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Immunohistochemical expression of C-*myc* oncogene, heat shock protein 70 and HLA-DR molecules in malignant cutaneous melanoma

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Abstract The clinical course of malignant melanomas is frequently unpredictable, although a number of prognostically useful variables can be identified. There is a need for additional markers of prognostic value. In a series of 60 malignant cutaneous melanomas, we analysed the immunohistochemical expression of c-*myc* proto-oncogene, heat shock protein 70 (HSP70) and HLA-DR molecules in order to investigate their prognostic significance. C-*myc*, HSP70 and HLA-DR were expressed in 43.3%, 56.6% and 38.3% of all melanoma cases, respectively. Advanced Clark levels (Clark III–V) were significantly associated with c-*myc* expression rate ($P<0.05$), HSP70 detection ($P<0.01$) and HLA-DR positivity ($P<0.01$). Increased Breslow thickness (>1.5 mm) was related to HLA-DR expression ($P<0.05$). High mitotic rate was closely associated with c-*myc* positivity ($P<0.05$), while HSP70 and HLA-DR expression separately correlated to clinical stage of the disease ($P<0.05$). The evaluation of these variables may be of immunological and prognostic significance. They were found to be associated with melanocyte subpopulations of the vertical growth phase which are arguably characterized by an increased invasive potential.

Key words C-*myc* · Heat shock protein 70 · HLA-DR · Malignant melanoma

Introduction

The behaviour of cutaneous malignant melanoma seems to be unpredictable. Remarkable progress has been made in investigating the complexity of cellular immunity and in uncovering the mechanisms through which melanoma cells evade immunological reactions. Additionally, melanoma progression is accompanied by an increase in genetic instability [27].

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Several publications reported an activation of oncogenes in malignant melanoma [1, 18, 35]. C-*myc* proto-oncogene is mapped on chromosome 8 and various abnormalities concerning its activated expression accompany the progression of many malignancies [20, 33]. Recent data support the suggestion that c-*myc* oncoprotein influences the presence of determinants of immunological importance on melanoma cells [34].

Heat shock proteins (HSP) are produced after cell exposure to a variety of environmental and pathophysiological stressful conditions [5, 30]. The group of the 70-kDa family (HSP70) mainly seems to protect all proteins from the damage caused by various stressful stimuli and is of particular interest, as those proteins may be implicated in the antigen presentation mechanism of the immune anti-tumour response by their association with antigenic peptides derived from cellular proteins [12, 15, 21, 40, 43]. It has been postulated that HSP70 are involved in protein-oncogene interactions including these of the protein products of the human c-*myc* oncogene and the p53 tumour suppressor gene. Furthermore, elevated HSP70 expression may be an indicator of biological stress experienced by tumour cells in some carcinomas and may also predict patients' clinical outcome [12].

It is well known that the production of a cell mediated local specific immune response to a malignant tumour is directly regulated by the major histocompatibility complex (MHC) class II (HLA-DR, -DP and -DQ) [26]. MHC class II molecules are immunoregulatory glycoproteins expressed on the surface of lymphocytes, macrophages and some endothelial cells, where they have a critical role in antigen recognition and presentation [7]. The MHC class II molecules have also been demonstrated on various malignant tumours, including malignant melanoma [10]. Little evidence, however, exists for a prognostic significance [2, 22].

We studied the immunohistochemical expression of these markers in a series of melanomas and analysed their relationship to clinicopathological variables of known prognostic significance.

