

# Estrogen Receptor Expression in Cutaneous Melanoma

## *A Real-Time Reverse Transcriptase–Polymerase Chain Reaction and Immunohistochemical Study*

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**Objective:** To evaluate estrogen receptor (ER) expression in human melanoma tissues and in the adjacent healthy skin with the aim of explaining whether the *ERα*:*ERβ* expression ratio has a role in neoplastic progression.

**Design:** Prospective study.

**Setting:** Department of Dermatology, University of Florence, Florence, Italy.

**Patients:** Fourteen patients, 12 with cutaneous melanoma (6 women and 6 men) and 2 with melanocytic nevi (1 woman and 1 man).

**Main Outcome Measures:** Using quantitative reverse transcriptase–polymerase chain reaction and immunohistochemical analysis, we analyzed *ERα* and *ERβ* messenger RNA (mRNA) and *ERβ* protein expression in

cutaneous melanoma and in the healthy skin surrounding the lesions.

**Results:** All melanocytic lesions expressed detectable levels of *ERα* and *ERβ* mRNA as well as *ERβ* protein. Dividing melanoma cases into 2 groups according to Breslow thickness, we found lower *ERα* and *ERβ* mRNA levels and lower *ERβ* protein levels in thicker, more invasive tumors.

**Conclusions:** These observations suggest a role for ERs in the metastatic process of melanoma cells, pointing at the possibility of using *ERβ* expression as a prognostic indicator of melanoma. 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 happens for other tumors.

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**T**HE ROLE OF ESTROGENS IN the cause and progression of many cancers is well documented.<sup>1</sup> The effects of estrogens are mediated by estrogen receptor  $\alpha$  (*ERα*) and estrogen receptor  $\beta$  (*ERβ*), which are members of

terms of gene regulation and biologic responses or that they could contribute to the selective actions of 17- $\beta$ -estradiol and of other estrogenic molecules on target cells.<sup>1,3</sup> Various studies have shown either a decreased expression of *ERβ* messenger RNA (mRNA) and *ERβ* protein or an increased *ERα*:*ERβ* mRNA ratio in tumor vs normal tissues in several cancers, including breast, ovary, colon, and prostate tumors.<sup>4,5</sup>

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the nuclear steroid receptor superfamily. Estrogen receptor  $\alpha$  and *ERβ* classically mediate their action by ligand-dependent binding to the estrogen-response element, leading to transcriptional regulation of target genes.<sup>1</sup> 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).<sup>2</sup>

These differences suggest either that the 2 receptors could have distinct functions in

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The question of whether melanoma tumors express the 2 ERs is intensely debated, even though epidemiologic data clearly show a survival benefit for female patients with metastatic melanoma vs male patients.<sup>6,7</sup> Some investigators have suggested a survival advantage in premenopausal over postmenopausal women, but in another large multivariate analysis, the female advantage was equally strong in

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**Table 1. Patient Characteristics**

Patient No./ Sex/Age	Site	Diagnosis	Clark Level	Breslow Thickness, mm	Note
1/F/69	Back	Clark nevus	NA	NA	Junctional melanocytic nevus with focal cytologic atypia
2/F/92	Leg	In transit melanoma metastasis	NA	NA	Absence of distal metastases
3/M/65	Back	Melanoma	III	0.5	NA
4/M/72	Scalp	Melanoma	IV	3.5	Locoregional lymph node metastases
5/M/72	Back	Melanoma	IV	1.0	NA
6/F/67	Back	Melanoma	IV	1.9	Locoregional lymph node metastases
7/F/78	Arm	Melanoma	II	0.22	NA
8/M/87	Shoulder	Melanoma	IV	2.7	Locoregional lymph node metastases
9/M/68	Chest	Melanoma	I	In situ	NA
10/M/74	Back	Melanoma	II	0.7	NA
11/F/70	Shoulder	Melanoma	III	0.6	NA
12/F/69	Arm	Melanoma	V	4.5	NA
13/F/58	Mammary region	Melanoma	IV	5.5	NA
14/M/50	Back	Clark nevus	NA	NA	Junctional melanocytic nevus with focal cytologic atypia

Abbreviation: NA, not applicable.

both premenopausal and postmenopausal groups.<sup>8</sup> The mechanisms that underlie this apparent female survival benefit in melanoma has not, as yet, been thoroughly investigated; neither have its implications been exploited in connection with the prevention of the metastatic process. It must be stressed, moreover, that skin has its own capacity to produce steroids, including estrogens, starting from cholesterol.<sup>9-11</sup>

For all of these reasons, we embarked on a project of evaluating ER expression in human melanoma tissues and in the adjacent healthy skin, with the aim of explaining whether the *ERα:ERβ* expression ratio has a role in neoplastic progression.

