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Ets-1 transcription factor is widely expressed in benign and malignant melanocytes and its expression has no significant association with prognosis

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Ets-1 transcription factor has been associated with tumor progression in various carcinomas, but its expression in malignant melanoma was only recently described. The study was conducted in two steps: exploratory and confirmatory. In the first step, we studied 69 primary melanomas, 28 metastatic melanomas, 10 usual intradermal nevi and 13 various melanocytic skin lesions. In the second step, an additional group of 98 patients with follow-up of up to 200 months was also evaluated. Immunohistochemical analysis of formalin-fixed/paraffin-embedded tissues was performed using 1G11 antibody and polymer conjugate for visualization. While Ets-1 was variably expressed in 83% primary melanomas in exploratory and 69% in the confirmatory group, the expression of Ets-1 was also found in normal benign melanocytes and all nevi. Analysis of the exploratory group revealed lower expression of Ets-1 in primary melanomas than in common nevi ($P=0.048$, Mann–Whitney *U*-test) and metastatic melanomas expressed significantly less Ets-1 than primary melanomas ($P=0.015$, Mann–Whitney *U*-test). There was a negative correlation between Ets-1 expression and the largest dimension of the primary tumors ($r=0.23$, $P=0.034$, Spearman's correlation rank test), but no correlation with the depth of tumor invasion (Breslow thickness) or the presence of ulceration was found. Analyses of the confirmatory group revealed no association between Ets-1 expression with disease-specific survival or time to treatment failure. However, a statistical trend was found for worse outcome for those primary melanomas that had strong expression (*H*-score >100) of Ets-1 ($P=0.054$). Ets-1 is expressed in benign melanocytes probably due to their neural crest origin. We conclude that Ets-1 expression cannot be used to differentiate between benign and malignant melanocytic lesions and it has no definite association with clinical outcome. At the same time, its role in tumor progression in some cases of malignant melanoma cannot be entirely excluded.
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Malignant melanoma originates from melanocytes derived from the neural crest.[1] It is the most deadly form of skin cancer, whose incidence is rapidly increasing worldwide.[2] To date the most reliable prognostic marker of malignant melanoma is the vertical thickness (Breslow thickness) of the primary tumor.[3,4] Although progression of malignant melanoma occurs through a series of well-defined steps, the knowledge of genetic and phenotypic background for the disease is still rather scarce. The proto-oncogene *ETS-1* belongs to the ETS family of transcription factors that also include, among others, ets-2, elf-1, fli-1 and PU-1.[5–7] Ets-1 plays an important role in cancer progression due to its ability to activate transcription of metastasis-, angiogenesis- and invasion-associated genes.[8–12]

In a limited study on 10 melanomas and 24 benign melanocytic lesions, Keehn *et al*[13] recently reported expression of the Ets-1 in melanocytic lesions and suggested that Ets-1 expression might be an important pathogenic mechanism and predictor of aggressive biologic behavior of cutaneous melanoma.

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Herein, we have investigated whether expression of Ets-1 differs through the spectrum of melanocytic lesions and whether its expression in primary melanoma is associated with disease-specific survival and time to treatment failure.

Materials and methods

In the first step, formalin-fixed, paraffin-embedded tissue sections from 69 primary melanomas, 28 metastatic melanomas, 10 usual intradermal nevi and 13 various melanocytic skin lesions (two Spitz nevi, one Halo nevus, two common blue nevi, one cellular blue nevus and seven dysplastic nevi with moderate atypia) were randomly collected from the archives of the pathology department, The Norwegian Radium Hospital. The median age of the patients was 62 years (range 19–93 years). Of the primary tumors, 53 were classified as superficial spreading and 15 as nodular. The primary tumor thickness median was 1.25 mm (range 0.30–7.00 mm) for superficial spreading tumors and 3.9 mm (range 1.50–6.00 mm) for nodular melanoma. The largest tumor dimension (diameter of the tumor) was also recorded.