Materials and methods

Our study comprised 60 cutaneous malignant melanomas. The mean age of patients was 40,56 years (range: 25–73 years, median age: 45 years) and the male/female ratio was 39/21 (1.85). All cases were examined histologically in the Department of Pathology of Athens University during the years 1988 to 1993. All malignant melanomas were classified according to sex, age, anatomical location, clinicopathological type (18 nodular, 37 superficial spreading, 2 lentigo maligna melanomas, 3 acral lentiginous melanomas), tumour cell type (24 epithelioid, 19 spindle, 10 mixed, 7 other types), growth phase pattern [18 without radial growth phase, 42 with radial growth phase), mitotic rate according to McGovern [mean percentage of mitoses of melanoma cells counted on high-power fields; either 10 at least in large melanomas or 5 throughout the tumour in small melanomas (absent in 5, low in 26 and high mitotic activity in 19 of our cases)] [28] and Clark levels of invasion (6 in Clark I, 15 in Clark II, 19 in Clark III, 12 in Clark IV, 8 in Clark V level of invasion) [13]. We used polarized light to distinguish Clark IV from Clark III levels.

All neoplasms were classified according to Breslow tumour thickness (18 cases of Breslow <0.75 mm, 15 of 0.75–1.5 mm, 18 of 1.6–4 mm, and 9 of >4 mm) [8], melanin pigmentation presence, presence and degree of host inflammatory response [along the dermis-epidermis junction in in situ (Clark I) or microinvasive (Clark II) cases and within tumour stroma (tumour infiltrating lymphocytes) in cases with vertical growth phase (Clark III, IV and V)] [17, 30], presence of partial or complete radial growth phase immunologic regression [14], presence of ulceration (present in 15 cases), presence of microscopic satellites [17] (loco-regional metastases were observed in 19 cases), clinical disease stage [16] [34 in stage I (disease confined to the local site), 19 in stage II (regional nodes invaded), 7 in stage III (distant metastases presence)].

A three-step immunoperoxidase staining technique was used on paraffin-embedded, 4 μm-thick tissue sections from primary lesions. After deparaffinization through graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 0.1% hydrogen peroxide in methanol for 20 min. Immunostaining was performed using the avidin-biotinylated horseradish peroxidase (ABC-HRP) method (Dako, Denmark). As primary antibodies we used the monoclonal mouse IgG antibody to c-*myc* oncoprotein (Ab-1, Oncogene Science) at a dilution of 1/100, the polyclonal rabbit anti-HSP70 Ab (A500, Dako) at a dilution 1/300 and the monoclonal mouse anti-human HLA-DR alpha-chains Ab (TAL.1B5, M746 Dako) at a dilution of 1/100 with an overnight incubation. Aminoethylcarbazole was used as chromogen counterstained with Mayer's haematoxylin. Tumour sections subjected to the whole procedure except for incubation with the primary antibody were used as "substitute" controls. Skin tissue sections from areas not particularly exposed to ultraviolet solar radiation were used as negative controls for all markers. Such samples were chosen as it is known that UVc (220–280 nm) rays are responsible for many genetic and biochemical alterations and may act as stress factors influencing the markers expression in our cases [39]. Several sections of totally benign melanocytic lesions such as intradermal naevi (n=10) were also tested for all markers but no positive immunostaining was observed. Previously c-*myc* positive breast cancer tissue sections and human tonsil sections were used as positive controls for c-*myc* and HLA-DR respectively, whereas sections of skin suffering from severe radiodermatitis were used as positive controls for HSP 70 detection.

All immunostained slides were analysed and scored in a blinded fashion by two different observers without knowledge of clinicopathological data. In each section at least ten high power fields (×400) were examined under light microscopy and mean percentages of c-*myc*, HSP70 and HLA-DR positive melanoma cells among the total of malignant cells were separately calculated. Immunostaining in stromal cells and adjacent epidermis was also taken into account. As in several of our reference studies, cases with c-*myc* or HLA-DR malignant cell percentages lower than 5% were considered as negative. Cases with HSP70 immunopositive melanoma cells lower than 10% were evaluated as HSP70 negative. Each marker was independently studied in all cases. Positive immunostaining of all markers was easily estimated as melanin presence was generally reduced in all selected cases. Therefore, section bleaching was not necessary.

Statistical analysis was undertaken using Student's t-test and chi-squared test for association (Yates' correction factor), as well as using the correlation coefficient (r). All the results were considered at the 5% level of statistical significance.