## METHODS

### PATIENT CHARACTERISTICS

The expression of *ERα* and *ERβ* was investigated in 14 patients, 12 with cutaneous melanoma (6 women and 6 men) and 2 with junctional melanocytic nevi with focal cytologic atypia excised at the Department of Dermatology of the University of Florence (1 woman and 1 man). Of the cutaneous melanoma cases, 1 was melanoma in situ; 2 were Clark level II; 2, level III; 5, level IV; 1, level V; and 1, cutaneous metastasis of melanoma. The characteristics of these patients (mean [SD] age, 70.8 [10.5] years; range, 50-92 years) and of the melanocytic lesions are listed in **Table 1**. The female patients, all in menopause for at least 10 years, never had any kind of hormone replacement therapy. Similarly, male patients never received any kind of hormonal therapy. None of the patients included in the study was obese.

The melanomas were excised following an existing protocol,<sup>12</sup> and written consent was obtained from all patients. Fragments of the melanoma and the surrounding healthy skin were removed from the surface of the excision area by means of 2-mm biopsy punch and sent for mRNA extraction. The area of the neoplasia to be subjected to biopsy was chosen by dermoscopic analysis to select a significant part. Subsequently, excised cutaneous specimens were formalin fixed and paraffin embedded for conventional histopathologic examination.

Biopsies were performed on the sentinel lymph nodes in the 6 patients with melanomas thicker than 1 mm. In 3 of these cases (patients 4, 6, and 8), lymph node locoregional metastases were found.

### RNA PREPARATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total cellular RNA was extracted from melanoma and healthy skin sealed in RNAlater (Ambion Inc, Austin, Texas) by using RNAwiz RNA Isolation Reagent (Ambion Inc) according to the manufacturer's instructions. Briefly, the biopsy specimens were disintegrated and homogenized in 1 mL of denaturing solution. The lysate was mixed with chloroform and centrifuged. After phenol and chloroform extraction, the RNA was precipitated from the aqueous phase with isopropanol, washed with 75% ethanol, and air dried. The pellet was then dissolved in ribonuclease-free water and stored at -80°C. Since no RNA isolation method is capable of consistently producing RNA without contaminating DNA at the detection level of reverse transcriptase-polymerase chain reaction (RT-PCR), to avoid the amplification of genomic contaminants, RNA samples were pretreated with 2 U of deoxyribonuclease I (DNase I) (of the DNA-free kit (Ambion Inc) at 37°C for 30 minutes to remove any genomic DNA. The DNase was then inactivated by adding 0.1 volume of DNase inactivation reagent and removed by spinning at 10 000g for 90 seconds. The yield of RNA was calculated by spectrophotometry, and to check RNA quality, 1 µg of each RNA sample was analyzed by agarose gel electrophoresis. The 1% agarose gel containing 0.125-µg/mL ethidium bromide, after a run of 40 minutes at 90 V, was visualized in UV light.

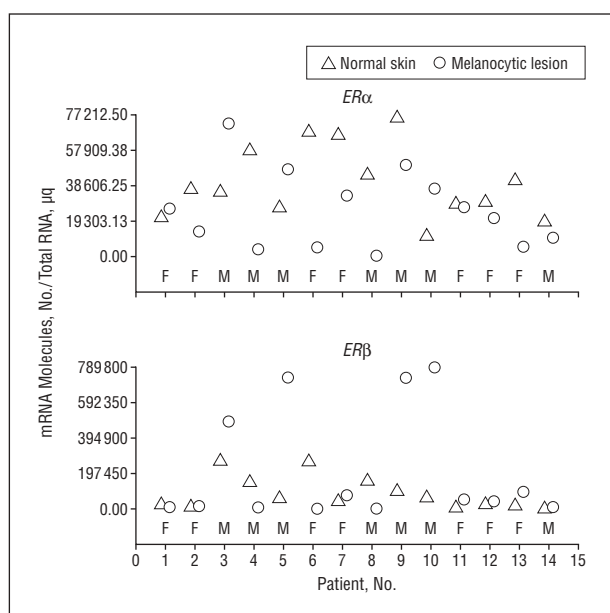
First-strand complementary DNA (cDNA) was synthesized with 1 µg of total RNA in a final volume of 20 µL using the RETROscript Reverse Transcription kit for RT-PCR (Ambion Inc), oligo(dT), and deoxyribonucleotide triphosphates (dNTPs), according to the manufacturer's protocol.

