Expression of the Apoptosis Inhibitor Protease Inhibitor 9 Predicts Clinical Outcome in Vaccinated Patients with Stage III and IV Melanoma

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Abstract

Purpose: There have been reports of successful treatment of metastatic melanoma patients with active specific immunotherapy (ASI) using irradiated autologous tumor cell vaccination. It is still unknown why some patients respond and others do not. Tumor cells can evade the immune system, for example through interference with antigen presentation by down-regulation of MHC molecules or expressing proteins interfering with cytotoxic lymphocyte—induced apoptosis like the granzyme B antagonist protease inhibitor 9 (PI-9).

Experimental Design: PI-9 expression was detected in melanoma cell lines. To investigate if PI-9 is important in the response to ASI, paraffin-embedded tissues from stage III or IV melanoma patients were stained.

Results: PI-9 is expressed in melanoma cells and expression in metastasized melanoma cells is, in this group of patients, an adverse prognostic marker with regard to overall and disease-free survival. Moreover, loss of MHC-1 expression frequently occurs during tumor progression but is not associated with poor clinical outcome. Interestingly, melanoma patients with a favorable clinical outcome after ASI therapy usually have high percentages of activated (granzyme B—positive) tumor-infiltrating lymphocytes at time of first diagnosis and low percentages of activated lymphocytes at time of recurrent tumor.

Conclusions: Expression of PI-9 in metastatic melanoma cells is associated with unfavorable clinical outcome whereas MHC-1 down-regulation is not. Although it cannot be proven that PI-9 expression is directly responsible for failure of immunotherapy, these data suggest that expression of PI-9 could be an important immune escape mechanism and that modulation of this inhibitor may enhance the efficacy of immunotherapy.

Melanoma is a highly malignant, increasingly common tumor for which treatment failure is a well-known problem. Although melanoma accounts for only 4% of all skin cancers, it causes the greatest number of skin cancer—related deaths worldwide. There has been a worldwide increase in incidence of melanoma over the last four decades with the highest incidence in Australia and New Zealand (1).

Early detection and complete surgical excision of melanoma is the best manner to reduce mortality. In case of a regional lymph node metastasis (stage III), 5-year survival is dependent on the number of involved lymph nodes (2). Despite a large number of clinical trials to test anticancer strategies, the median survival time of advanced melanoma patients (stage IV) is still no longer than 6 to 10 months (3).

Novel immune therapeutic strategies aiming at the induction, enhancement, and prolongation of the immune response against malignant melanoma cells are currently being developed and applied in clinical practice (4). The aim of these therapies is to induce a tumor-specific immune response, which will eliminate the tumor cell often by means of apoptosis induction. Although some therapies seem promising, the results obtained are variable and it is still unknown why some patients respond and others do not.

We have previously shown that active specific immunotherapy (ASI) is probably beneficial in a fraction of stage III and stage IV melanoma patients when given in the adjuvant setting (5). In these patients, the delayed-type hypersensitivity (DTH) response against the tumor cell vaccine strongly correlated with survival, suggesting that the immune response is involved in outcome following ASI therapy.
Cytotoxic lymphocytes are able to induce tumor cell apoptosis. Cytotoxic lymphocytes comprise two effector cell populations with the ability to eliminate tumor cells by induction of apoptosis: CTLs and natural killer (NK) cells. CTLs become activated following recognition of tumor-specific antigens when presented in the context of the proper MHC-1 complex, whereas NK cells kill the target cell in absence of MHC-1 (6). Down-regulation of MHC-1 molecules on the surface of the tumor cell prevents recognition by CTLs and is frequently associated with invasive and metastatic tumor phenotypes (7, 8).

Despite recognizing target cells in different ways, the cytotoxic mechanisms of the two cell populations are identical. CTLs and NK cells mediate cell death by two distinct pathways: the secretory pathway or through a membrane receptor-ligand interaction involving members of the tumor necrosis factor family (9–11). The secretory pathway is triggered after exocytosis of “cytotoxic” granules containing perforin and several serine proteases, of which granzyme B is essential for rapid DNA fragmentation in the target cell followed by apoptosis (12). Both activated CTLs and NK cells express granzyme B, which can be detected using standard immunohistochemistry using granzyme B–specific antibodies (13). Target cell death can also be induced by members of the tumor necrosis factor family, such as membrane-bound Fas ligand on the cytotoxic lymphocyte that interacts with the receptor Fas on the target cell (14).

