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High expression of FOXP3 in primary melanoma is associated with tumour progression

A.L. Gerber,[1] A. Münst,[1] C. Schlapbach,[1] M. Shafighi,[2] D. Kiermeir,[2] R. Hüsler[2] and R.E. Hunger[1]

[1]Department of Dermatology and [2]Department of Plastic Surgery, Inselspital, University Hospital Bern, Bern, Switzerland

Correspondence
Robert E. Hunger.
E-mail: robert.hunger@insel.ch

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Conflicts of interest
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Summary

Background The antitumour immune response plays an important role in the prognosis of melanoma. High numbers of circulating regulatory T cells have been associated with rapid disease progression.
Objectives To assess the influence of forkhead box protein (FOXP)3, CD1a and langerin expression on the prognosis of primary melanoma.
Methods We analysed 185 primary melanomas by immunohistochemical staining for expression of the regulatory T-cell marker FOXP3 and the dendritic cell markers langerin and CD1a, and correlated marker expression with clinical outcome.
Results Disease-free survival and overall survival were significantly longer in patients expressing low levels of FOXP3 in the primary melanoma, whereas they were associated with high expression of CD1a. The negative prognostic value of FOXP3 expression was independent of the Breslow tumour thickness. Langerin expression did not correlate with the clinical outcome.
Conclusions High expression of FOXP3 in the primary melanoma may be used as an additional independent prognostic marker for early tumour progression in patients with melanoma.

What's already known about this topic?

- Breslow tumour thickness is the most important prognostic marker for melanoma.
- The immune system, and especially regulatory mechanisms, are important for tumour control and tumour progression.
- The transcription factor forkhead box protein (FOXP)3 is specific for regulatory T cells.

What does this study add?

- High expression of FOXP3 in the primary melanoma is associated with reduced time to disease progression and reduced overall survival, independent of the tumour thickness.
- FOXP3 immunohistochemical staining is easy to perform and may be used to detect patients at high risk for tumour progression.

The best primary-tumour-related prognostic marker for malignant melanoma is the Breslow tumour thickness.[1] Other important prognostic markers are mitotic rate, for thin tumours, and the stage of the sentinel lymph node. As collection of the sentinel lymph node requires surgery and leads to multiple surgery-related complications,[2] one important issue in melanoma research is to define new, more accurate prognostic markers relying only on the analysis of the primary tumour.

The immune system plays a crucial role in controlling cutaneous melanoma. New data show that the induction of a strong immune response in patients with melanoma may improve survival.[3] Interestingly, blocking normally occurring mechanisms responsible for the downregulation of immune responses has been shown to improve melanoma outcome efficiently.[4]

Regulatory T cells (Tregs) are a subpopulation of T cells that have the vital property of downregulating harmful

immune-mediated inflammation. They have been shown to express the surface markers $CD4^{+}CD25^{high}$ and are specified by the expression of the transcription factor forkhead box protein (FOXP)3.[5] Here we report that high expression of FOXP3, as assessed by immunohistochemical staining of primary melanoma, is associated with early progression of disease in malignant melanoma.

Materials and methods

Patients and tissue samples

Archived tissue samples of consecutive melanomas between 2000 and 2006 from patients at the Department of Dermatology, University of Bern, Inselspital, Bern, Switzerland were included in the study. All of the procedures in this study were in accordance with the ethical standards of the Ethical Committee of the Canton of Bern (KEK) on human experimentation, and with the Helsinki Declaration of 1975, as revised in 1983. The patient and melanoma characteristics are summarized in Table 1. All patients with melanoma were followed up at the Department of Dermatology, and the clinical follow-up data were collected in a melanoma database using standard file maker software.

