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Review

Immunologic functions as prognostic indicators in melanoma

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ABSTRACT

Outcome in melanoma patients with advanced disease is poor and systemic treatment seems to benefit only a subset of patients. Predictive markers identifying these patients are currently not available. Early studies showed an association of immune-related side effects such as vitiligo and autoimmune thyroiditis with response to IL-2 or IFNα treatment. However, conflicting data have been reported as well, mentioning the effect of a higher rate of immune-related toxicities during prolonged administration of the drug in responders. The review discusses the prognostic significance of autoimmunity during various forms of immunotherapy and stresses the importance of correcting for guarantee-time bias. In addition, other immune-related factors which have been associated with melanoma prognosis such as, CRP, white blood cell count, absolute lymphocyte count and human leukocyte antigen will be reviewed as well. A better understanding of the immune system and the host-tumor interactions should ultimately lead to more effective treatment. A major challenge expected to be addressed in future is proving ways to uncouple tumor immunity from autoimmunity.

1. Introduction

Current prognostic biomarkers based on the conventional American Joint Committee on Cancer (AJCC) staging system (TNM) include Breslow tumor thickness, presence of ulceration and extent of nodal involvement for primary cutaneous melanoma, and furthermore, site of distant metastases and serum lactate dehydrogenase (LDH) in metastatic melanoma (Balch et al., 2009). In melanoma, prognostic markers are needed to subdivide traditional tumor stages into subsets of patients behaving differently in order to achieve personalized treatment. Since systemic treatment is still disappointing for the overall patient group, the identification of a predictive factor to select patients benefitting treatment is therefore beneficial.

Various systemic treatment regimens occasionally cured patients with widespread metastatic, but only DTIC is a worldwide approved therapy (Eggermont and Kirkwood, 2004; Eggermont and Schadendorf, 2009). Also, fotemustine is approved in some countries, due to the observation that it prolonged time to occurrence of brain metastases (Avril et al., 2004). Cytokine-based therapy with IL-2, based on its ability to produce durable responses, is approved for patients with metastatic melanoma in the USA but not in Europe, (Atkins et al., 1999). Twenty-one phase III trials evaluating the addition of interferon α (IFN) or of IL-2 alone or of the combination

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of IL-2 and IFN to mono or combination chemotherapy, showed improved response rates, at the cost of significant toxicity, but failed to provide proof for survival benefit (Eggermont and Schadendorf, 2009). Strikingly, combining chemotherapy with immunotherapy has not been very successful either. However recently, a relevant prolongation of survival was demonstrated with ipilimumab in patients with metastatic melanoma (Hodi et al., 2010).

Interestingly, the phenomenon of autoimmunity observed during various forms of immunotherapy, IL-2, IFN and anti-CTLA-4 therapy, has been linked to treatment response (Atkins et al., 1988; Gogas et al., 2006; Phan et al., 2001). To understand the link between tumor immunity and autoimmunity in melanoma and to explore its implication on prognosis and treatment outcome remains a challenge (Ramirez-Montagut et al., 2003). Here we review several immune-related factors which have been associated with melanoma prognosis, thereby focusing on the potential predictive value of these factors in patients receiving immunotherapy.

2. C-Reactive protein

High serum levels of C-reactive protein (CRP) were associated with shortened survival in metastatic melanoma and resistance to treatment with interleukin-2 (Tartour et al., 1996, 1994). It is a serum marker, which could discriminate melanoma patients entering AJCC stage IV from patients remaining in AJCC stages I, II or III (Deichmann et al., 2004).

Findeisen et al. showed that serum amyloid A (SAA) and CRP combined were also useful prognostic markers in early-stage melanoma (Findeisen et al., 2009). Serum mass spectrometry revealed a peak at m/z 11.680 differentiating between stage I en IV melanoma, which could later be identified as SAA. In univariate analysis SAA and CRP were prognostic marker in 276 stage I-III melanoma patients ($p = .04$ and $p = .006$ respectively) and in 103 stage IV patients (both $p < .0001$). Multivariate analysis including the well-known prognostic markers and the serum markers; S100b, CRP, LDH and SAA, revealed sex, stage, tumor load as well as S100b, CRP, and SAA as prognostic markers in stage I–IV disease with an interaction between CRP and SAA. A significant prognostic discrimination was found for the combination of these two markers in stage I-III ($p = .01$) and stage IV ($p < .0001$).