In the second step, *ERα*, *ERβ*, and *β-actin* (as housekeeping gene) expression was qualitatively evaluated by amplifying RT products (2-5 µL) using a set of specific primers for the genes of interest. The PCR were performed in separate tubes with 6 pmol of each primer and puReTaq Ready-To-Go PCR Beads (Amersham Biosciences Corp, Piscataway, New Jersey) containing 10mM TRIS-hydrochloride (pH 9.0),

**Table 2. *ERα* and *ERβ* Messenger RNA Levels in Melanocytic Lesions and in Healthy Skin<sup>a</sup>**

Patient No.	Diagnosis	Clark Level	Breslow Thickness, mm	<i>ERα</i>		<i>ERβ</i>	
				Healthy Skin	Melanocytic Lesion	Healthy Skin	Melanocytic Lesion
1	Clark nevus	NA	NA	20 382	22 456	24 983	22 435
2	In transit melanoma metastasis	NA	NA	37 543	17 567	23 900	26 987
3	Melanoma	III	0.5	34 705	72 465	265 210	483 900
4	Melanoma	IV	3.5	57 603	3636	148 125	65 825
5	Melanoma	IV	1.0	26 003	47 115	57 240	728 350
6	Melanoma	IV	1.9	67 586	48 893	261 650	24 505
7	Melanoma	II	0.22	65 716	32 931	42 037	75 582
8	Melanoma	IV	2.7	44 183	323	156 100	1799
9	Melanoma	I	In situ	75 213	49 711	98 237	730 575
10	Melanoma	II	0.7	10 784	36 747	63 680	787 800
11	Melanoma	III	0.6	31 590	26 755	4467	52 017
12	Melanoma	V	4.5	21 900	20 783	18 567	39 283
13	Melanoma	IV	5.5	42 290	4973	17 220	93 528
14	Clark nevus	NA	NA	24 433	9981	4189	9026

<sup>a</sup>Levels of *ERα* and *ERβ* were measured by real-time quantitative polymerase chain reaction and are reported as number of molecules of specific messenger RNA per microgram of total RNA.



**Figure 1.** Levels of *ERα* (A) and *ERβ* (B) messenger RNA (mRNA) in melanocytic lesions and healthy skin of our study patients. Levels of *ERα* and *ERβ* mRNA were measured in melanocytic nevi (patients 1 and 14), melanoma (patients 3-13), and metastatic cutaneous melanoma (patient 2) by real-time quantitative polymerase chain reaction. All data are reported as number of molecules of specific mRNA per microgram of total RNA.

50mM potassium chloride, 1.5mM magnesium chloride, 200µM each dNTP, and 2.5 U of puReTaq™ DNA polymerase (Amersham Biosciences Corp) in a final volume of 25 µL. Parallel RT-PCR without added RT were performed for each sample to confirm that the PCR products resulted from cDNA rather than from genomic DNA. The PCRs were performed with a standard thermal profile and appropriate annealing temperature for 35 cycles. The RT-PCR products (10-15 µL) were electrophoresed on agarose gels and stained with ethidium bromide; they were excised, purified using the Quiaex II kit (Qiagen, Valencia, California), and directly sequenced with an automated detection system (ABI-PRISM 3100; PE Applied Biosystem, Foster City, California) to confirm correct amplification of cDNA.

Expression levels of *ERα* and *ERβ* mRNA in each sample were analyzed by real-time quantitative PCR with a Stratagene Mx-3000P Detection System (Stratagene, La Jolla, California) and were normalized using *β-actin*. Upstream and downstream primers and internal oligonucleotides (Taqman probes) dual labeled (a fluorochrome [6-fluorescein-5-carboxamido (6-FAM)] at the 5' end and a black hole quencher [BHQ1] at the 3' end) and specific for each cDNA were designed by PROLIGO Primers & Probes (Proligo, Paris, France). Particular attention was paid in customizing primers to detect cDNA of both ER genes owing to the great homology in their nucleotide sequences. Primers and probes for *ERα* were the following: forward primer 5'-TGATGAAAG-GTGGGATACGA-3', reverse primer 5'-AGCTCTCATGTCTC-CAGCAG-3', and probe 5'-(6-FAM)AGACCGAAGAGGAGG-GAGAATGTGAA(BHQ1)-3'. For *ERβ*, we used forward primer 5'-GTATGCGGAACCTCAAAAGAG-3', reverse primer 5'-GTTCCCACTAACCTTCCTTTTC-3', and probe 5'-(6-FAM) CCTGGTGAAGCAAGATCGCTAGAAC(BHQ1)-3'. For *β-actin* we used forward primer 5'-AGCCTCGCCTTTGCCGA-3', reverse primer 5'-CTGGTGCCTGGGGCG-3', and probe 5'-(6-FAM)CCGCCGCCGTCCACACCCG(BHQ1)-3'. Reaction conditions were optimized until the standard deviation of duplicate determinations of the standard curve of threshold cycle for the samples was less than 3%. Expression levels of *ERα*, *ERβ*, and *β-actin* genes are expressed as the number of molecules of specific mRNA per microgram of total RNA and reported as mean (SD) values. The PCR mixture contained 5 µL of di QuantiTect Probe PCR Master Mix (Qiagen GmbH, Hilden, Germany), 0.5µM of each primer, and 100nM of the Taqman probe in a final volume of 20 µL. Every amplification reaction was performed in triplicate with the following thermal profile: a first step at 95°C for 10 minutes for HotStarTaq DNA Polymerase (Qiagen, Valencia) activation, followed by 40 cycles of 3 steps (95°C for 15 seconds, 60°C for 30 seconds, and 76°C for 30 seconds). Product amount was measured by interpolation from standard curve of threshold cycle values generated from known initial concentrations of cDNA.