Immunohistochemistry

Immunostaining was performed using the streptavidin–biotin complex/alkaline phosphatase method, as previously described, with the following modified pretreatment procedures for antigen retrieval.[6] The following monoclonal antibodies were used as first-stage reagent: mouse anti-FOXP3 (abcam22510; Abcam, Cambridge, U.K.), mouse anti-CD1a (M3571; DakoCytomation, Glostrup, Denmark) and mouse antilangerin (CD207, clone 310F.02; Dendritics, Dardilly, France). Paraffin-embedded sections 2–3 μm thick were dewaxed and rehydrated, and 10-min pressure cooking was used as pretreatment [Dako Target Retrieval Solution pH 6·0 (DakoCytomation) for CD1a, 10 mmol L^{-1} Tris with 1 mmol L^{-1} ethylenediaminetetraacetic acid buffer pH 9·0 for FOXP3, and 10 mmol L^{-1} citric buffer pH 6·0 for langerin]. They were then incubated with the primary antibody or an isotype-matched control antibody for 1 h at room temperature, followed by treatment with a biotinylated secondary antibody and streptavidin–biotin complex/alkaline phosphatase (K0376; DakoCytomation). All sections were developed in fresh fuchsin naphthol (K0624; DakoCytomation) and counterstained with haematoxylin. Quantitative analysis of marker expression on at least 2 mm^2 of tissue (FOXP3, langerin and CD1a) was performed by two different methods: (i) by counting under a conventional light microscope and (ii) by using the digital image analysis software NIS-Elements BR 2·30 (Nikon, Tokyo, Japan), as previously described.[7]

Table 1 Patient and tumour characteristics

Characteristic	Value
Number of patients analysed	185
Age (years), mean (range)	56·6 (26–87)
Male	53·5%
Female	46·5%
Fitzpatrick skin type[27]	I, 7·0%; II, 66·1%; III, 26·9%; IV–VI, 0%
Number of melanoma tissues analysed	185
Tumour thickness (mm), mean ± SD	1·51 ± 1·78
Tumour type	
Superficial spreading melanoma	59·5%
Nodular melanoma	32·2%
Acral lentiginous melanoma	3·3%
Lentigo maligna melanoma	3·9%
Amelanotic melanoma	1·1%
Ulceration	11·4%

Statistics

Descriptive statistics were used to summarize the demographic and tumour characteristics of the patients with melanoma. To compare the two counting methods (counting under a microscope vs. counting by image analysis), the Pearson correlation coefficient r was computed and the scatter plot of the two counting methods was inspected.

For the survival analysis, Cox proportional hazards models were used to model the influence of FOXP3 and age (in years), sex and skin type (treated as numerical quantities for simplicity) on different survival times (always in days). Specifically, the following survival times were analysed: (i) locoregional metastasis-free survival (LRMFS); (ii) distant metastasis-free survival (DMFS); (iii) overall survival (OS) and (iv) disease-free survival (DFS); i.e. events are death or locoregional or distant metastases. A significance level $\alpha = 0{\cdot}05$ was chosen, and R 2.13.0 was used for computations (www.r-project.org). Exact 95% confidence intervals for the proportion surviving were obtained with Blaker's method, computed with R.

Results

The demographic, clinical and tumour data of all analysed patients with melanoma are listed in Table 1. In total 185 primary melanomas from 185 patients were analysed, and the median follow-up time was almost 8 years (2895 days).

Semiserial tissue sections of all primary melanomas were immunohistochemically stained with antibodies against FOXP3, langerin and CD1a. Marker expression was analysed using an image analysis system as described above. Examples of tissue sections stained with FOXP3-specific antibodies are shown in Figure 1. In a first step, marker expression was assessed by counting positive cells under a dissecting