Stam et al. previously assessed the effects of interferon α2b on the acute-phase response in a small subset of stage IIb/III melanoma patients participating in the EORTC 18952 trial (Stam et al., 2002). They found significant increases in ferritin levels and less pronounced decreases in CRP levels at end of induction and at 6 months in the treated group as compared to baseline levels. We recently extended the analysis in order to explore the association of ferritin and CRP changes over time with clinical response to IFN therapy. Ferritin levels in the IFN-treated patients were increased from end of induction and remained high during the treatment period. CRP levels varied slightly during the course of the study but no significant differences between treated and untreated patients were observed. Some patients in the IFN group had decreased CRP levels at end of induction. However, none of these changes were predictive for treatment response in patients receiving IFN (submitted data).

3. White blood cell count

Initially in patients with metastatic renal cell carcinoma receiving immunotherapy with IL-2 or IFN, baseline elevated neutrophil counts in peripheral blood were associated with poor survival (Negrier et al., 2002). A study by Schmidt et al. in 321 stage IV melanoma patients reported similar results for patients treated with IL-2-based immunotherapy (Schmidt et al., 2005). In univariate analyses, elevated neutrophil counts ($p < .001$) and elevated monocyte counts ($p < .001$) were identified as prognostic factors. Entering elevated neutrophils (or monocytes) in a multivariate analysis comparable results were found ($p = .02$). A validation study in stage IV melanoma patients accrued to the EORTC 18952 biochemotherapy trial confirmed these findings (Schmidt et al., 2007). Pretreatment elevated neutrophils count was an independent prognostic factor for reduced overall survival (HR = 1.5; $p = .02$), and a high leukocyte count was an independent prognostic factor of both reduced overall survival (HR = 1.7; $p < .001$) and reduced progression-free survival (HR = 1.5; $p = .008$).

Recently six cases of paraneoplastic granulocytosis were described in a series of 626 patients with metastatic melanoma (Davis et al., 2010). These patients were found to have unexplained leukocytosis with neutrophilia. After extensive work-up for infectious disease, serum granulocyte colony-stimulating factor (G-CSF) level was determined and was abnormally elevated in all patients. The degree of leukocytosis directly correlated to the level of serum G-CSF. In three patients the onset of paraneoplastic granulocytosis did not occur with the appearance of metastatic melanoma but at later point in the course of the disease. It is unknown whether G-CSF-secreting melanoma tumors are indicative for a more aggressive tumor phenotype or whether these tumors respond differently to therapy.

4. Absolute lymphocyte count

An early report by Bernengo et al. already provided evidence that melanoma patients with normal lymphocyte counts had a better prognosis compared to those with reduced lymphocyte counts (Bernengo et al., 1983). Furthermore, in patients with metastatic melanoma receiving IL-2, absolute lymphocyte count immediately after therapy was significantly higher in responders compared with non-responders (Phan et al., 2001). Moreover a higher change in lymphocyte count, as compared to baseline value, was documented in responders. Generally, rebound lymphocytosis peaked 2–5 days after cessation of IL-2. A positive association between lymphocyte count and response was also reported by some others (Rosenberg et al., 1998; West et al., 1987).

Recently, in 51 patients with advanced melanoma who were treated with ipilimumab 10 mg/kg, clinical benefit was correlated with absolute lymphocyte count (Ku et al.). Patients underwent laboratory testing before each ipilimumab administration. In order to correlate absolute lymphocyte count (ALC) to outcome, patients were stratified based on a cutoff of $\geq$1000/μL (high ALC) versus <1000 cells/μL (low ALC). At baseline there was a borderline significant trend toward improved overall survival for the high ALC group ($p = .06$),

which remained after correction for LDH. After the first ipilimumab dose, patients with high ALC had a significantly improved overall survival ($p < .01$), however this association was most pronounced after the second dose of ipilimumab, with a median overall survival of 11.9 months versus 1.4 months and $p < .0001$. Similar results were found when corrected for LDH. Although this study comprised a relatively small number of patients and no detailed multivariate analysis was performed, the results might have strong implications in future trials and clinical practice. These data suggest that patients with ALC <1000 cells/μL do not benefit from ipilimumab treatment and could be spared of further toxicity.