Recent studies have shown that tumor cells can become resistant to cytotoxic lymphocyte–induced apoptosis, even if they are properly recognized, by expressing proteins that interfere with the two cytotoxic lymphocyte–induced apoptosis pathways. Among these proteins is the intracellular serine protease inhibitor 9 (PI-9), which inhibits granzyme B efficiently and plays a role in protection of cytotoxic lymphocyte against their own granzyme B (15, 16). Interestingly, expression of PI-9 has been detected in various types of lymphomas (17), certain carcinomas (18), and in melanoma cell lines (19). In lymphomas, PI-9 expression was strongly related with both grade and poor clinical outcome (17–20).

In this study, we investigated stage III and IV melanoma patients treated with ASI therapy on whether expression of PI-9 and/or loss of MHC-1 expression in melanoma cells is a prognostic marker predicting overall and disease-free survival. Moreover, we investigated whether the numbers of activated tumor infiltrating CTLs and NK cells are related to clinical outcome in these melanoma patients.

From 28 of 81 patients, paraffin-embedded tissue was available of the primary tumor and the metastasis. These tissues were used for this study. Paraffin-embedded samples of the metastasis used for ASI therapy were present in our own archives. Biopsy samples from primary tumors were kindly provided by the departments of pathology from the following hospitals: Medical Center Alkmaar, Alkmaar; Hospital Gooi Noord, Huizen; University Maastricht, Maastricht; Hospital de Heel, Zaanland; University Medical Center, Utrecht; Kamerenland, Haalten; Antonie van Leeuwenhoek hospital, Amsterdam; Laboratory Pathology Oost-Nederland, Enschede; Gelre Hospital, Apeldoorn; Pathology Hospital, Iala clinics, Zwolle; and Spaarne Hospital, Hoofddorp. From the remaining patients, 10% sufficient tissue material was left of the primary tumor or the metastasis.

From each patient, the following characteristics were used from the medical records: age at diagnosis, date of diagnosis, sex, stage, site of first diagnosis, therapy, response, occurrence and date of relapses after therapy, and cause and date of death. The overall survival and disease-free survival were determined from the time of ASI until death related to melanoma or until end of follow-up or until date of disease relapse, respectively. Patient characteristics are summarized in Table 1.

Cell lines. The following human melanoma cell lines were derived at the Ludwig Institute for Cancer Research, Lausanne Branch, Switzerland: Me 305, Me 304, Me 290, Me 285A, Me 280RA, Me 275, Me 260LN, Me 252, Me 237, Me 235, Me 215, and Me 204A. NAB-MEL and M22-Mel.3.0 were a gift from F. Jotereau (Nantes, France) and T. Boon (Brussels, Belgium). All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% glutamine, and 1% penicillin and streptomycin. COS-7 cells, transfected with a pDNA3.1/Hygro plasmid containing cDNA encoding full-length human PI-9 (21), were used as a positive control for PI-9 protein expression. Briefly, 2 × 10^5 COS-7 cells were grown in 25 cm² culture flasks for 24 hours in DMEM with 10% heat-inactivated fetal bovine serum, antibiotics, and glutamine. Transfection was done with FuGENE 6 transfection reagent. For preparation of cell lysates, cells were trypsinized and washed twice in PBS. Cell lysates were prepared in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% glycerol, 0.1% Nonidet P-40] supplemented with a protease inhibitor cocktail (Roche Biochemicals, Basel, Switzerland). Cell debris and nuclei were removed by centrifugation at 10,000 × g for 10 minutes and protein concentration was determined by the Bradford assay (Pierce, Rockford, IL) and stored at −20°C.

Antibodies. The antibodies used were monoclonal antibody granzyme B7 (mouse immunoglobulin G2a) specific for human granzyme B (Sanbio, Uden, the Netherlands; ref. 22); monoclonal antibody PI-9-17 (mouse immunoglobulin G1, VUmc Amsterdam, the Netherlands) specific for human PI-9 (21); polyclonal anti-CD3 (DakoCytomation, Glostrup, Denmark); monoclonal antibody HCA2 reactive with HLA-A locus products and monoclonal antibody HC10 preferentially recognizing HLA-B/C locus products (23), both kindly provided by Prof. Dr. J. Neefjes (Netherlands Cancer Institute, Amsterdam, the Netherlands).