Results

C-myc immunohistochemical expression

C-*myc* oncoprotein was expressed in the melanoma cell cytoplasm of 26 cases (43.3%) (Fig. 1). The percentage of c-*myc* positive tumour cells ranged from 5% to 80% [mean±SEM ($\bar{x}$±SEM): 35.6%±4.6%, 95% confidence interval (95% CI): 26.4%–44.7%]. C-*myc* positivity positively correlated at a statistically significant level with late Clark level of invasion (P<0.05), increased mitotic rate (P<0.05), vertical growth phase (P<0.05) and HSP70 positivity incidence (P<0.01) (Table 1). Furthermore, in the 20 cases where c-*myc* and HSP70 simultaneous expression was observed, c-*myc* positivity rates significantly correlated with HSP70 positivity presence (r≅0.8) (Fig. 2) and both markers were topographically distributed in the same parts of each neoplasm. In 8 out of the 37 superficial spreading melanomas, adjacent epidermis demonstrated focal c-*myc* positivity which, was focused on normal melanocytes as well as keratinocytes but was of a comparatively weaker staining intensity than that of the tumour cells.

HSP70 immunohistochemical expression

Thirty-four (56.6%) out of the 60 melanomas tested positive for HSP70 immunohistochemically. The percentage of HSP70 positive melanoma cells ranged from 40% to

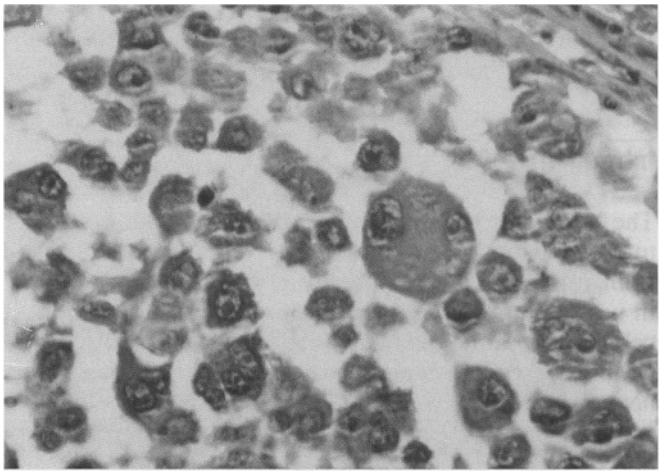

Fig. 1 C-*myc* cytoplasmic positivity in giant melanoma cells (ABC-HRP, ×400)

Table 1 Relation between c-*myc* expression and the variables examined

Variables	No of cases	C-*myc* positive cases	C-*myc* negative cases	*P*-value concerning c-*myc* expression
Sex				
Male	39	15	24	$P>0.10$
Female	21	11	10	
Clark level				
I	6	1	5	$P<0.05$
II	15	3	12	
III	19	9	10	
IV	12	6	6	
V	8	7	1	
Breslow thickness (in mm)				
<0.75	18	8	10	$P>0.10$
0.75–1.5	15	6	9	
1.6–4	18	7	11	
>4	9	5	4	
Mitotic rate				
Absent	15	3	12	$P<0.05$
Low	26	10	16	
High	19	13	6	
Clinicopathological classification				
Without radial growth phase	18	12	6	$P<0.05$
With radial growth phase	42	14	28	
Clinical stage				
I	34	13	21	$P>0.10$
II	19	9	10	
III	7	4	3	
HSP70 expression				
Positive	34	20	14	$P<0.01$
Negative	26	6	20	
HLA-DR expression				
Positive	23	13	10	$P>0.10$
Negative	37	13	24	