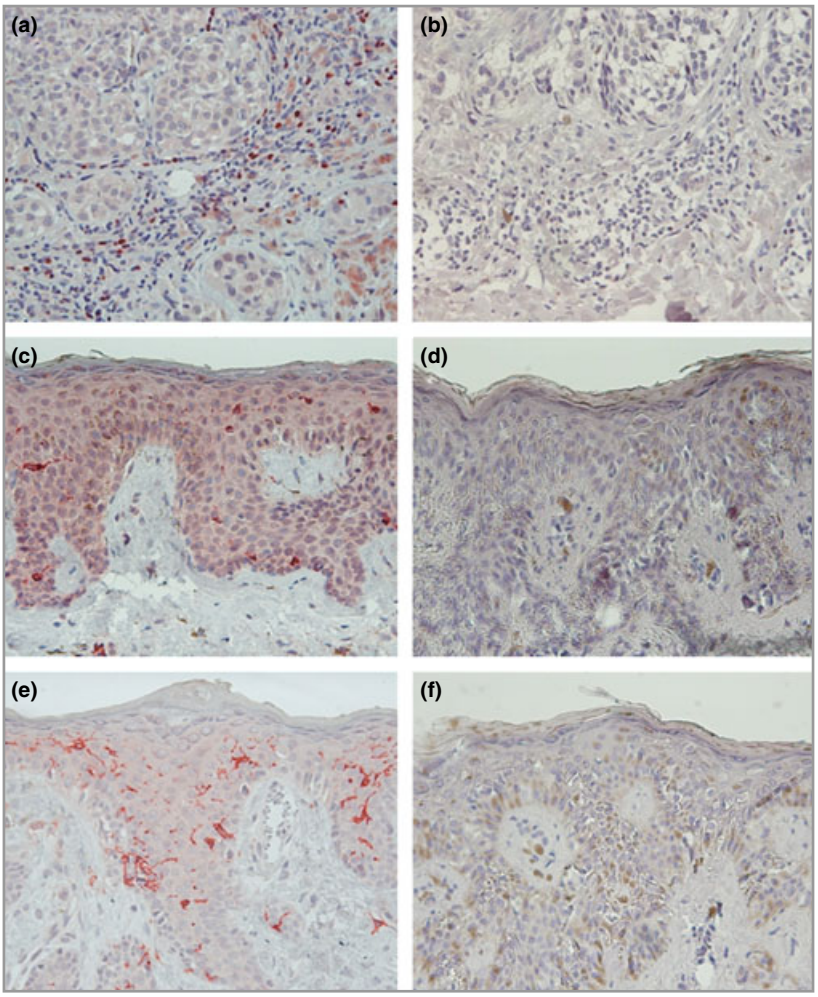

Fig 1. Examples of primary melanoma stained with antibodies specific for forkhead box protein (FOXP)3, langerin and CD1a. Representative samples of immunohistochemically stained tissue sections of primary melanomas for (a) FOXP3, (c) langerin and (e) CD1a, and their corresponding negative controls: (b) FOXP3, (d) langerin and (f) CD1a.

microscope, and in parallel by a digital image analysis system. As shown in Figure 2, the results obtained by the two systems are highly correlated (Pearson r = 0·974). All statistical analyses were carried out with both datasets, but as the results are very similar only the results of the analysis of the computer-counted data are reported in this article.

Previous publications[8–10] postulated a correlation of disease outcome with the distribution pattern of FOXP3. Based on these findings the sections were subdivided into perivascular, diffuse and peritumoral infiltrates, according to the expression pattern of FOXP3. However, there was no statistically significant correlation of these parameters with disease outcome. This may, at least in part, be due to the fact that subdivision of the samples leads to low statistical power of the data.

To visualize the influence of FOXP3, langerin and CD1a expression on the clinical outcome, the patients were assigned to 'high-expression' and 'low-expression' groups, the threshold being the median level of the respective marker expression (patients with values above the median formed the 'high' group and the rest the 'low' group). As shown in Figure 3, patients with low FOXP3 expression in the primary tumour had a significantly better clinical outcome ($P < 0·05$), as demonstrated by DFS and time to death (OS). Although DFS and

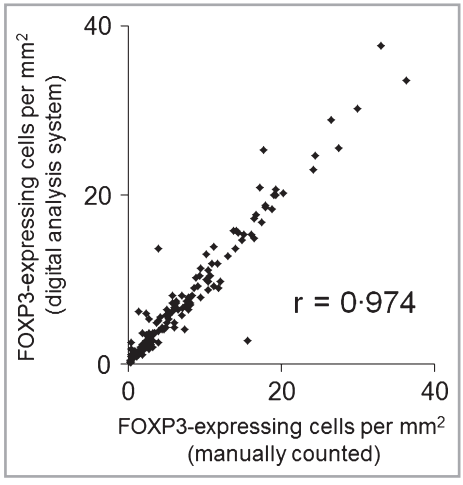

Fig 2. Forkhead box protein (FOXP)3-expressing cells per mm^2 tissue section, assessed using a digital analysis system (*y*-axis) compared with conventional counting under a light microscope (*x*-axis). *r* indicates the Pearson correlation coefficient.

time to death were better in the CD1a high-expression group, the differences were not statistically significant and the level of langerin expression could not be shown to have an