In the second step, we included formalin-fixed/paraffin-embedded tissues from additional 98 patients with median follow-up for patients alive of 156 months (range 68–200 months). In total, 62 were classified as superficial spreading and 36 as nodular melanomas. The median age was 54 years (range 25–83 years). For superficial spreading tumors, the median tumor thickness was 1.20 mm (range 0.11–10.00 mm), whereas the mean depth of nodular tumors was 4.00 mm (range 1.23–15.00 mm). Overall, the mean depth of invasion was 2.88 mm (range 0.11–15.00 mm). Treatment of patients was according to WHO standard guidelines.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was cut at 5 μm, dried overnight at 60°C and deparaffinized in xylene. Subsequently, sections were rehydrated through graded alcohols into water. Heat-induced epitope retrieval was achieved by boiling sections in the EDTA buffer at pH 8.9 in the Electrolux microwave oven (Stockholm, Sweden) at 1000 W for 20 min (4×5 min). After boiling, sections were allowed to cool at room temperature for 20 min, rinsed thoroughly with water and placed in Tris-buffered saline (TBS) for 5 min. Endogenous peroxidase was blocked with peroxidase block solution provided in the EnVison + ® kit (DakoCytomation, Glostrup, Denmark) for 5 min and slides rinsed/washed with TBS. Slides were then incubated with 1G11 antibody (1:20, NovoCastra Laboratories, New Castle upon Tyne, UK) for 30 min at room temperature. Visualization was performed using EnVision + ® (DakoCytomation, Glostrup, Denmark) method according to the manufacturer's instructions. Appropriate positive and negative controls were used. In addition, strong nuclear staining in benign small lymphocytes was used as internal positive control.

Only nuclear staining was recorded. Very faint cytoplasmic staining was also detected in some cases, but was not recorded. Ets-1 immunostaining showed great variability from case to case and in some cases, there was some variability in different areas of the tumor, but there was no definite order in the distribution of the positive cells with respect to horizontal or vertical orientation. Expression of Ets-1 was quantified using *H*-score (histo-score) system, according to the method described by McCarty *et al*,[14] which considers both the intensity and percentage of cells staining at each intensity. Cells were counted using $\times 400$ magnification and a cell counter. The score was calculated as follows: *H*-score = (%3 + cells $\times$ 3) + (%2 + cells $\times$ 2) + (%1 + cells $\times$ 1). Overall, the cases were also designated as 'positive' if more than 10% of the tumor cells showed expression of the antigen.

Statistical Methods

Disease-specific survival for the patients with 'Ets-1-positive' *vs* 'Ets-1-negative' primary melanomas was calculated by the Kaplan–Meier survival estimates and the log-rank test from the date of diagnosis until last contact or death from disease. Time to treatment failure (TTF) was calculated from the date of first complete remission to the date of last contact, if alive and none relapsed, or to relapse or death, whichever came first. Results obtained by *H*-score were also analyzed by using the Cox proportional hazards model. The χ^2, linear-to-linear association and Mann–Whitney *U*-tests were used whenever appropriate for comparison of subgroups. The association between the intensity of expression with depth of invasion (Clark levels); tumor thickness according to Breslow and diameter of tumors was studied with the Spearman rank correlation. Statistical significance was established at the $P<0.05$ level. Analyses were performed in SPSS 11.5.

Results

Results of the analysis of the exploratory group are summarized in Table 1. All common intradermal nevi and dysplastic nevi expressed high levels of Ets-1. The percent of positive cells was larger than 80% in all nevi. Similar Ets-1 expression was also found in Halo nevus and Spitz nevi, while blue nevi had comparable numbers of cells positive, but the intensity of the staining was less pronounced. Ets-1 was also expressed in benign intraepidermal melanocytes (Figure 1). Primary melanomas had lower expression of Ets-1 than common nevi ($P=0.048$, Mann–Whitney *U*-test) and metastatic melanomas

Table 1 Ets-1 expression in benign and malignant melanocytic lesions (exploratory group, $n=120$)

Ets-1	*Diagnosis*			
	Common intradermal nevus	*Dysplastic nevus*	*Primary melanoma*	*Metastatic melanoma*
Negative	0	0	12 (17)	9 (32)
Positive	10 (100)	7 (100)	57 (83)	19 (68)
Total	10 (100)	7 (100)	69 (100)	28 (100)

Primary melanoma *vs* common nevi ($P=0.048$, Mann–Whitney *U*-test) and primary melanomas *vs* metastatic melanoma ($P=0.015$, Mann–Whitney *U*-test).

