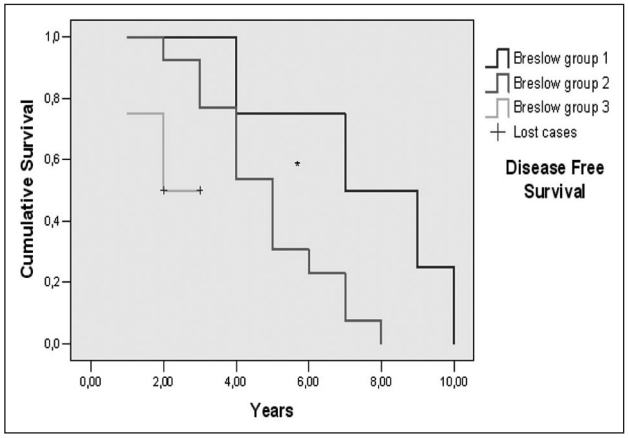

Figure 6. — Kaplan-Meier curves of disease-free survival stratified by Breslow thickness in primary melanomas (*p = 0.003) (Breslow group 1: depth < 1mm, Breslow group 2: depth 1-4 mm, Breslow group 3: depth > 4mm).

Follow up was feasible for 53/60 patients (88.3%) for a mean period of 8.2 ± 2.8 years [1-11]. As expected, mean survival rate was noticeably decreased in cases of nodal metastatic disease (p = 0.002). On Cox multivariate analysis, a mitotic index > 4/mm^2 in the primary tumor was a significant, unfavorable prognostic factor for overall survival independently of patients' age and sex as well as of the location of the tumor, the histological type, and the presence of lymphatic invasion (Table 3). Breslow thickness of the primary tumor was not an independent factor for overall survival, but only for disease-free survival rates (Figure 6).

ERb immunoreactivity levels in the primary tumors did not affect overall survival. However, when ERb expression patterns were studied in the specific subpopulations examined, a survival advantage was evident in cases of intense