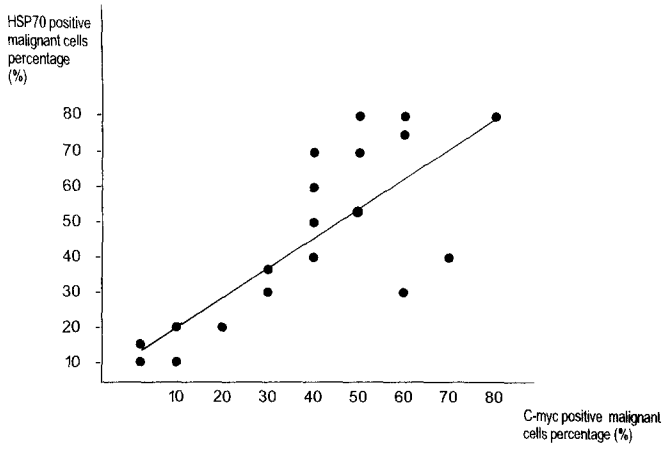

Fig. 2 Statistical correlation between c-*myc* positivity and HSP70 presence ($r \cong 0.8$)

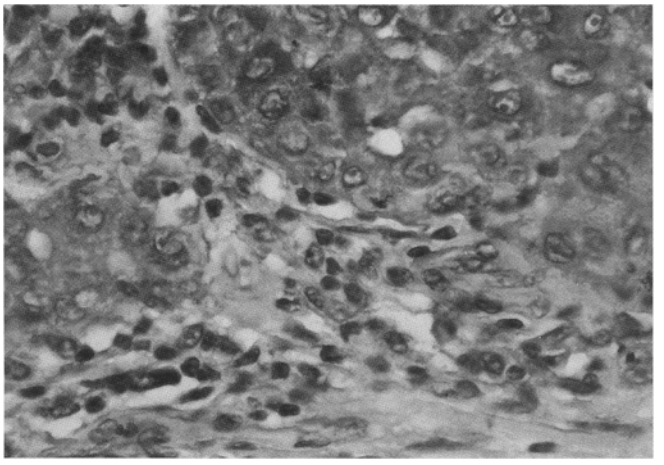

Fig. 3 Focal HSP70 positive expression in malignant melanocytes (ABC-HRP, ×300)

85% ($\bar{x}$±SEM: 45.4%±4.75%, 95% CI: 35.9%–54.9%). Cytoplasmic immunostaining was demonstrated apart from tumour cells (Fig. 3) in activated lymphocytes and stromal macrophages. In 10 of the 34 positive cases a decreased expression was noted in adjacent keratinocytes and not morphologically involved melanocytes of the surrounding normal epidermis. Giant cell subpopulations, when noticed, demonstrated stronger HSP70 staining intensity. HSP70 positivity was significantly associated with advanced Clark level of invasion ($P<0.01$) and late clinical stage ($P<0.05$) (Table 2).

HLA-DR immunohistochemical expression

HLA-DR positive expression on tumour cells was detected in 23 (38.3%) of the examined melanomas among which the 5 cases with complete immunological regression were included and the percentage of HLA-DR positive malignant cells ranged from 5% to 30%. HLA-DR immunohistochemical expression was cytoplasmic with focal peripheral intensification (Fig. 4) and was observed apart from tumour cells, on membranes of inflammatory and stromal cells (lymphocytes, macrophages) (Fig. 4) as well as in the surrounding epidermis of 7 HLA-DR(+) cases. HLA-DR positivity or negativity of each case was considered according to melanoma cells HLA-DR expression. However, in 10 out of the 37 HLA-DR "negative" cases, DR immunopositivity was detected only on lymphocytes and stromal histiocytes, either melanophages or not. HLA-DR expression on malignant cells significantly correlated with advanced Clark level ($P<0.01$), increased Breslow thickness ($P<0.05$), late clinical stage ($P<0.05$) and suggestively with absence of radial growth phase (nodular histological type) ($0.10>P>0.05$) (Fig. 6) (Table 3).