5. Autoimmunity

5.1. *IL-2*

Hypothyroidism was the first described autoimmune phenomenon associated with a favorable tumor response after treatment with IL-2 and lymphokine-activated killer cells (Atkins et al., 1988). Tumor regression occurred in five out of seven (71%) patients with laboratory evidence of hypothyroidism in contrast to 5 of the 27 euthyroid patients (19%). Similar observations were reported by others, yet these studies involved a limited set of patients (Scalzo et al., 1990; Weijl et al., 1993). Also, Rosenburg et al. described the strong correlation between the occurrence of vitiligo in patients with metastatic melanoma and response to IL-2 therapy (Rosenberg and White, 1996). The occurrence of vitiligo and thyroid dysfunction was evaluated by Phan and colleagues in 372 patients receiving IL-2 treatment (Phan et al., 2001). Responders were more likely to develop thyroid dysfunction and vitiligo. The authors limited their evaluation to the presence of thyroid dysfunction and or vitiligo by day 60 in order to correct for the possible confounding factor that long-term immunologic side effects were due to prolonged IL-2 administration. This could occur in responders since they continued on therapy. Thyroid dysfunction was not associated with treatment response anymore, which is in agreement with two previous studies showing the association of a higher incidence of thyroid dysfunction with prolonged IL-2 treatment but no relation with clinical response (Krouse et al., 1995; Kruit et al., 1993). Since it takes time to develop vitiligo, it is difficult to differentiate between a true association with treatment response and the appearance of vitiligo as a result of prolonged IL-2 treatment (in responders). A similar study by Boasberg et al. demonstrated in a multivariate analysis that vitiligo was predictive for improved survival (HR: 0.50, $p = .04$). Median time to onset of vitiligo was 35 days (ranging 24–202 days). When time to occurrence of vitiligo was taken into account, using it as a time-dependent covariate, vitiligo was not a significant predictor of survival (HR: 0.55, $p = .09$) (Boasberg et al., 2006).

5.2. *IFN*

Gogas et al. were the first to evaluate prognostic significance of autoimmune antibodies and or clinical signs of autoimmunity (vitiligo) in melanoma patients treated with adjuvant high dose interferon (HDI) (Gogas et al., 2006). Antithyroglobulin, antinuclear or anticardiolipin antibodies were detected in 24 percent of the patients, and only 3 patients (2%) developed vitiligo without the occurrence of autoantibodies. Autoimmunity during treatment was associated with a significantly improved relapse-free and overall survival. Another study in patients treated with low dose interferon (LDI), also showed a correlation with autoimmunity and a significantly better RFS ($p = .05$) and a trend toward improved OS ($p = .07$) (Satzger et al., 2007). The results of abovementioned trials appeared promising, however, both studies analyzed the effects of autoimmunity on outcome in patients receiving IFN treatment, no observation group was evaluated. Therefore, the predictive value of autoimmunity still needed to be elucidated. Subsequently, a study performed by the EORTC and the Nordic melanoma group described the association of autoantibodies in patients receiving intermediate doses of interferon or no treatment with RFS (Bouwhuis et al., 2009). The analysis was performed in a subset of patients participating in the EORTC 18952 or the Nordic IFN trial (Eggermont et al., 2005; Hansson et al., 2007). At baseline, 33% of the patients in the EORTC 18952 and 35% of the patients in the Nordic IFN study tested positive for antithyroglobulin, antinuclear or anticardiolipin antibodies, however, no difference in RFS between patients with or without preexisting autoantibodies was observed. During follow-up, autoantibodies were more frequently detected in patients receiving interferon as compared to untreated patients, 36% (EORTC 18952) and 39% (Nordic IFN) versus 26% and 14% respectively in the observation arm of both trials. Seroconversion in IFN-treated patients correlated with improved outcome if a Cox model (model 1) in which antibody status was time independent was used (EORTC 18952, HR = 0.37, $p < .001$ and Nordic IFN, HR = 0.49, $p = .002$). When treating antibody status as a time-dependent variable, no strong association with RFS was found. Furthermore, results from a comparable side study from the ECOG 2696 trial were in agreement with the previous study (Stuckert et al., 2007).