#### IMMUNOHISTOCHEMICAL ANALYSIS

All cases that underwent RT-PCR also underwent immunohistochemical analysis. A representative specimen, 4 µm thick, from each formalin-fixed and paraffin-embedded skin lesion was

**Table 3. Estrogen Receptor Gene and Protein Expression According to Melanoma Lesion Breslow Thickness**

Estrogen Receptor	Breslow Thickness, mm		P Value <sup>c</sup>
	≤1.0	>1.0	
ER $\alpha$ <sup>a</sup>	41 931 (26 755-72 465)	11 270 (323-48 892)	.03
ER $\beta$ <sup>a</sup>	606 125 (52 017-787 800)	33 135 (1799-93 528)	.02
ER $\beta$ <sup>b</sup>	90 (25-90)	30 (10-50)	.04

Abbreviation: ER $\beta$ , estrogen receptor  $\beta$  protein.

<sup>a</sup>Levels of ER $\alpha$  and ER $\beta$  were measured by polymerase chain reaction and are reported as median (range) number of molecules of specific messenger RNA per microgram of total RNA.

<sup>b</sup>Levels of ER $\beta$  protein expression were measured by immunohistochemical analysis and are reported as median (range) percentage expression.

<sup>c</sup>Wilcoxon rank sum test.

evaluated. The slides were deparaffinized in Bio-Clear (Bio-Optica, Milan, Italy) and hydrated with a series of decreasing ethanol concentrations and finally distilled water. Antigen retrieval was routinely performed by immersing the slides in a thermostat bath containing preheated 10mM citrate buffer (pH 6.0) for 40 minutes at 97°C followed by cooling for 20 minutes at room temperature. To block endogenous peroxidase activity, slides were treated with 3.0% hydrogen peroxidase in distilled water for 10 minutes. After blocking nonspecific antigen with normal horse serum (UltraVision; LabVision division of Thermo Fisher Scientific Inc, Fremont, California), we incubated the sections for 30 minutes at room temperature with mouse monoclonal antibody against the C-terminus epitope of ER $\beta$ , clone EMR02 (Novocastra Laboratories Ltd, Newcastle, England), diluted 1:50 in antibody diluent (Ventana Medical Systems, Tucson, Arizona). Staining was achieved using a biotin-conjugated antimouse and antirabbit secondary antibody (UltraVision) and streptavidin-peroxidase (UltraVision). Bound antibody was detected using 3,3'-diaminobenzidine (Dako, Glostrup Denmark) as chromogen. Nuclei were slightly counterstained with Mayer hematoxylin. The negative control procedure was performed by substituting a nonimmune serum for the primary antibody at the same concentration. As a positive control, we used a fibromatosis sample certainly positive for ER $\beta$ . The control sections were treated in parallel with the samples in the same run. Only definite nuclear staining was regarded as positive; cases were scored by the percentage of tumor cells that stained, and staining was further classified as 1+ (≤20%), 2+ (21%-50%), or 3+ (>50%).

### STATISTICAL ANALYSIS

Data analysis was performed by means of SPSS software (release 10.0) (SPSS Inc, Chicago, Illinois). Comparisons of mRNA and protein levels between groups were performed with the non-parametric 2-sample Wilcoxon rank sum test. A P value less than .05 was chosen to indicate a significant difference.

### RESULTS

Using RT-PCR, we analyzed ER $\alpha$  and ER $\beta$  mRNA expression in cutaneous melanomas, melanocytic nevi, and healthy skin surrounding the lesions examined. All melanocytic cutaneous lesions expressed detectable levels of ER $\alpha$  and ER $\beta$  mRNA, with levels varying from patient to patient (**Table 2** and **Figure 1**). It is interesting to note that the same variability existed between the individual patient's healthy skin surrounding the lesion and the melanocytic cells. In particular, the levels of ER $\alpha$  did not differ between benign (Clark nevus) and malig-

nant melanocytic lesions but rather with melanoma thickness (Breslow thickness).