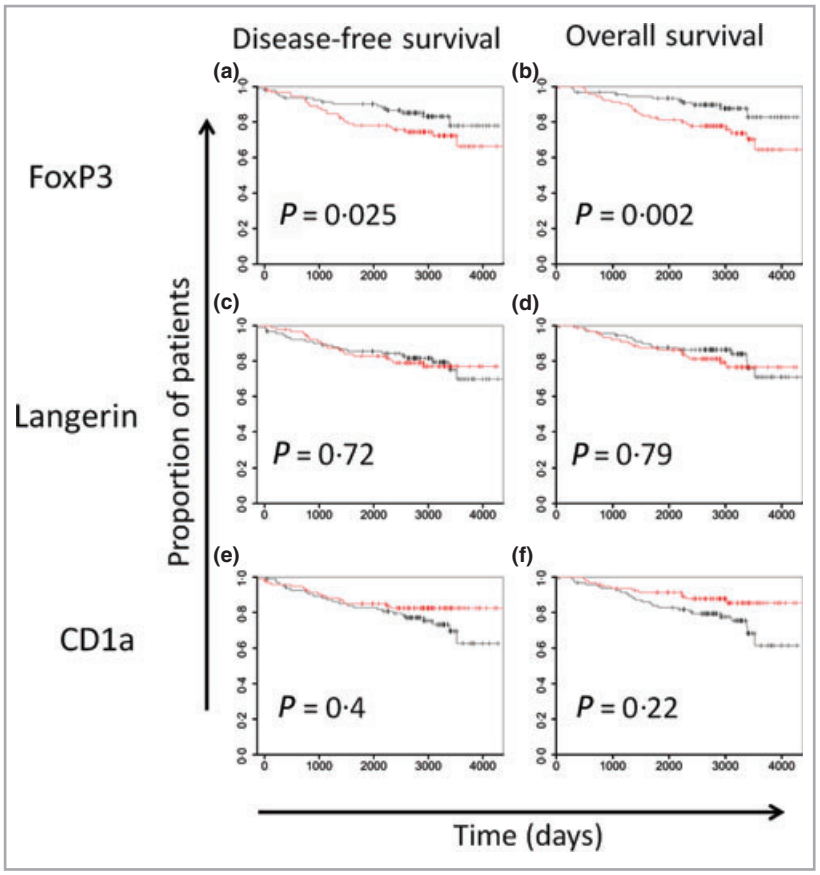

Fig 3. The clinical outcome is significantly better for patients with melanoma with low expression of forkhead box protein (FOXP)3 in the primary tumour. Kaplan–Meier curves for disease-free (a, c, e) and overall survival (time to death) (b, d, f) are shown. The patients are assigned to two groups, the threshold being the median of the expression level of the respective markers (i.e. FOXP3, langerin or CD1a). Patients with values above the median form the 'high' group (red line) and the rest the 'low' group (black line).

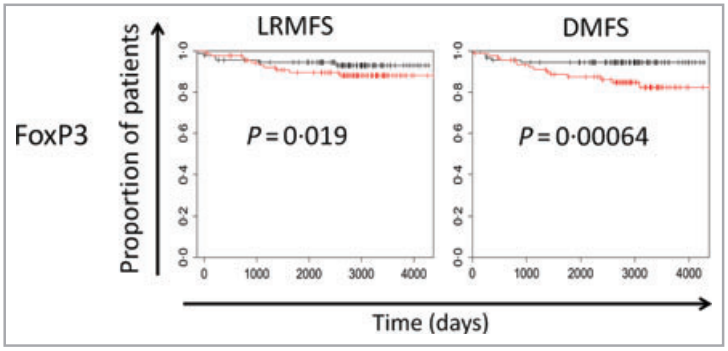

Fig 4. (a) Locoregional metastasis-free survival (LRMFS) and (b) distant metastasis-free survival (DMFS) are significantly better for patients with melanoma with low expression of forkhead box protein (FOXP)3 in the primary tumour, as demonstrated by Kaplan–Meier curves. The patients are assigned to two groups, the threshold being the median of the FOXP3 expression level. Patients with values above the median form the 'high' group (red line) and the rest the 'low' group (black line).

influence on either clinical parameter. In addition, Figure 4 shows also that LRMFS and DMFS were both statistically significantly longer in patients with low expression of FOXP3. As a control we analysed the survival data with the two most important prognostic factors for clinical outcome, i.e. Breslow tumour thickness and ulceration. As shown in Figure 5, in our high-expression patient cohort tumour thickness and ulceration were also important prognostic factors, as expected.[11] However, there was no correlation of FOXP3 expression with Breslow tumour thickness (Fig. 6a), and, as shown in Figure 6b, the differences in 5-year survival rate were comparable for FOXP3 high- and low-expression tumours.

As the FOXP3 dataset showed the best correlation with the clinical outcome, we performed additional statistical analyses. Table 2 shows the estimated coefficients from the Cox proportional hazards model for four parameters of tumour progression: LRMFS, DMFS, OS and DFS (P-values in parentheses).