Guarantee-time bias is an important confounder when analyzing the prognostic value of any potential biomarker in serial measurements and should be taken into account (Anderson et al., 1983; Kalbfleisch and Prentice, 2002). Satzger et al. did not perform landmark analyses or time-dependent Cox model analyses to correct for guarantee-time bias and therefore the observation that autoimmunity is beneficial in patients treated with LDI is difficult to interpreter (Satzger et al., 2007). Moreover, conflicting data were reported when correction was made, autoimmunity remained a strong independent prognostic marker in the Gogas's study but it lost significance in the side studies from the EORTC 18952, Nordic IFN and ECOG 2696 trials (Bouwhuis et al., 2009; Gogas et al., 2006; Stuckert et al., 2007). Although the same enzyme-linked immunosorbent assays (Quanta Lite, Inova Diagnostics) and titers were used in the studies by Gogas et al. and the EORTC/Nordic Melanoma group collaboration, major differences were found for the occurrence of autoantibodies at baseline. 33% and 35% of the patients from the EORTC 18952 study and from the Nordic IFN trial respectively had preexisting autoantibodies, against 1.5% reported by Gogas et al. This seems very low since antinuclear antibodies for instance are commonly detected in healthy persons, with reported prevalences ranging 4–35%, and prevalences up to 40% in cancer patients (Imran et al., 2003; Shovman et al., 2005; Solans-Laque et al., 2004; Tan et al., 1997). The higher seroconversion rate in the EORTC 18952 and Nordic IFN trials as compared with Gogas's

study and the ECOG 2696 trial, could be explained by a longer treatment duration in the EORTC 18952 and Nordic IFN trial and a longer sampling period in these trials. Interestingly, the median time to seroconversion was only 3 months in the study by Gogas et al. compared to 6–12 months in the trials reported by the EORTC/Nordic melanoma group. A possible explanation for this difference could be the fact that the induction treatment comprised a higher dosage and was delivered intravenously (vs. subcutaneously) in the study by Gogas et al. Maybe only clinical manifestations of autoimmunity resemble "true autoimmunity" and should be used to identify possible treatment responders. This seems less likely since autoimmune diseases are often preceded and/or accompanied by the occurrence of autoantibodies. Also, from the 52/200 (26%) of the IFN-treated patients with signs of autoimmunity in the study of Gogas, only three (2%) patients had clinical manifestations of autoimmunity (vitiligo) without autoantibodies. Therefore, using autoantibodies as an index of immune response seems reasonable.

Recently the association of autoimmune antibodies in patients receiving long-term (5 years) treatment with pegylated (PEG)-IFN or no treatment (observation) and prognosis, was described in patients accrued to the EORTC 18991 trial (Bouwhuis et al.). Results were comparable, a correlation with improved outcome (in all patients) according to the (biased) Cox model, yet upon correction for guarantee-time bias using the time-dependent Cox models, the occurrence of autoantibodies lost significance. The effect of seroconversion on outcome in observation patients or PEG-IFN treated patients separately were similar, suggesting that seroconversion is also not a predictive serological marker for treatment outcome.