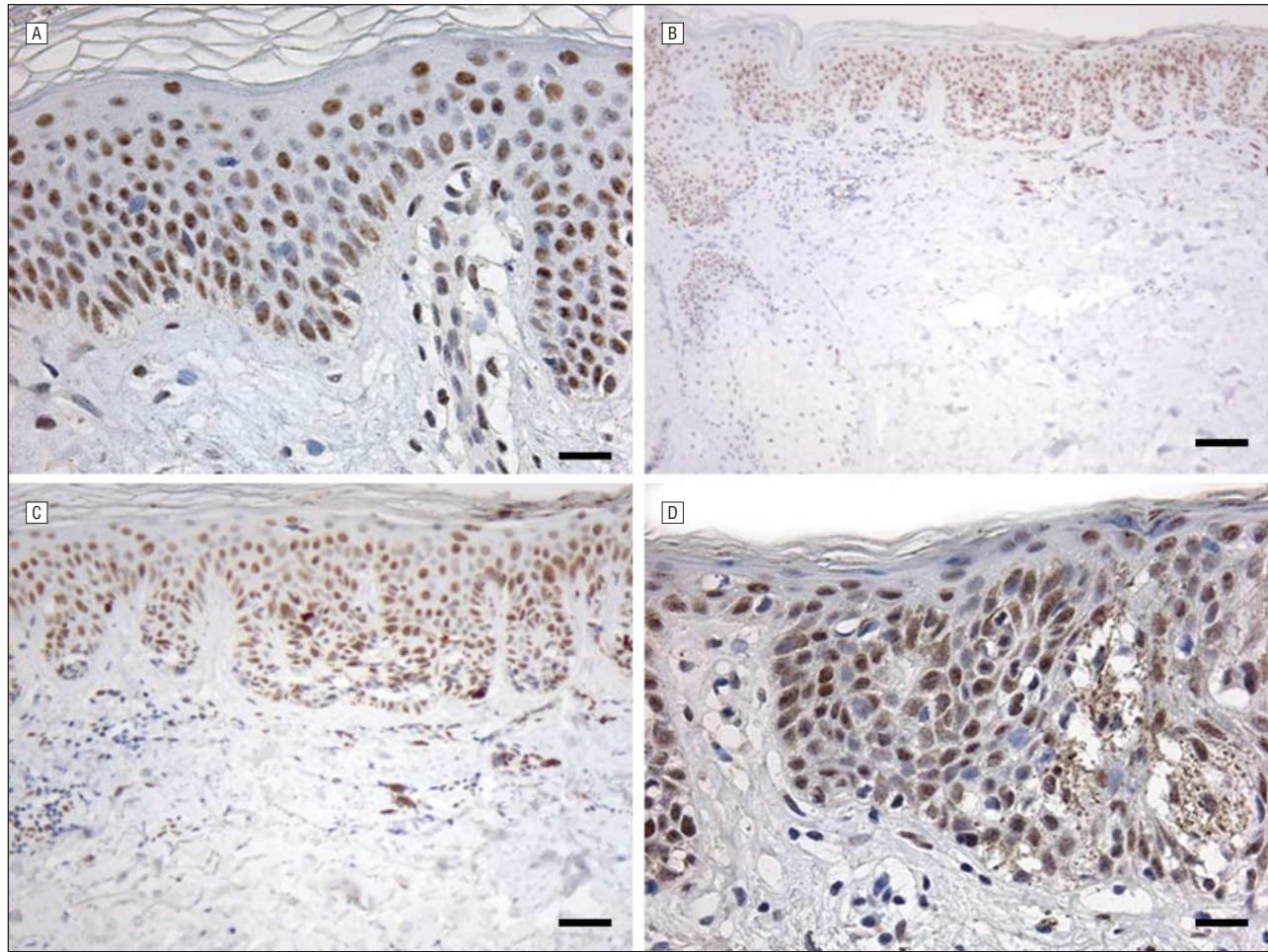
Conversely, ER $\beta$  expression appears to vary significantly in the various groups studied. Indeed, **Figure 1** shows that the levels of ER $\beta$  are practically superimposable for the benign melanocytic tissue and the healthy skin surrounding it (patients 1 and 14), being particularly low compared with melanoma. Moreover, 8 of 11 patients with melanoma (patients 3, 5, 7, and 9-13) showed higher levels of ER $\beta$  in the melanoma cells than in the healthy skin surrounding the lesions. The remaining 3 patients with melanoma (patients 4, 6, and 8) showed extremely low levels of ER $\beta$  expression in the melanoma cells and higher levels in the healthy skin surrounding the lesions. It is of great interest to note that these were the patients who already showed signs of lymph node metastasis during neoplastic progression. These same patients (patients 4, 6, and 8) also showed low levels of ER $\alpha$  in the tumoral tissue.