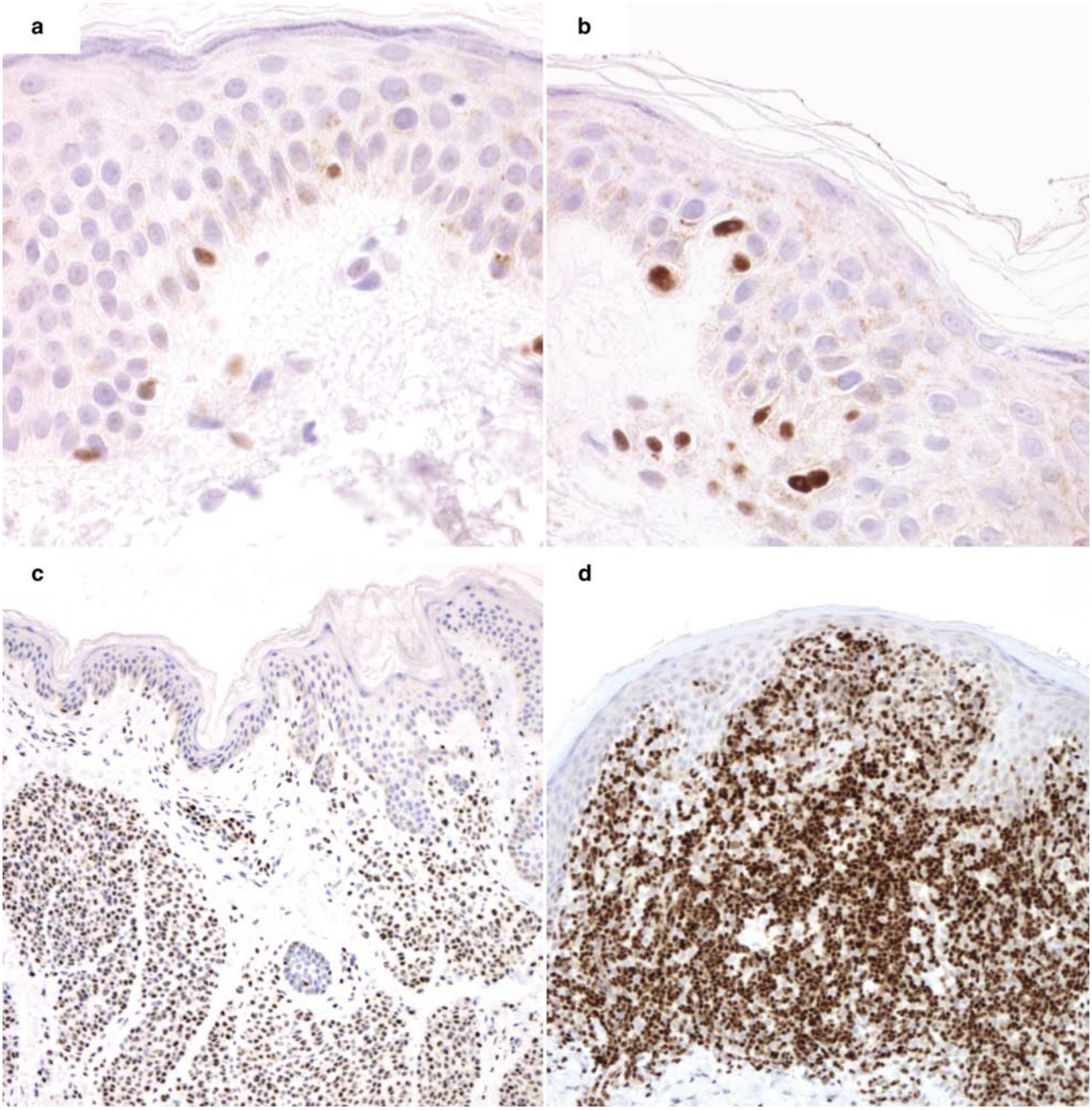

Figure 1 Intraepidermal benign melanocytes show variable expression of Ets-1. Some have weak (**a**) and some strong (**b**) expression ($\times 400$ magnification). Usual benign nevi have moderate to strong expression. Intraepidermal nevus (**c**) and Halo nevus (**d**) are shown ($\times 200$ magnification) (Ets-1 immunostaining).