5.3. *Anti-CTLA-4*

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an important inhibitor of T cell activation (Greenwald et al., 2005; Teft et al., 2006). Blocking CTLA-4 with neutralizing antibodies, ipilimumab or tremelimumab, is therefore a promising approach to augment antitumor immune responses. Treatment with ipilimumab has been more extensively investigated, showing objective response rates ranging from 5% to 17% (Attia et al., 2005; Downey et al., 2007; Hersh et al.,; Hodi et al., 2010; O'Day et al., 2010,; Weber et al., 2009; Wolchok et al., 2010). Attia et al. were the first to describe the correlation of severe autoimmune side effects such as dermatitis and colitis in 56 stage IV melanoma patients, with clinical response to ipilimumab (Attia et al., 2005). Five of the 14 patients with immune-related adverse events (irAEs) exhibited a clinical response, whereas only 2 of the 42 patients without autoimmune side effects responded ($p = .008$). This association has also been reported by others (Downey et al., 2007; Weber et al., 2008). A recent study by Wolchok et al. elicited a dose-dependent effect of ipilimumab on efficacy and safety in patients with advanced melanoma (Wolchok et al., 2010). Best overall response rate (the proportion of patients with a complete or partial response, according to modified WHO criteria), was 11.1% for 10 mg/kg ($n = 72$), 4.2% for 3 mg/kg ($n = 72$), and 0% for 0.3 mg/kg ($n = 73$). IrAEs of grade 3–4 arose in none of the patients in the 0.3 mg/kg group, in 5 patients of the 3 mg/kg group and in 18 patients of the 10 mg/kg group. The most common grade 3–4 adverse events were gastrointestinal immune-related events and generally managed successfully with steroids. This study, however, did not explore the association between the occurrence of irAEs and clinical response. Another study reported for patients receiving ipilimumab (10 mg/kg) that patients suffering grade 3–4 irAEs at week 24 had a significantly higher clinical response rate as compared to those with grade ≤ 2 irAEs ($p < .01$) (Ku et al.). Improved overall survival was shown by Hodi et al. in a large phase III study in metastatic melanoma for patients receiving ipilimumab (3 mg/kg) plus glycoprotein 100 (gp100) peptide vaccine compared to patients treated with gp100 alone (HR = 0.68, $P < .001$) (Hodi et al., 2010). The frequency of grade 3 or 4 irAEs was 10–15% in the ipilimumab group and 3% in the gp100 alone group, all occurring during the induction and reinduction periods. Among the 94 patients who survived for 2 years many experienced residual effects such as vitiligo ($n = 12$) and endocrine immune-related adverse events requiring hormone-replacement therapy ($n = 8$).

Tremelimumab is a newer agent which is generally tolerable and has demonstrated antitumor activity (Camacho et al., 2009; Kirkwood et al., 2010). Although ipilimumab and tremelimumab have not been compared directly, it seems that response rates with tremelimumab are lower, which might be explained by suboptimal dosing. Notably is the fact that these patients also developed less immune-related toxicities as compared to ipilimumab treated patients. The only evidence for a correlation between treatment response and irAEs in 30 patients receiving tremelimumab ($p = .05$) was found by Reuben et al. (Reuben et al., 2006). Five patients suffered from grade 3 diarrhea and 1 patient from grade 3 dermatitis but no grade 4 adverse events were observed. A more recent trial evaluating efficacy and safety in patient treated with tremelimumab, 15 mg/kg, every 90 days, reported an objective response rate of 6.6%, with all responses being durable (Kirkwood et al., 2010). Grade 3–4 adverse events included diarrhea (11%), fatigue (2%) and colitis (4%). Furthermore, Camacho et al. showed for the tremelimumab 15 mg/kg arm a treatment-related adverse event rate of 13% including; diarrhea, rash, pruritus, fatigue and nausea, and a serious adverse event rate of 9% (Camacho et al., 2009). Rates for immune-related adverse events were not mentioned. It might be possible that if tremelimumab dosing would be intensified, the number of immune-related adverse event rates and the clinical response rates could be increased.