Finally, let us point out that all markers were associated with adverse prognostic determinators. In our 42 cases with radial growth phase, immunopositivity for all three markers was not homogeneously distributed

Table 2 Relation between HSP70 expression and the variables examined

Variables	No of cases	HSP70 positive cases	HSP70 negative cases	*P*-value concerning HSP70 expression
Sex				
Male	39	22	17	$P>0.10$
Female	21	12	9	
Clark level				
I	6	1	5	$P<0.01$
II	15	4	11	
III	19	12	7	
IV	12	10	2	
V	8	7	1	
Breslow thickness (in mm)				
<0.75	18	9	9	$P>0.10$
0.75–1.5	15	8	7	
1.6–4	18	10	8	
>4	9	7	2	
Mitotic rate				
Absent	15	10	5	$P>0.10$
Low	26	15	11	
High	19	9	10	
Clinicopathological classification				
Without radial growth phase	18	11	7	$P>0.10$
With radial growth phase	42	23	19	
Clinical stage				
I	34	14	20	$P<0.05$
II	19	14	5	
III	7	6	1	
HLA-DR expression				
Positive	23	16	7	$P>0.10$
Negative	37	18	19	

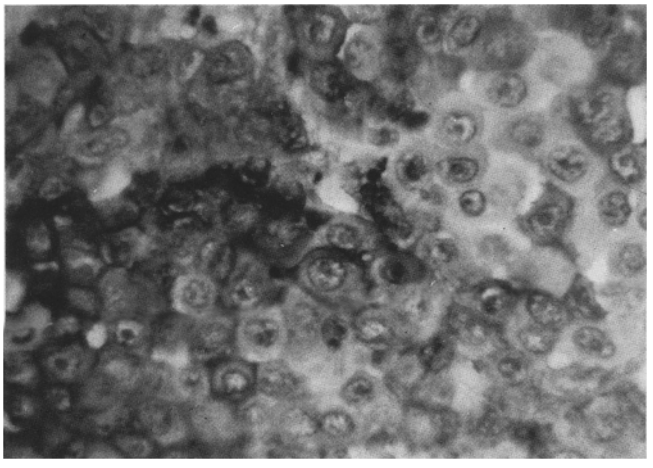

Fig. 4 HLA-DR membrane and cytoplasmic positivity in several epithelioid melanoma cells (ABC-HRP, ×300)

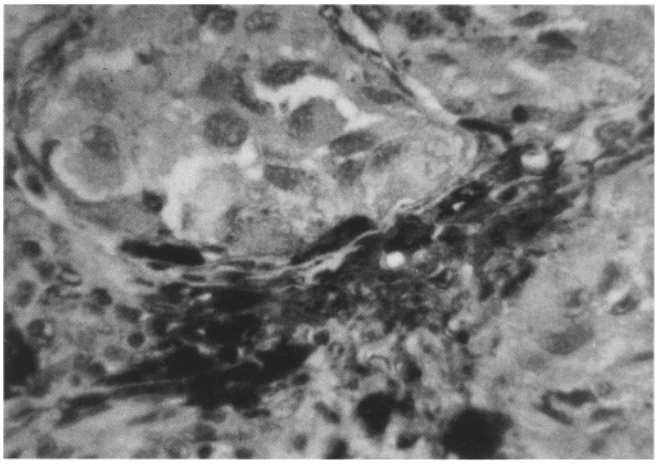

Fig. 5 HLA-DR positive expression in stromal cells of a malignant melanoma (ABC-HRP, ×300)