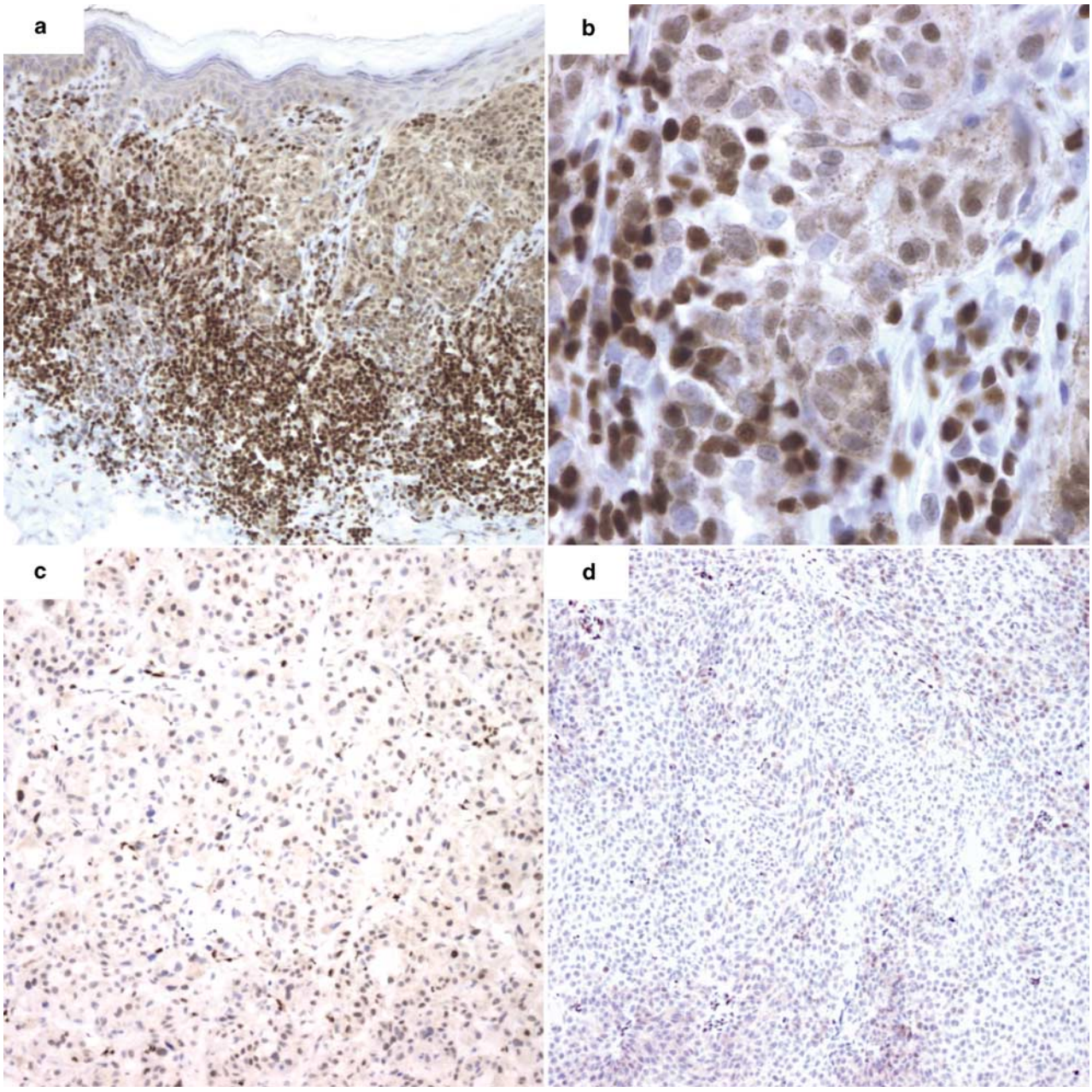

Figure 2 Strong expression of Ets-1 is present in peritumoral lymphocytes, while primary melanoma expresses weak to moderate Ets-1 (**a**) (× 200 magnification), (**b**) (× 400 magnification). Overall, expression of Ets-1 was much lower in metastatic melanoma as shown in the two representative cases (**c** and **d**) (× 400 magnification) (Ets-1 immunostaining).