6. Is it possible to uncouple tumor immunity and autoimmunity?

The immunologic concepts behind concomitant tumor immunity and autoimmunity are complex (Ramirez-Montagut et al., 2003; Uchi et al., 2006). Although tumor immunity and autoimmunity use similar mechanisms, uncoupling is possible (Turk et al., 2002). Various mechanisms have been described (Bowne et al., 1999; Hara et al., 1995; Trcka et al., 2002). Results from a vaccination (AdhDCT) study in mice suggested that tumor immunity and autoimmunity could be separated by modulating the STAT4/STAT6 signaling axis (Zhang et al., 2009). In Stat4-deficient mice Th1 development and IFNγ production are impaired while Th2 functions are intact. On the contrary, Stat6-deficient T cells cannot differentiate into IL-4 producing Th2 cells but develop into IFNγ producing Th1 cells. Vaccination in Stat6-deficient mice,

resulted in antitumor immunity and the occurrence of autoimmunity (vitiligo). However, after depletion of CD8+ T cells, tumor protection was lost but occurrence of vitiligo was not affected, indicating that tumor immunity was dependent upon STAT6 signaling. The opposite was observed in Stat4-deficient mice following depletion of CD8+ T cells. The mice did not develop vitiligo yet antitumor immunity was preserved. Similar studies are wanted for other agents like IL-2, IFN and anti-CTLA-4.

7. Human leukocyte antigen

Serological typing for human leukocyte antigen (HLA) class I and class II antigen expression has previously shown to be associated with melanoma prognosis and treatment response.

An early report by Lee et al. suggested that the risk of melanoma incidence or progression could be influenced by HLA-DQB1*0301 (Lee et al., 1994). Stage I/II melanoma patients with positive HLA-DQB1*0301 status were at an increased risk of developing recurrent disease compared to stage-matched patients lacking this allele (Lee et al., 1996). Also HLA-DRB1*1101 was associated with disease recurrence in patients with localized melanoma (Lee et al., 2002). Clinical response in patients with metastatic melanoma receiving IL-2 was described for patients expressing HLA-DQ1 (Rubin et al., 1995). Another report in a larger set of patients could not confirm this beneficial effect of HLA-DQ1, instead, demonstrated an association of homozygosity of HLA-DR and tolerance to IL-2 treatment (Marincola et al., 1995). In a small study of 32 melanoma patients Scheibenbogen et al. reported an association between HLA-B44 and HLA-Cw7 and response to IL-2, however in a later analysis of 54 patients, the same authors did not find an association between HLA-B44 and response, while HLA-Cw7 remained marginally correlated (Scheibenbogen et al., 1994). Recently, HLA typing in 284 high-risk melanoma patients receiving high-dose adjuvant interferon revealed that the 55 patients expressing HLA-Cw*06 had a better relapse-free and overall survival compared to the 229 patients with a negative HLA-Cw*06 status (Gogas et al., 2010). When controlling for disease stage in a multivariate analysis, the *p*-values for the association of HLA-Cw*06 with RFS and OS were 0.02 and 0.04 respectively.

Abovementioned studies provide evidence that human leukocyte antigen is associated with melanoma prognosis and treatment response, however many contradicting reports have been published. Especially the older studies included only a limited set of patients and validation in a larger set of patients often failed to confirm the previous findings. Results could be different since patients of various ethnic backgrounds have been analyzed. Also, HLA typing techniques have been improved over the years. Moreover, one should keep in mind that in these kind of analyses multiple testing should be corrected for. Larger studies are needed to clarify the role of HLA in melanoma prognosis. Ideally, both treated and untreated patients are analyzed to assess its potential predictive value as well.

8. Conclusions

After years of evaluating different prognostic markers in melanoma we still have not found a marker predicting response to immunotherapy. Since the overall treatment results are disappointing it is necessary to identify patient populations benefitting a certain treatment. With promising new drugs such as ipilimumab it is essential that we continue this search. Although the appearance of autoimmune related toxicities were described to be associated with response to ipilimumab, this association should be confirmed by upcoming phase III trials.

The advances in basic understanding of the immune system and the host-tumor interactions should ultimately lead to more effective and tailor-made treatment. Immunotherapy is associated with considerable toxicity therefore one of the future's challenges will be to induce more potent tumor immunity balancing autoimmune side effects.

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