Western blot analysis. Proteins (20 μg/lane) were resolved by 10% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane (Hybond ECL nitrocellulose membrane, Amersham Pharmacia Biotech, Freiburg, Germany) by electroblotting. Nonspecific binding was blocked by incubation in TBS, 0.5% Tween 20, and 5% (w/v) dry milk. The membrane was incubated with primary antibody (PI-9-17, 1 μg/μL, 1:100) in blocking solution for 2 hours at room temperature. The bound primary antibody was visualized with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA) and chemiluminescence development reagent (ECL system, Amersham) according to the instructions of the manufacturers.

Immunohistochemistry. Immunohistochemical staining was done as previously described (17, 24). Briefly, paraffin-embedded 4 μm tissue sections were deparaffinized with xylene and alcohol, and endogenous peroxidase was blocked by incubation for 30 minutes.
with 0.3% (v/v) H₂O₂ in methanol. For granzyme B, PI-9-17, and HCA10, antigen retrieval was done with 10 mmol/L Na-citrate (pH 6) and, for CD3 and HCA2, with 10 mmol/L Tris, 1 mmol/L EDTA (pH 9). For granzyme B, CD3, HCA2, and HC10, the microwave was used (700 W for 5 minutes and 360 W for 10 minutes) and, for PI-9, the pressure cooker (21 minutes at 120°C) was used for boiling. Following antigen retrieval, primary antibodies and secondary antibodies were applied, and immunostaining was done with aminoethyl carbazol substrate solution (Zymed laboratories, Inc., San Francisco, CA) using horseradish peroxidase–conjugated streptavidin-biotin-complex and further signal enhancement by the catalyzed reporter deposition method (DakoCytomation) for PI-9-17 and granzyme B7, or using the Envision™ horseradish peroxidase system (DakoCytomation), following the protocol supplied by the manufacturer for CD3, HC10, and HCA2.

**Interpretation of the staining.** Quantification of absolute numbers of CD3 and granzyme B–positive lymphocytes in the reactive infiltrate present in tumor sections was done using a commercially available interactive video overlay–based measuring system (QPROMIT, Leica, Cambridge, United Kingdom) as previously described (25). Per tumor slide, 100 fields of vision were randomly selected using an automatic scanning stage. With granzyme B being the ultimate cytotoxic lymphocyte activation marker, the percentage of activated CTLs was determined by dividing the number of granzyme B–positive lymphocytes by the number of CD3-positive lymphocytes. Staining was blindly scored by two pathologists (J.A.K and J.J.O).

For PI-9, a case was scored positive when cytoplasmic and/or nuclear staining of tumor cells was observed. PI-9–positive reactive T-lymphocytes and dendritic cells present within the tumor cell area acted as positive internal control (17). Cases without such staining were regarded as not interpretable (n = 2). Tumor cells were identified based on morphology. The cases were divided into five categories: <5%, 5% to 25%, 25% to 50%, 50% to 75%, and 75% to 100% of the tumor cell population positive for PI-9. Staining with HCA2 and HC10 predominantly showed a membranous staining pattern. Positive tumor cells were quantified by dividing the cases into three categories: 0, negative; 1, lower expression than lymphocytes (weak expression); and 2, expression equal to lymphocytes. Staining of lymphocytes acted as internal control. Cases without positive staining of lymphocytes were regarded as not interpretable and were therefore not included (eight cases).

### Table 1. Patient and tumor characteristics per patient

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Survivors

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Nonresponders

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### Abbreviations:
- DoD, death of disease; ni, not interpretable; nk, not known.
- *Interpretation of PI-9 expression: 0, <50% tumor cells; 1, ≥50% tumor cells.
- †Interpretation of MHC-1 expression: 0, negative; 1, less intense staining as compared with normal lymphocytes (weak expression); 2, staining intensity equal or stronger than normal lymphocytes.
- ‡% activated CTLs is determined as (number of granzyme B–positive cells per measurement area / number of CD3-positive cells per measurement area) × 100%.
Expression of the Granzyme B Inhibitor PI-9 in Melanoma

Analysis of clinical data and statistical methods. Survival curves were constructed with the Kaplan-Meier method. Differences between curves were analyzed using the log-rank test. Qualitative variables were analyzed by Pearson’s \( \chi^2 \) test. The Spearman’s test was used to analyze correlations between different variables. All \( P \) values were based on two-tailed statistical analysis. \( P < 0.05 \) was considered significant. All analyses were done using the SPSS statistical software (version 11.5, SPSS, Inc., Chicago, IL).