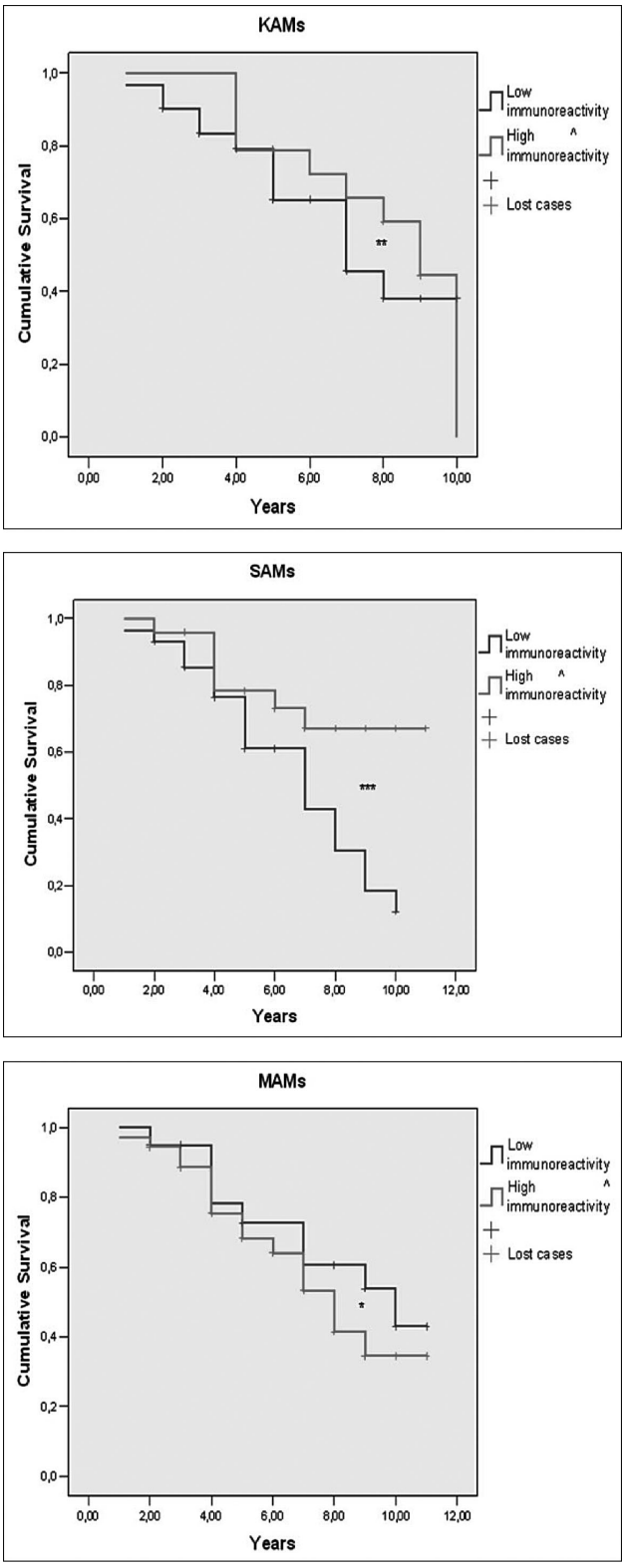

Figure 7. — Kaplan-Meier survival curves based on the immunoreactivity of the cellular compounds studied (*p = 0.580, ** p = 0.368, ***p = 0.013, ^ Low immunoreactivity < 50%, High immunoreactivity ≥ 50%) (KAMs: keratinocyte-associated melanoma cells, SAMs: stroma-associated melanoma cells, MAMs: melanoma-associated melanoma cells).

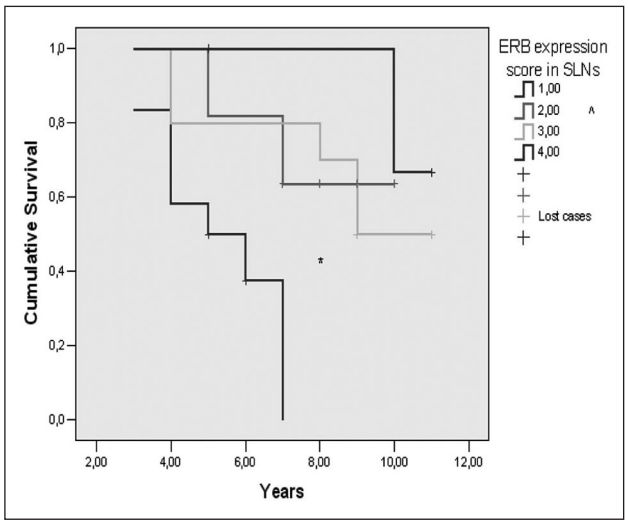

Figure 8. — Kaplan-Meier survival curves estimated by ERb expression in SLNs (*p = 0.002) (^ Score 0: < 5% positive staining; Score 1: 5-25% positive staining; Score 2: 26-50% positive staining; Score 3: 51-75% positive staining; Score 4: ≥ 76% positive staining).

SAMs immunostaining (≥ 50%) in the primary lesions (p = 0.013), but not in KAMs or MAMs subpopulations (Figure 7). On the other hand, when ERb immunostaining was studied in SLN tissues, survival rates were favored in all cases of ERb staining patterns > 25% (p = 0.002) (Figure 8).

Discussion

The current study was designed in purpose to explore the potential relationship between ER expression in primary melanomas and SLNs and malignant melanocyte physiology and pathophysiology, especially during the metastatic process through the lymph route. ERa was observed only in a few cases; however, these negative ERa data are entirely consistent with previous studies examining ERa expression in primary melanomas [9, 11].

ERb was the predominant estrogen receptor found in all melanomas and SLNs examined in our series. Although previous studies have confirmed that varying degrees of nuclear expression for immunoreactive ERb are observed in various forms of melanocytic lesions, including melanomas [10, 12], the present study provides novel information regarding ERb expression in SLNs of malignant melanomas.