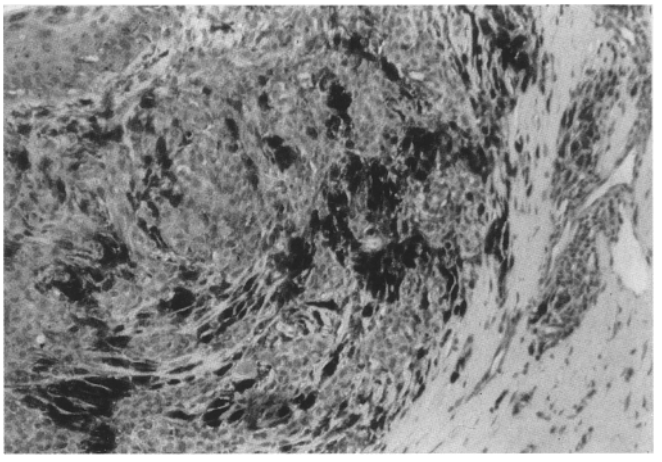

Fig. 6 HLA-DR expression in a nodular malignant melanoma (ABC-HRP, ×150)

throughout each positive lesion but was more or less associated with vertical growth phase melanoma cells when such a phase existed. In general, radial growth phase cells were only sporadically positive.

Discussion

An immunohistochemical approach preserves morphological information and allows the detection of antigenic molecules on malignant or on stromal and inflammatory components of the lesion. As far as malignant melanocytes are concerned, the selective localization of positive staining in distinct subclones of malignant cells is of significance, as neoplastic cells of radial growth phase melanomas may differ antigenically from vertical growth phase melanocytes which probably form the metastatic cellular compartment of the tumour [17, 32].

Irreversible genetic alterations such as oncogene activation by point mutations or gene amplification, are the initiating stages of human carcinogenesis [36]. In the c-

Table 3 Relation between HLA-DR expression and the variables examined

Variables	No of cases	HLA-DR positive cases	HLA-DR negative cases	*P*-value concerning HLA-DR expression
Sex				
Male	39	16	23	$P>0.10$
Female	21	7	14	
Clark level				
I	6	2	4	$P<0.01$
II	15	2	13	
III	19	5	14	
IV	12	8	4	
V	8	6	2	
Breslow thickness (in mm)				
<0.75	18	3	15	$P<0.05$
0.75–1.5	15	4	11	
1.6–4	18	10	8	
>4	9	6	3	
Mitotic rate				
Absent	15	5	10	$P>0.10$
Low	26	9	17	
High	19	9	10	
Clinicopathological classification				
Without radial growth phase	18	10	8	$P>0.05$
With radial growth phase	42	13	29	
Clinical stage				
I	34	8	26	$P<0.05$
II	19	10	9	
III	7	5	2	

myc positive cases of our study the overactivation of this proto-oncogene is obviously implicated in the malignant transformation of epidermal melanocytes. However, when local invasive activity transverses the epidermal basement membrane, c-*myc* positivity tended to be a feature of the typical vertical ("tumourigenic") compartment of the lesion. This finding is in keeping with the fact that a much higher incidence of chromosomal abnormalities is apparent in vertical growth phase melanoma cells [3]. The quantitatively increased c-*myc* expression characterized the typical vertical growth pattern in which solid clusters of cells are filling papillary dermis (Clark III) or infiltrate the reticular dermis (Clark IV) or subcutaneous fat (Clark V). In microinvasive cases in which poorly circumscribed, "non tumourigenic", lesion cells are scattered in papillary dermis without filling it (Clark II) or in those with melanocytes restricted to the epidermis (Clark I), the percentage of c-*myc* positive cells was found to be significantly lower.

The nodular melanomas of our survey, expressed c-*myc* oncogene significantly more frequently. It has been postulated that nodular melanomas differ histogenetically and morphologically from other types [29]. However, taking into account that Clark levels of invasion define a stepwise progression of biological properties associated with malignant behaviour of melanoma cells, one could argue that c-*myc* expression is generally associated with vertical growth phase melanomas, irrespective of histological type. Considering the statistical correlation of c-*myc* with high mitotic activity, we may assume that the activation of this protooncogene is related to increased biological agressiveness; a thought consistent with the high c-*myc* presence in melanocytes with high metastatic potential [4].