If we divide melanoma cases in 2 groups according to Breslow thickness, ie, thin lesions (≤1 mm, 6 cases) vs thicker melanomas (>1 mm, 6 melanomas), we observe that both ER $\alpha$  and ER $\beta$  mRNA expression correlate with melanoma thickness. In particular, thin melanomas show significantly higher ER mRNA levels than thicker lesions (**Table 3**).

Finally, the patient who had melanoma with metastases in transit but with no distal metastases at the time of examination (patient 2) showed extremely low levels of ER $\beta$  both in the melanoma cells and in the healthy skin surrounding the lesion.

Immunohistochemical analysis, carried out on tissues from the same patients, showed that epidermal keratinocytes overlying and adjacent to benign and malignant melanocytic lesions were diffusely ER $\beta$  positive, although with varying staining intensity (**Figure 2A**). No significant differences in distribution and staining in the epidermis were noted between melanocytic nevi, melanomas associated with disease progression, and nonprogressed melanomas. Concerning melanocytic nevi, in 1 case, the immunostaining was judged not evaluable since the lesion was associated with prominent regression phenomena, and the number of residual melanocytes was too scarce to be evaluated (case 14). The other junctional melanocytic nevus with focal cytologic atypia displayed strong ER $\beta$  expression in more than 80% of melanocytes (3+) (**Table 4**, **Figure 2B-D**). Similarly, the in situ





**Figure 2.** Immunohistochemical staining for estrogen receptor  $\beta$  protein. A, Strong and diffuse nuclear staining in epidermal keratinocytes adjacent to a melanocytic nevus (original magnification  $\times 40$ ; scale bar, 20  $\mu\text{m}$ ). B, Low magnification shows positive staining in a junctional melanocytic nevus with focal cytologic atypia (original magnification  $\times 10$ ; scale bar, 100  $\mu\text{m}$ ). C, Lentiginous epidermal hyperplasia with positive melanocytes (original magnification  $\times 20$ ; scale bar, 50  $\mu\text{m}$ ). D, Nuclei of epidermal keratinocytes and atypical melanocytes show positive staining (original magnification  $\times 40$ ; scale bar, 20  $\mu\text{m}$ ).