expressed significantly less Ets-1 than primary melanomas ($P=0.015$, Mann–Whitney *U*-test) (Figure 2). There was a negative correlation between Ets-1 expression and diameter of the primary tumors ($r=0.23$, $P=0.034$, Spearman's correlation rank test), but no correlation with depth of tumor invasion or the presence of ulceration was found.

Results of the analysis of the confirmatory group are summarized in Table 2 and survival plots (Figures 3–5). The group of the patients, which was evaluated for the outcome, had 69% tumors with Ets-1 expression, which was similar to 83% in the exploratory group. No difference in disease-specific survival was found between patients with Ets-1-positive and Ets-1-negative malignant primary melanoma ($P=0.98$, Kaplan–Mayer log-rank test) (Figure 3). However, patients with tumors that had high expression of Ets-1 (*H*-score >100) showed a trend for shorter disease-specific survival ($P=0.054$), but not for the TTF ($P=0.81$) (Figure 4). Also, an association of older age (>65 years) and shorter disease-specific survival ($P<0.0001$) and TTF ($P=0.049$) was found (Figure 5).

Table 2 Ets-1 expression in malignant melanoma (confirmatory group, $n = 98$)[a]

Variable	*Ets-1 (%)*		*Total*
	Positive	*Negative*	
Age (in years)			
25–45	8 (27)	22 (73)	30 (100)
46–65	17 (38)	28 (62)	45 (100)
66–83	5 (22)	18 (78)	23 (100)
Sex			
Male	11 (28)	29 (72)	40 (100)
Female	19 (33)	39 (67)	58 (100)
Type			
Superficial Spreading	16 (26)	46 (74)	62 (100)
Nodular	14 (39)	22 (61)	36 (100)
Depth of invasion[b]			
<2.88 mm	21 (30)	49 (70)	70 (100)
≥ 2.88 mm	9 (33)	18 (67)	27 (100)
Ulceration			
No	19 (31)	43 (69)	62 (100)
Yes	8 (26)	23 (74)	31 (100)

[a]No significant associations were found.
[b]2.88 mm = mean depth of invasion.

Discussion

The overexpression of Ets-1 found in different human cancers has been associated with invasiveness and the degree of malignancy.[15–17] Ets-1 expression is not restricted to the tumor cells since its mRNA is also found in the stromal fibroblasts of invasive tumors.[18,19] Ets-1 is not expressed in quiescent endothelial cells in adult tissues, but it is induced by proliferating and migrating endothelial cells but not after these cells have reached confluence.[20,21] The transcription factor Ets-1 is expressed in many different migratory cell types, suggesting that it may play an important role in regulating motility. Even though it was only recently described in melanomas,[13] Ets-1 is upregulated in the cranial neural folds and dorsal neural tube approximately 4–6 h prior to commencement of neural crest migration and continues to be expressed by migrating cranial neural crest cells and subsequently by some neural crest-derived tissues.[22,23] Ets-1 was also demonstrated in the neural crest of the mouse embryo.[24] Hence, the expression of Ets-1 in intraepidermal benign melanocytes is consistent with their neural crest origin and perhaps does not suggest neoplastic transformation in melanocytic lesions. Lower expression of Ets-1 in melanoma than in nevi as well as lower expression in metastatic than in primary tumors may suggest partial loss of the neural crest phenotype. Similarly, c-KIT, a tyrosine kinase receptor encoded by the c-KIT proto-oncogene, has been found to play a pivotal role in normal growth and differentiation of