Previous data support the suggestion that c-*myc* protein stimulates the expression of HSP70 by transacting on the HSP70 promotor [42]. Moreover, the presence of both of our immunological markers (HSP70, HLA-DR) in malignant and inflammatory as well as other stromal cells possibly indicates some interactions between different cell populations. As HSP70 expression correlated with tumour progression we may speculate that HSP70 cell expression is connected with possible protection of malignant cells against host cytotoxic reactions [see 23, 24]. It has also been hypothesized that HSP70 acts as a "coverage" for surface membrane antigens [19], among which some tumour-associated antigens may be included.

Our finding that HSP70 was expressed in several lymphocytic elements is in keeping with data indicating that the 72/74 peptide binding protein, which is related to HSP70, can be detected on the surfaces of immunoglobulin positive B-cells [43]. In addition, this observation may be supported by the remarkable similarity in the aminoacid sequence between HSP70 and grp-78 (glucose-regulated protein-78) which binds to the immunoglobulin heavy chains [45]. It has also been reported that immunoglobulins can be sporadically expressed on melanocytes as a result of endocytosis.

The higher proportion of HSP70 positive cases in advanced clinical stages ($P<0.05$) may be the consequence of the effect of the aggressive neoplastic microenvironment on HSP70 expression on melanoma cells. Local hypoxaemic conditions, acting as stress factors, potentially induce the biochemical alterations which lead to the elevated production of HSP70.

A fundamental finding in our study was the intimate association of malignant melanocytes DR positive expression (Fig. 4) with adverse prognostic indicators. The latter is reinforced by recent evidence suggesting that HLA-DR expression on melanoma cells is not a favourable prognostic sign [9, 38]. It has been argued that HLA-DR positive malignant melanocytes suppress the immune response via the occupancy of the T-cell receptors (TCR) of activated T-cells by immunogenic epitopes which are presented in association with HLA-DR molecules by non-professional antigen presenting cells (melanocytes and not macrophages) [10, 11]. So, in the case of DR positive melanoma cells, the unresponsiveness and subsequent anergy of Th1 cells caused by their TCR ineffectual occupancy, seems to be responsible for the relation of DR positive expression to melanoma cell aggressive behaviour [25]. In our 5 cases with complete ra-

dial growth phase regression, almost all of the very few remaining melanocytes were DR positive. This reinforces the possibility that, in general, regression plays a permissive role in the development of the metastatic subclone by obliterating radial growth phase cells [17], while allowing the growth of vertical growth phase cells which are not efficiently recognized by the host response, probably due to their DR positivity.

Nevertheless, the recognition of tumour elements, when presented as exogeneous antigens by professional, DR(+), antigen presenting cells to Th1 lymphocytes, induces the cellular immune response by promoting the development of T cytolytic CD8(+) cells. Th1 activated lymphocytes also secrete interleukins 2 and 3, tumour necrosis factor α (TNF-α), lymphotoxin (TNF-β) and predominantly gamma interferon (IFN-γ) which stimulates the expression of Fc receptors on macrophages, thus enhancing antibody mediated cellular cytotoxicity as well [37]. So, DR expression, when noted on tumour infiltrating inflammatory components (Fig. 5) and generally on professional antigen presenting cells such as macrophages, is likely to have a different, favourable effect on tumour immunology. This favourable effect, however, might be ultimately overcome by malignant melanocytes' DR expression.

Researchers have recently focused on the development of specific immunotherapeutic approaches. Previous observations have suggested novel modes of therapeutic interventions by using the peptide-binding property of HSP70 for specific vaccination against cancer [6, 44]. Interleukin-2 treatment has been proved to be of special significance in melanoma therapy [25]. Since in DR-positive cases, IL-2 production by activated Th-cells is probably hindered, melanoma cells DR positive expression status may be of value in distinguishing those melanomas with a particular need for such kind of immunotherapy.

The identification of markers of immunological importance in combination with the study of oncogenes is promising in the understanding of this tumour's complicated pathobiology and clinical evolution, especially when these markers are associated with a vertical cellular compartment which is more likely to metastasize.

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