Results

Clinical characteristics. Patient characteristics were previously published (5) and the characteristics of 28 patients from the group selected for this study were summarized in Table 1 together with tumor characteristics. Twelve patients presented with stage III and 16 patients with stage IV melanoma at time of admittance to the ASI trial. Twenty-three patients had a relapse after ASI treatment and finally died. Five patients, all stage III at time of diagnosis, were alive and disease-free at time of last control. The median follow-up period of these five melanoma patients was 53.2 months. Stage III patients had a better survival than stage IV patients (\( P = 0.01 \), data not shown) as previously reported (5).

In this group of patients, the mean number of vaccinations given was four, ranging from two to nine. In two patients, the start of ASI therapy was postponed because of radiotherapy. Preceding ASI, four patients received systemic therapy (interleukin 2, IFN-\( \gamma \), and dacarbazine).

The DTH of 23 patients in this group varied between 0 and 105 mm. Twenty-two patients had a DTH response after ASI. In this selected group, the patients with a favorable clinical outcome have a significantly better DTH response (mean, 40.8 mm) than patients with an unfavorable outcome (mean, 12.8 mm; one-way ANOVA test, \( P = 0.02 \)).

Strong expression of protease inhibitor 9 in metastatic melanoma biopsies correlates with poor disease-free survival after immunotherapy. In 6 of 14 melanoma cell lines tested, PI-9 protein (42 kDa) was detected (Fig. 1). A nonspecific band was observed at 75 kDa in all lanes. COS-7 cells transfected with pcDNA3.1-PI-9 acted as positive control.

Subsequently, PI-9 expression was investigated using immunohistochemistry in melanoma biopsies (Fig. 2A-D). Expression of PI-9 was detected in tumor cells of 21 of 26 (80%; two were not interpretable) cases of primary melanoma and in 22 of 28 (79%) metastases. Biopsies of primary melanomas and metastases were scored semiquantitatively in five categories: <5%, 5% to 25%, 25% to 50%, 50% to 75%, and >75% PI-9–positive tumor cells. The percentage of PI-9 expressing tumor cells varied from none to >75%. In >50% of the tumor cells, 15 of 26 primary tumors and 12 of 28 metastases showed PI-9 expression. In 25 of 28 patients, expression of PI-9 could be compared between primary melanoma and metastasis. PI-9 expression varied between primary biopsies and metastases (Table 2).

To investigate whether PI-9 expression was related to outcome following ASI therapy, different cutoff values (5%, 25%, 50%, and 75%) were tested using the log-rank test. In primary biopsies, percentages of PI-9–positive tumor cells were not related to clinical outcome, regardless of the cutoff value used. For the metastases, 5%, 25%, 50%, and 75% cutoff levels all provided significant information with regard to disease-free survival time (3%, \( P = 0.04 \); 25%, \( P = 0.01 \); and 50% and 75%, \( P = 0.006 \)). Patients with zero or <50% PI-9–positive tumor cells were found to have a significantly longer disease-free survival (\( P = 0.006 \); Fig. 3A) and overall survival time after ASI therapy (\( P = 0.03 \), data not shown) than patients with >50% PI-9–positive tumor cells. When stage III and IV melanoma patients were separately analyzed, the prognostic effect of PI-9 was observed in both groups, although the effect in stage IV patients was less evident than in stage III patients and was not statistically significant (\( P = 0.009 \) for stage III patients, \( P = 0.1 \) for stage IV patients; Fig. 3B and C).

Loss of MHC-I expression does not preclude a favorable response to active specific immunotherapy. Expression of MHC-I on tumor cells was investigated in 28 patients. Staining on infiltrating lymphocytes was used as internal control. A significant correlation (\( P < 0.0001 \), Pearson’s \( \chi^2 \) test) was found between expression levels of HLA-A and HLA-B/C as determined by HCA2 and HC10 staining, respectively. Interpretable staining results were obtained for both the primary and the metastatic tumor material from 20 patients. Complete loss of MHC-I expression was observed in 4 of 19 patients in whom the primary tumor showed clear expression of MHC-I (see Table 2). In cases with intact MHC-I expression, relatively high percentages of activated CD3/granzyme B–positive CTLs were detected (Mann-Whitney \( U \) test, \( P = 0.02 \); Fig. 4A).

To investigate whether loss of MHC-I expression correlates with clinical outcome, patients were divided into two groups: one group with intact MHC-I expression in primary tumor and
metastasis, and one group with intact MHC-1 expression in the primary tumor but with loss or decreased expression of MHC-1 in the metastasis. The latter group of patients tended to have a better survival after ASI therapy than patients without loss of MHC-1 expression (log-rank test, \( P = 0.1 \); Fig. 4B).