In the models for DMFS, OS and DFS, FOXP3 had a significant and positive coefficient, showing that FOXP3 expression

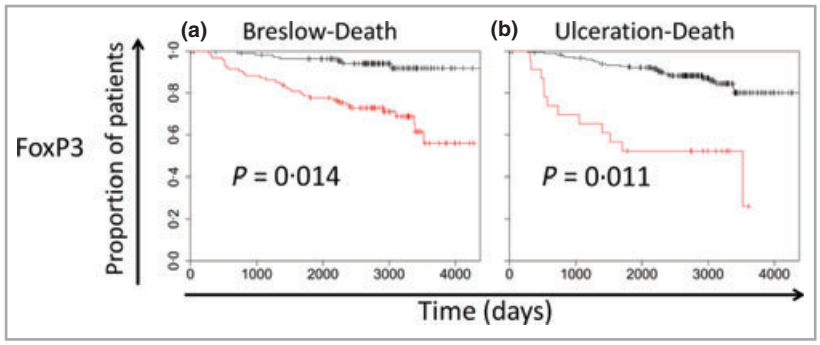

Fig 5. Breslow tumour thickness and ulceration of the primary tumour are important prognostic parameters in the study population. (a) Kaplan–Meier curves for melanoma-specific survival. The patients are assigned to two groups, the threshold being the median of the tumour thickness. Patients with a tumour thickness above the median form the 'high' group (red line) and the rest the 'low' group (black line). (b) Kaplan–Meier curves for overall survival for patients with (red line) and without (black line) ulceration of the primary melanoma.

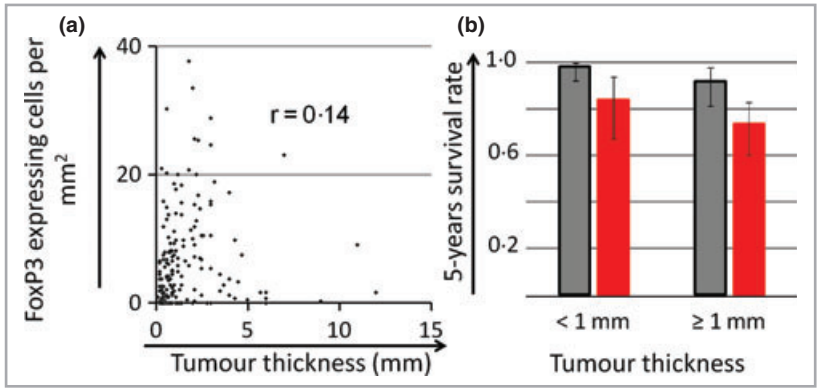

Fig 6. Forkhead box protein (FOXP)3 expression is not dependent on Breslow tumour thickness. (a) FOXP3 expression per mm^2 tissue section is plotted against tumour thickness. r indicates the Pearson correlation coefficient. (b) The 5-year survival rate is shown for patients with melanoma with high (red bars) and low (black bars) expression of FOXP3 in the primary melanoma. The patients are assigned to two groups, the threshold being the median of the level of FOXP3 expression. Error bars indicate 95% exact confidence intervals.

Table 2 Coefficients of the four survival analyses for forkhead box protein (FOXP)3

Variable	Locoregional metastasis-free survival	Distant metastasis-free survival	Overall survival	Disease-free survival
FOXP3	0·0576 (0·019)	0·0784 (0·00064)	0·05577 (0·002)	0·0402 (0·025)
Age	0·0169 (0·360)	0·0138 (0·470)	0·06464 (7·5e−5)	0·0512 (0·00021)
Sex	−0·2174 (0·680)	−0·3932 (0·460)	−0·70461 (0·083)	−0·5357 (0·140)
Skin type	−0·5453 (0·300)	0·0261 (0·960)	0·00498 (0·99)	−0·1155 (0·740)
LR test *P*-value	0·137	0·0238	4·81e−6	0·000173

Estimated coefficients from the Cox proportional hazards model, using the computer-counted FOXP3 marker value (in cell counts per mm^2), age (in years), sex (1 for women, 0 for men), and skin type (1–6, as a numerical quantity) to model four survival times separately (*P*-values in parentheses). LR, likelihood ratio.

is associated with a higher hazard ratio and therefore with a lower survival time. The model for LRMFS is not significantly better than a model that contains only a constant, and should thus be interpreted with caution. Note that age has a positive and significant coefficient when analysing OS or DFS, but no significant age effect can be found for DMFS.