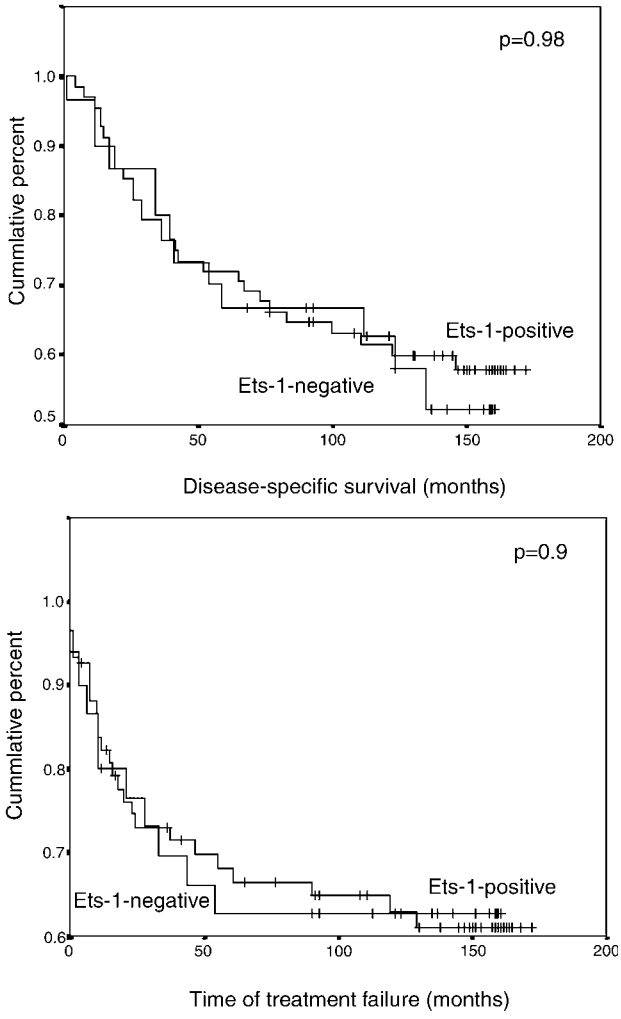

Figure 3 No difference in disease-specific survival or TTF is found between cases with or without Ets-1 expression.

embryonic melanoblasts, but its expression progressively decreases during local tumor growth and invasion of human melanomas.[25–27] Of note, another transcription factor, AP-2, which is also expressed in neural crest cell lineages during mouse embryogenesis, was found to be downregulated in malignant melanoma.[28] Loss of AP-2 correlated with loss of c-KIT expression in highly metastatic melanoma cells.[29] Furthermore, loss or inactivation of the p16 gene, the product of the p16/INK4a/CDKN2/MTS tumor suppressor gene, has been shown to be associated with the progression of melanoma.[30] It has been recently reported that Ets-1 as well as Ets-2 activate the promoter of the p16 INK4a gene, whose product inhibits Cdk4 and Cdk6 cyclin-dependent kinases, through an Ets-binding site and contributes to regulation of cellular senescence.[31] Our findings are in agreement with these previous studies.

Huang *et al*[32] were able to inhibit melanoma growth and metastasis by enforced c-KIT expression implying that downregulation of c-KIT is related to melanoma progression. Downregulation of Ets-1