**A high percentage of activated lymphocytes in the primary tumor is associated with a favorable prognosis.** CD3/granzyme B–positive activated lymphocytes were detected in all cases, demonstrating the characteristic granular cytoplasmic staining in activated CTLs (Fig. 2E and F). Absolute numbers of CD3- and granzyme B–positive cells in the tumor area varied considerably between patients and between the primary tumor and its metastasis (Table 1). The correlation between absolute numbers of CD3-positive lymphocytes and absolute numbers of granzyme B–positive lymphocytes in the primary tumor was \( R = 0.5 \) (Spearman’s test, \( P = 0.01 \)) and the correlation between absolute numbers of CD3-positive lymphocytes and absolute numbers of granzyme B–positive lymphocytes in the metastasis was 0.8 (Spearman’s test, \( P = 0.01 \)). In addition, the percentage of activated CTLs varied considerably between melanoma samples, with a median of 15% (Table 1).

To investigate whether the percentage of activated CTLs was related to clinical outcome following ASI therapy, biopsies were divided in cases with the percentage of activated CTLs under or above the median value of 15%. It seemed that patients with >15% activated CTLs in the primary tumor (\( n = 12 \)) had a significantly better prognosis than patients with <15% activated CTLs (\( n = 8 \), \( P = 0.007 \); Fig. 4C). The percentage of activated CTLs in the metastasis of the patients was unrelated to clinical outcome (data not shown). No significant differences were observed between percentages of activated CTLs when comparing primary melanoma biopsies with metastasis or when comparing PI-9–positive versus PI-9–negative cases (\( P > 0.05 \); Fig. 4D).

**Discussion**

In this study, we have shown that a high percentage of PI-9–positive tumor cells in melanoma metastases is associated with
poor clinical outcome following ASI therapy in this group of stage III and IV melanoma patients.

The serine protease inhibitor PI-9, an inhibitor of granzyme B–mediated apoptosis, has been shown to be an important apoptosis-regulating protein (16). It was previously detected in other tumor cells, including Hodgkin and non-Hodgkin lymphomas (17) and in tumor cell lines of melanoma, cervical carcinoma, breast carcinoma, and colon carcinoma (19). We also detected PI-9 expression in melanoma cell lines. We could confirm expression of PI-9 in biopsy samples of primary and metastatic melanoma. Our observation that expression of PI-9 in tumor cells of metastatic melanoma of stage III and IV melanomas is associated with an unfavorable clinical outcome following vaccination therapy suggests that expression of PI-9 is an effective immune escape mechanism of tumor cells. Moreover, determining expression of PI-9 in melanoma may be of use in predicting the clinical response to vaccination therapy.

Another mechanism by which tumor cells may evade the immune system is down-regulation of MHC-1 molecules. Decreased or complete loss of MHC-1 expression has previously been associated with an invasive and metastatic melanoma phenotype (7, 8, 26, 27). We found a decrease in the expression of MHC-1 in the metastasis as compared with the primary tumor, supporting the notion that loss of MHC-1 is a probable strong immune escape mechanism in melanoma. Strikingly, we found that complete loss or decreased levels of MHC-1 on the metastatic melanoma cells was not associated with an unfavorable outcome following ASI therapy. It should be reminded that autologous metastatic melanoma cells were used as vaccine for these patients. Thus, a proper antigen presentation in the context of MHC-1 complex by the tumor cells is apparently not a prerequisite for a favorable outcome or a strong local immune response as indicated by the strong DTH response at the site of vaccination. The involvement of dendritic cells, which, due to their capacity to take up antigens from apoptotic, necrotic, and intact tumor cells (28), can mediate cross-presentation, could potentially explain these results. Alternatively, lack of MHC-1 expression on melanoma cells might indicate that tumor cell killing is not mediated by CTls but rather by NK cells. Because patients with a favorable outcome after ASI therapy did not relapse, we could not test whether ASI therapy results in an increase of NK cells. However, previous reports have also shown that lymphokine-activated killer cells are strong effector cells, especially in melanoma patients (29–31). In addition, despite MHC-1 down-regulation

<table>
<thead>
<tr>
<th>Metastasis</th>
<th>MHC-1 negative*</th>
<th>MHC-1 positive*</th>
</tr>
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<tbody>
<tr>
<td>Primary tumor</td>
<td>MHC-1 negative</td>
<td>1</td>
</tr>
<tr>
<td>MHC-1 positive</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>PI-9 negative</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>PI-9 positive</td>
<td>7</td>
<td>8</td>
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*Interpretation of MHC-1 expression: negative, no expression of MHC-1 observed in melanoma cells as compared with lymphocytes; positive, tumor cells showed the same or weaker expression of MHC-1 as lymphocytes.