Consistently to the literature, the distribution and degree of ERb immunoreactivity in the present series was markedly different depending directly on the Breslow depth of the primary tumor and the cellular microenvironment within the lesion. The loss of ERb expression in cancer cells of progressing melanomas may represent a critical stage in estrogen-dependent tumor progression. In the present study, the decrease in ERb expression observed in thicker melanomas suggests that it may be relevant to the pathophysiology of malignant melanoma. Noteworthy, similar immunostaining patterns were documented when tissues of SLNs were examined. A constant finding was the decrease of ERb levels in SLNs associated with increasing Breslow thickness of the primary tumor. This was related to the concomitant decrease in the levels of ERb expression in thicker primary tumors as well. The present study demonstrated a strong association of ERb immunoreactivity levels and negative SLNs, as well as improved patients' survival. This could be a useful tool in the pathological evaluation of SLNs and further clinical assessment of patients with malignant melanomas.

Another captivating finding was that the degree of ERb expression in melanomas depended upon the specific microenvironment of melanoma cells and correlated with its metastatic potential. Melanoma cells in the epidermis (KAMs) were the most ERb immunoreactive compared to the dermal aggregates at the periphery (SAMs) and melanoma cells in large dermal nodules (MAMs). However, ERb immunoreactivity of MAMs only, although in low levels, increased in deeper and more aggressive melanomas. These findings suggest that estrogenic compounds could be influencing the interactions of melanocytes with neighboring cells including keratinocytes, fibroblasts, nerves, capillary and lymphatic endothelial cells, and the extracellular matrix, as indicated by other studies [13-15]. Although KAMs and MAMs represent two cellular compounds related traditionally to favored and poor prognosis, respectively, no correlation was found between their ERb immunostaining levels and SLN infiltration. On the other hand, SAMs were mostly identified in melanomas of one to four mm which represent the most controversial prognostic type of the tumor. Enchantingly, ERb immunoreactivity levels of SAMs were strongly associated with SLN status, indicating a possible crucial step in the metastatic process of the disease at this level. The present findings highlight the importance of carefully examining the microenvironment of melanocytic cells, especially in tumors of one to four mm Breslow thickness [16].

Malignant melanoma is the most aggressive form of skin cancer with a rapidly increasing incidence rate. Although a lot of debate has been conducted in relation to its hormonal behavior compared to other tumors, the role of estrogens in the progression of the disease remains unclear. Some findings that suggest a hormonal role in melanoma include older epidemiologic studies indicating a survival benefit for female patients with metastatic melanoma; the rarity of melanoma prior to puberty; and the peak incidence in women coinciding with the late child bearing years and the beginning of menopause [6]. However, larger, more recent population studies strongly indicate that survival is superior among female patients, independently to Breslow thickness, histological type, and tumor site, suggesting that other tumor-related variables are implicated in the progression of the disease [17, 18].

The data collected by the current study support the hypothesis that melanoma could be classified as an estrogen-

responsive tumor and that ERs expressed in both the primary tumor and the SLN could indicate molecular responses associated with the invasive capacity of melanoma. The effects of estrogens are mediated by different kinds of estrogen receptors (ERs) and probably, as it occurs in breast cancer, not all melanoma cells show the same types of ERs. Therefore, research should focus on melanoma and SLNs themselves, particularly on the subtypes of receptors for estrogens they express. Specific genetic polymorphisms of the ERα and ERβ genes in melanoma patients might correlate with a higher proportion of melanoma [19]. Furthermore, it is nowadays clear that the sole existence of two forms of the ERs is not sufficient to account for the diverse biological roles of estrogens and pharmacological activities of synthetic ER ligands [20]. The discovery of co-regulator complexes in nuclear estrogen receptor action seems to enable the ERs to communicate with the general transcription apparatus, possess the catalytic activities required for chromatin modification, and capacity to integrate extracellular signals and translate them into transcriptional and biological events [21].

Further validation of these results may lead to altered targeted therapies as well as more radical surgical therapy (therapeutic lymph node dissection) even in primary stages of melanomas with disease-negative SLNs which exhibit altered expression of ERbs.

Conclusion

The current study indicates a role for the estrogen receptors expressed in the sentinel node of malignant melanomas during the metastatic process. These results pinpoint at the possibility of using ERb expression as a prognostic indicator of melanoma spreading through the lymph route. The possibility of distinguishing proliferative melanomas, which are associated with dismal prognosis, from the so-called dormant melanomas opens up novel avenues in tailoring individual treatments, as already occurs for other tumors.

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