**Table 4. Immunohistochemical Expression for Estrogen Receptor  $\beta$  Protein (ER $\beta$ ) in the Whole Series**

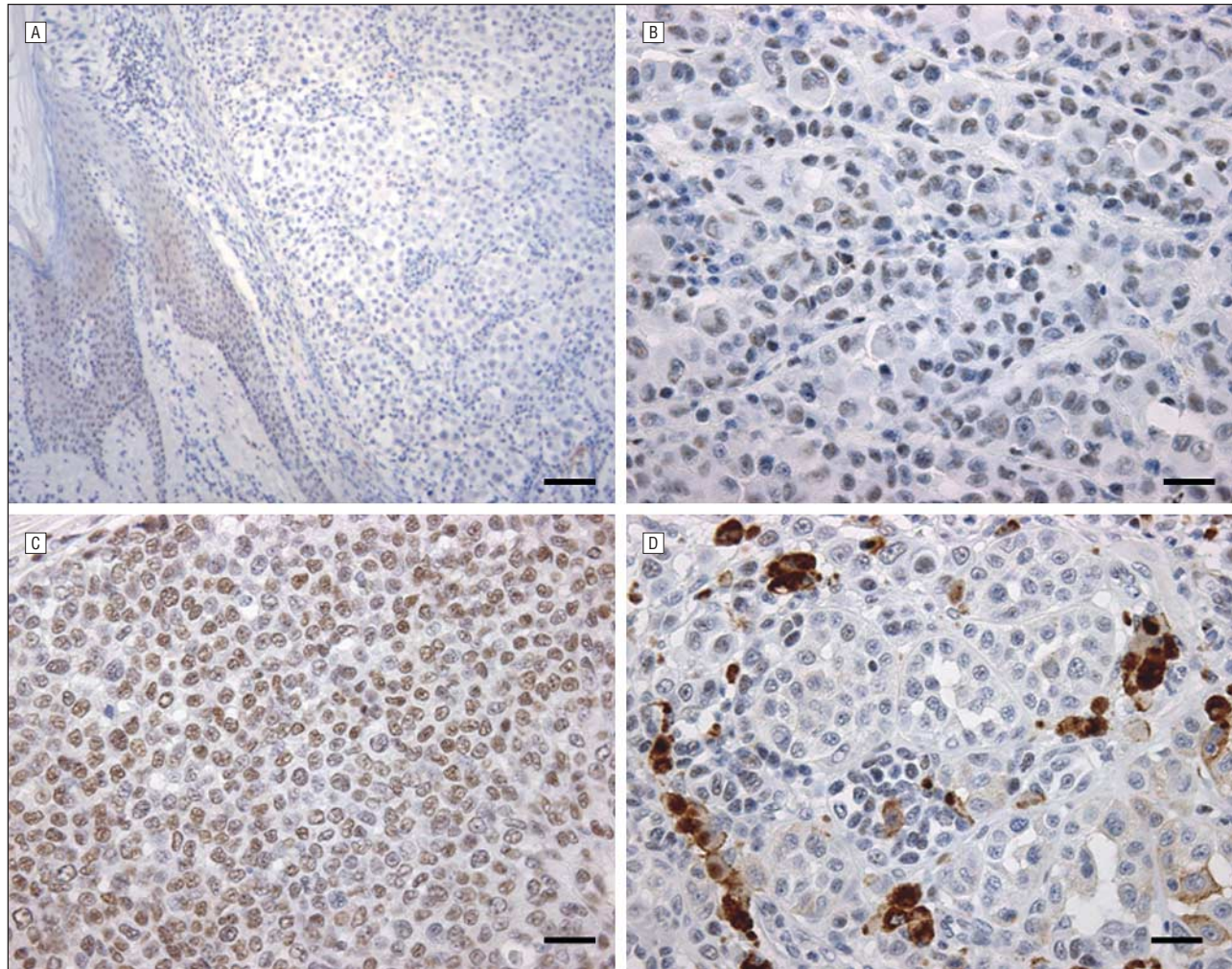
No.	Diagnosis	Clark Level	Breslow Thickness, mm	Disease Progression	ER $\beta$ Expression, %
1	Clark nevus	NA	NA	NA	80 (Strong)
2	In transit melanoma metastasis	NA	NA	Absence of distal metastases	30 (Weak)
3	Melanoma	III	0.5	NA	90 (Strong)
4	Melanoma	IV	3.5	Locoregional lymph node metastases	50 (Weak)
5	Melanoma	IV	1.0	NA	90 (Strong)
6	Melanoma	IV	1.9	Locoregional lymph node metastases	10 (Weak)
7	Melanoma	II	0.22	NA	25 (Weak)
8	Melanoma	IV	2.7	Locoregional lymph node metastases	20 (Weak)
9	Melanoma	I	In situ	NA	90 (Strong)
10	Melanoma	II	0.7	NA	90 (Moderate)
11	Melanoma	III	0.6	NA	40 (Weak)
12	Melanoma	V	4.5	NA	40 (Weak)
13	Melanoma	IV	5.5	NA	30 (Strong)
14	Clark nevus	NA	NA	NA	NE

Abbreviations: NA, not applicable; NE, not evaluable.

melanoma showed strong ER $\beta$  expression in more than 90% of melanocytes (3+). Among 10 primary invasive melanomas evaluated, 3 cases displayed moderate to strong 3+ expression (>50% of positive cells), 5 cases

were 2+ (21%-50% of positive cells), and 2 cases showed a weak 1+ ER $\beta$  expression (ie,  $\leq 20\%$  of positive cells) (**Figure 3**). The 2 cases showing 1+ ER $\beta$  staining were associated with disease progression (cases 6 and 8), con-





**Figure 3.** Immunohistochemical staining for estrogen receptor  $\beta$  protein. A, Low magnification of a nodular melanoma shows normal epidermal and adnexal epithelium with positively stained keratinocytes (left) and negative melanoma cells (right) (original magnification  $\times 20$ ; scale bar, 50  $\mu\text{m}$ ). B, Moderate staining in most of the melanoma cells (original magnification  $\times 40$ ; scale bar, 20  $\mu\text{m}$ ). C, Strong and diffuse nuclear staining in all melanoma cells (original magnification  $\times 40$ ; scale bar, 20  $\mu\text{m}$ ). D, Staining-negative cells in the context of a vertical growth phase melanoma (original magnification  $\times 40$ , scale bar, 20  $\mu\text{m}$ ).

sistent with results obtained by RT-PCR on *ER $\beta$*  mRNA expression. Interestingly, in most primary tumors we noted a decrease in nuclear *ER $\beta$*  staining in the invasive dermal clonal aggregates morphologically consistent with the tumor vertical growth phase in comparison with the in situ and radial growth phase. The cutaneous melanoma metastasis (case 2) showed weak 2+ *ER $\beta$*  staining (positivity in 30% of cells). Thin melanomas ( $\leq 1$  mm) were associated with significantly higher *ER $\beta$*  protein expression than thicker lesions ( $> 1$  mm) ( $P = .04$ ).

#### COMMENT

Malignant melanoma is the most aggressive form of skin cancer with a rapidly increasing incidence rate. Despite the wide variety of therapeutic approaches tested over the years, metastatic disease is still associated with a dismal prognosis owing to the minimal success of systemic therapy.