The finding that high expression of FOXP3 in primary melanoma is associated with a shorter DFS and time to death, independent of the tumour thickness, whereas high CD1a expression is associated with a better prognosis, supports the notion of an important role of the host immune surveillance in melanoma control.

Discussion

Tumour growth is a complex process that involves tumour–host interactions through multiple cellular and molecular factors in the tumour microenvironment. In these interactions,

the immune system is a double-edged sword. On the one hand, immune cells have been reported to control the malignant cells; on the other hand they may also exert tumour-promoting effects. $CD8^+$ T cells recognizing melanoma-specific antigens have been described for some time, and transfer of *in vitro* expanded $CD8^+$ T cells has been shown to exert therapeutic effects in patients with melanoma.[12] It has been reported that the infiltration of different human cancers, e.g. ovarian[13] and colorectal,[14] with $CD8^+$ T cells is associated with a favourable prognosis. Furthermore, natural killer cells, dendritic cells and macrophages have also been reported as independent good prognostic indicators in different human cancers.[10] Conversely, high numbers of neutrophils and plasma cells in melanomas are associated with a poor prognosis,[15] and a subclass of antibodies (IgG4) has been reported to impair antimelanoma immune response.[16]

In this context it is also interesting to note that malignant tumours have been shown to create an immunosuppressive microenvironment to escape immune surveillance and promote tumour development.[17,18] $CD4^+CD25^{high}$ Tregs, which express the Treg-specific transcription factor FOXP3, participate in antitumour immune response by dampening the T-cell-mediated immune response against the tumour cells. It has been reported that high numbers of circulating Tregs are associated with rapid tumour progression in experimental animal models of melanoma[19] and in patients with melanoma.[20] In patients with melanoma, the presence of FOXP3-positive cells in the primary tumour has also been associated with a higher frequency of metastases in the sentinel lymph node.[21] Our finding that tumour progression is statistically significantly accelerated in patients with a high proportion of FOXP3-expressing cells in the primary melanoma demonstrates that local immunosuppressive mechanisms are crucial already in the early stages of disease progression.

FOXP3 is expressed mainly by $CD4^+CD25^{high}$ cells, the so-called Tregs. However, other T-cell populations have also been described as expressing FOXP3 to a certain extent.[22] Therefore, we cannot exclude that some of the FOXP3 cells are activated T cells and not Tregs. As in routine immunohistochemical analysis it is not feasible to perform double or even triple staining with CD25 and CD4, and as the expected frequency of non-Tregs expressing FOXP3 is very low, we focused our study on single staining for FOXP3. Analysing the morphology of the FOXP3-expressing cells, we could not find any evidence that FOXP3 is expressed by melanoma cells.

DMFS and LRMFS were both significantly better in patients with low FOXP3 expression in the primary melanoma. Interestingly, FOXP3 expression correlated more closely with DMFS than with LRMFS, as shown in Figure 4. These results further suggest that survival is more influenced by distant than by locoregional metastases.[23] The finding that FOXP3 expression correlates better with DMFS than with LRMFS may further indicate that suppression of the immune response is especially important during the formation of distant metastases. Nevertheless, the coefficients of both survival analyses showed only slightly different values (0·0784 vs. 0·0576; Table 2).

Our findings support the notion that immunosuppression is a key process in melanoma progression. Interestingly, the influence on the clinical outcome of CD1a or langerin expression in the primary melanoma (both molecules are important in mounting an immune response) was not statistically significant. In a recent paper it was demonstrated that subsets of human skin-resident dendritic cells may suppress skin inflammation by inducing Tregs through production of interleukin-10.[24] This indicates that it may be necessary to assess different dendritic cell subpopulations, i.e. immunosuppressive vs. immunostimulating, to find a correlation with the clinical outcome.

Recently, multiple articles[10,25] have been published on the prognostic and predictive value of immune infiltrates in cancer, and data collected from large cohorts of human cancer have demonstrated that the immune classification of malignant tumours adds additional information to the currently used tumour staging method (American Joint Committee on Cancer/Union for International Cancer Control tumour thickness, nodes, metastasis classification).[26] FOXP3 is a further candidate that may increase outcome predictions in melanoma.

Taken together, our data further confirm the notion that immunosuppressive mechanisms are important factors for rapid tumour progression. Furthermore, the detection of high numbers of FOXP3-expressing cells may be used as a marker for rapid tumour progression. Further studies are needed to assess whether this marker may be helpful for routine clinical use.

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