† Interpretation of PI-9 expression: negative, <50% tumor cells; positive, >50% tumor cells.

Table 2. PI-9 and MHC-1 expression during tumor progression

![Comparison of disease-free survival time according to the percentage of PI-9–positive tumor cells in melanoma metastases. A, survival curves for all patients included in the study. Patients with >50% PI-9–positive tumor cells have a significantly worse disease-free survival (P = 0.006). B, survival curves of PI-9 in stage III patients (P = 0.009). C, survival curves of PI-9 in stage IV patients (P > 0.05).](image-url)
and the resulting resistance to CTLs, mice could be successfully immunized against MHC-1–negative melanoma and this response was critically dependent on CD4+ T cells and NK cells (32).

We found that high percentages of activated CTLs in the primary melanoma were associated with a favorable outcome. A high percentage of activated CTLs at time of diagnosis might reflect an immunogenic tumor, which is supported by the significant correlation between intact MHC-1 expression and a high percentage of activated CTLs. Interestingly, four of the five surviving patients showed a high percentage of activated CTLs in primary tumor but a low percentage of activated CTLs in the metastasis. A possible explanation can be that in these patients the antitumor cell response has become ineffective and maybe reawakened after ASI therapy. This is also suggested in a recently published report (33). In patients with low or absent MHC-1 expression, low numbers of activated CTLs may result from inappropriate CTL activation due to absence of proper antigen presentation.

Only one patient with high numbers of activated CTLs in the metastasis (n = 9) showed a favorable response after ASI therapy. Assuming that activated CTLs represent a specific antitumor cells response, and despite the intact MHC-1 expression in five of seven (one was not interpretable) cases, melanoma cells have apparently found an effective way to escape from this CTL response. Expression of PI-9 can maybe explain this in three of eight cases. Failure of CTLs to induce apoptosis in the other five patients may result from the presence of other apoptosis inhibiting factors like c-Flip (34) or the loss of apoptotic protease activating factor 1 expression (35).

We conclude that in this group of stage III and IV melanoma patients, (a) expression of PI-9 in metastatic melanoma cells is associated with unfavorable clinical outcome following ASI therapy; (b) loss or decrease of MHC-1 expression on melanoma cells frequently occurs during tumor progression; and (c) loss of MHC-1 expression on the tumor cells is not associated with unfavorable clinical outcome following ASI therapy. Although further investigation is needed to determine whether PI-9 expression is indeed responsible for the failure of tumor vaccination treatment, these data suggest that melanoma cells use various immune escape mechanisms and that specific immune escape mechanisms may determine the clinical response to ASI vaccination therapy.

**Fig. 4.** Correlation of MHC-1 expression with presence of activated CTLs in tumors and patient survival. A, correlation between MHC-1 expression in the metastasis and percentage of activated CTLs. Patients without MHC-1 expression on the tumor cells have a significantly lower percentage of activated CTLs (P = 0.02). Box plots are based on the median, quartiles, and extreme values. The box represents the interquartile range that contains 50% of the values. The whiskers are lines that extend from the box to the highest and lowest values. A line across the box indicates the median. B, overall survival time following ASI therapy in patients with complete loss or down-regulation of MHC-1 expression in the metastasis as compared with patients with equal expression in primary tumor and metastasis. Although the observed difference is not significant, these data suggest that melanoma cells use various immune escape mechanisms and that specific immune escape mechanisms may determine the clinical response to ASI vaccination therapy. C, comparison of overall survival time in relation to the percentage of activated CTLs infiltrating the primary tumor (P = 0.007). A high percentage activated CTLs in the primary tumor is associated with a favorable outcome. D, correlation between percentage of activated CTLs and expression of PI-9. In the metastasis, no difference in percentage of activated CTLs is seen between patients with <50% or >50% PI-9–positive tumor cells (P > 0.05).
References

Expression of the Apoptosis Inhibitor Protease Inhibitor 9 Predicts Clinical Outcome in Vaccinated Patients with Stage III and IV Melanoma


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