In contrast to other tumors, the role of estrogens in the initiation and progression of melanoma remains unclear. Some findings that suggest a hormonal role in melanoma include (1) epidemiologic data indicating a survival ben-

efit for female patients with metastatic melanoma; (2) the rarity of melanoma prior to puberty; and (3) the peak incidence in women coinciding with the late childbearing years and the beginning of menopause. These epidemiologic data were supported by studies using melanoma cell lines grown in experimental animals.<sup>13</sup> However, no significant changes in estrone or androstenedione levels were noted for male or female patients with disease progression.<sup>14</sup> The data collected support the hypothesis that melanoma could be classified as an estrogen-responsive tumor and that ERs could mediate molecular responses associated with the neoplastic transformation of normal benign nevi.

Following these considerations, tamoxifen, an ER antagonist used routinely in the treatment of breast carcinoma, has been used in the past in the treatment of patients with metastatic melanoma either as a single agent or, more commonly, in combination with other chemotherapeutic agents.<sup>15</sup> At present, the evidence resulting from these studies does not support the use of tamoxifen for the treatment of metastatic melanoma.

The aim of our study was to investigate whether ERs were present in melanocytic lesions, if their levels were

different from those of the healthy skin surrounding them, and if their expression was linked to the level of melanoma invasiveness. We found that all of the samples of the melanocytic lesions examined expressed both ERs, though at different levels and not always with a clear-cut link with the clinical features of the lesions themselves. In particular, in the samples of melanoma the levels of expression differed from those of the healthy skin surrounding the lesions, suggesting a potential role for ERs and/or for their ligands in the origin and progression of this neoplasm. Indeed, by classifying the patients into 2 groups according to melanoma thickness ( $\leq 1.00$  mm vs  $> 1.00$  mm), we found that levels of ERs, both  $\alpha$  and  $\beta$ , were closely and statistically correlated with Breslow thickness. To our knowledge, this is the most significant prognostic factor yet found for this tumor.

Expression of ER $\beta$  is similar between melanocytic nevi and the healthy skin surrounding them, but it differs between melanoma lesions and the healthy skin surrounding them. In 8 of 11 of our study patients with melanoma, ER $\beta$  levels were higher in the melanoma cells than in the normal cells. None of these 8 tumors showed distant invasiveness. Conversely, in 3 melanoma tissues, characterized by lymph node metastasis, ER $\beta$  expression in the tumor tissues was much lower than in the surrounding normal skin cells. The highest level of ER $\beta$  mRNA was found in the melanoma in situ.

These initial observations, to be confirmed by further case histories, could suggest a role for ERs in the metastatic process of melanoma cells; they might regulate the invasive capacity of melanoma. This knowledge opens up the possibility of using ER expression as a prognostic indicator of melanoma. The possibility of distinguishing proliferative melanomas linked to dismal prognosis from the so-called dormant melanomas opens up novel avenues in tailoring individual preventive treatments, as already happens for other tumors.

To our knowledge, our is the first study analyzing ERs in melanocytic lesions both with PCR and immunohistochemical analysis, an interesting observation being the significant correlation between the 2 types of analysis. In line with our observations, Schmidt et al,<sup>16</sup> using only PCR analysis, reported ER $\beta$  protein expression in all melanocytic lesions studied, including benign nevi, dysplastic nevi, and melanomas of various thickness, with an inverse correlation between ER $\beta$  expression and Breslow depth.

In conclusion, despite the relatively small number of patients and so our data requiring further confirmation by more case studies, we can hypothesize that the expression of ERs, ER $\beta$  in particular markedly decreases in the metastatic phase of melanoma. In future studies we will try to understand the molecular mechanisms that underlie this decrease, if ER $\beta$  modulates the cell cycle and/or apoptosis, and if it is linked to a modification of adhesion of melanoma cells.

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**Author Contributions:** Dr de Giorgi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* de Giorgi, Massi, Tanini, Boddi, Brandi, and Lotti. *Acquisition of data:* de Giorgi, Gozzini, Aragona, Sestini, and Paglierani. *Analysis and interpretation of data:* de Giorgi, Mavilia, Massi, Brandi, and Lotti. *Drafting of the manuscript:* de Giorgi, Massi, Gozzini, Aragona, and Sestini. *Critical revision of the manuscript for important intellectual content:* de Giorgi, Mavilia, Massi, Tanini, Paglierani, Boddi, Brandi, and Lotti. *Statistical analysis:* Massi, Aragona, Paglierani, Boddi, and Brandi. *Study supervision:* de Giorgi, Mavilia, Massi, Gozzini, Tanini, Sestini, Brandi, and Lotti.

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