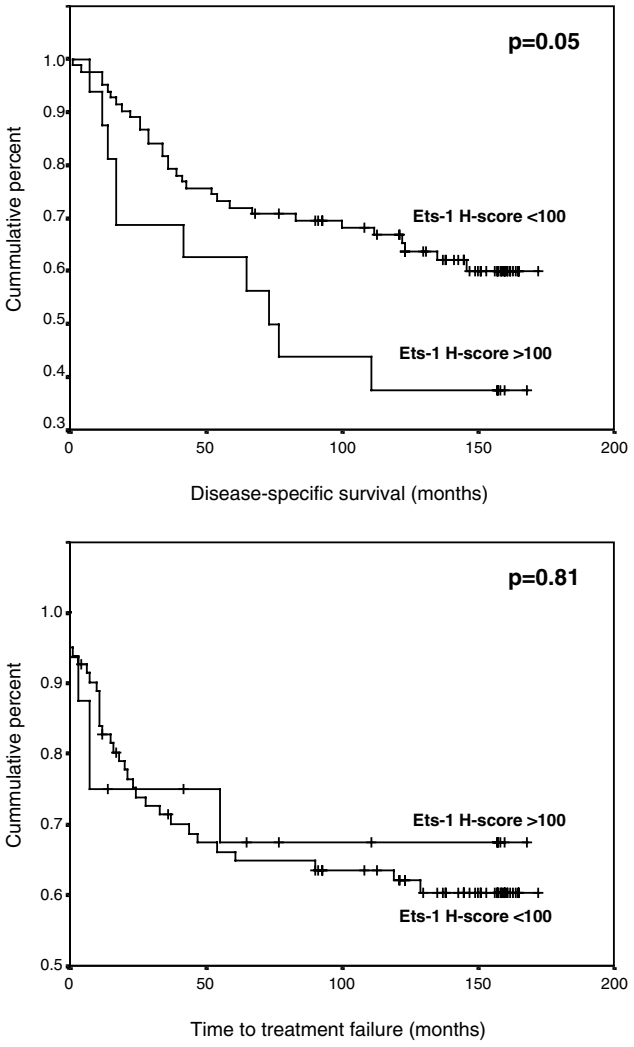

Figure 4 A trend for shorter disease-specific survival was found for the primary melanomas with strong expression of Ets-1.

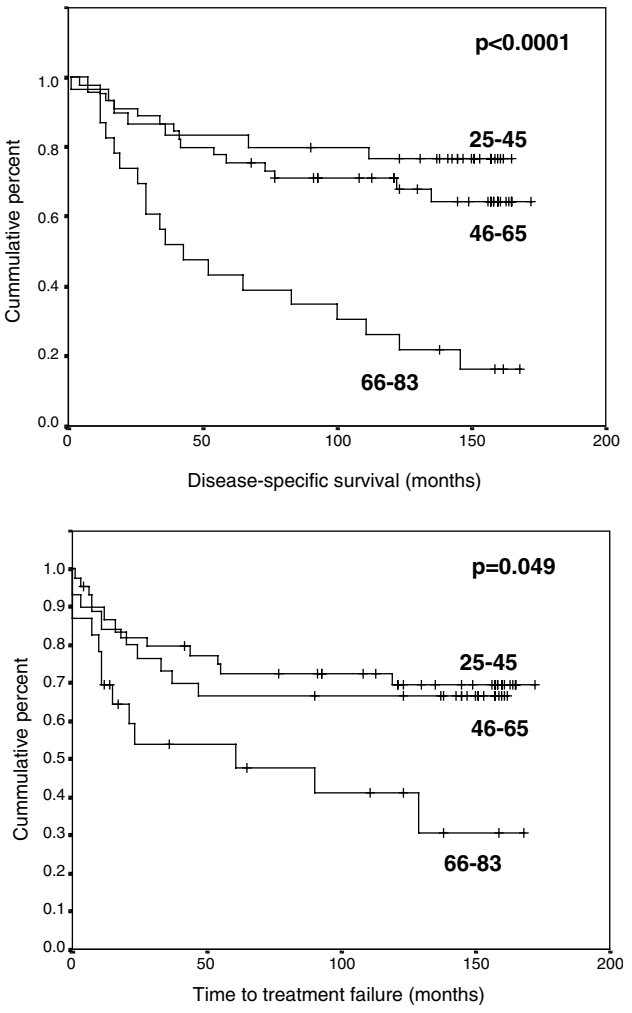

Figure 5 Older age is a strong negative prognostic factor in malignant melanoma.

does not appear to have the same connotation because we found a statistical trend showing that primary melanomas with strong expression of Ets-1 have worse outcome. This finding suggests multifactorial role of Ets-1 in biology of malignant melanoma. It cannot be excluded that Ets-1 still may have an important function in aggressive melanomas by its role in angiogenesis and tumor invasion by regulation of genes encoding for enzymes involved in degradation of the extracellular matrix.[33–35] Our finding that older age is a strong negative prognostic factor in malignant melanoma is also in agreement with previously published studies.[36,37]

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