Role of tumor-infiltrating immune cells and HLA expression in influencing tumor progression and the effectiveness of anticancer treatment

PhD thesis

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1. Introduction

The advent of immune checkpoint inhibitors has significantly improved outcomes for patients with advanced, inoperable, and high-risk operable melanoma. However, there remains a portion of patients who have progression of disease despite this treatment. In order to increase the rate of responders to therapy, research into biomarkers that can predict therapeutic effect is of primary importance.

Based on previous results of the research group, it was confirmed that the density of certain tumor-infiltrating immune cells is associated with the response to ipilimumab therapy. In addition, HLA I expression was associated with the intratumoral density of certain immune cell markers, as well as the response to ICI therapy and patient survival.

Based on these results, the aim of our further research was to determine whether the loss of HLA I expression could be a mechanism of resistance to ipilimumab therapy. In addition, we examined the predictive role of HLA I and II expression in metastatic melanoma patients receiving PD-1 inhibitor therapy.

2. Objectives

Study I

Thesis: Could the loss of HLA class I expression be behind the resistance that develops with ipilimumab therapy in melanoma patients?

(1) The first aim of the study was to explore potential changes in HLA-I expression level during the therapy, comparing pre-treatment and post-treatment metastatic samples.

(2) The second aim of the study was to examine infiltration of pre- and post-treatment tumor samples by CD8+ T cells and NK cells.

Study II

Thesis: Is HLA class I and II expression a predictive biomarker in melanoma patients receiving PD-1 inhibitors?

(1) The aim of the present study was to explore tumor cell HLA class I and class II expression as potential predictive markers in melanoma patients treated with PD-1 inhibitors, analyzing the association of expression results with therapeutic response and survival.

3. Materials and methods

3.1. Patients and tumor samples

Study I

We obtained archived paraffin blocks of sequential (pre- and post-treatment) tissue samples of patients with metastatic melanoma treated with ipilimumab between 2010 and 2015. Sample collection was restricted to metastases surgically removed within a 2 years range before or after ipilimumab treatment.

Altogether 29 metastases from 6 patients were studied, 18 pre-treatment and 11 post-treatment surgical samples.

Progression-free survival (PFS) and overall survival (OS) were calculated from the commencement of ipilimumab treatment till the last follow-up, tumor progression or death, respectively.

Study II

We included in our study all patients who received at least 3 cycles of PD-1 inhibitor treatment for stage IV melanoma in National Institute of Oncology between 2015-2022 and had a pre-treatment tumor sample of adequate quality.

Archived paraffin blocks of pretreatment surgical samples from 40 tumor resection samples of lymph node (n=70) and skin/subcutaneous metastases (n=42), obtained within 4 years of starting anti-PD-1 therapy were selected; the study cohort consisted of 40 patients (1–9 examined lesions per patient). Response assessment was based on immune-related response criteria (irRC) (70); patients with complete or partial remission as best overall response were considered responders in the evaluation. Among the clinical parameters of the patients, we examined the correlations of age, gender, BRAF mutation status, LDH level and ECOG status with the outcome of the disease.

3.2. Monoclonal antibodies

For detecting MHC class I molecules, HCA2 recognizing B2M-free HLA-A (excluding -A24), -B7301, and -G heavy chains; HC10 recognizing B2M-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, HLA-B (excluding -B5702, -B5804, and -B73) and HLA-C heavy chains; the β2-microglobulin (B2M) specific monoclonal antibody NAMB-1; and the anti-pan HLA class I EMR8-5 were utilized; HLA-DR,DQ,DP was detected using the monoclonal antibody LGII-612.14. We used the mouse anti-human CD8 mAb and the mouse anti-human NKp46 mAb to determine CD8+ T cells and

NK cells. The dilution was 1:1000 for the HC10, HCA2, EMR8-5, LGII-612.14 antibodies,1:300 for the NAMB-1, 1:50 for the CD8 and 1:100 for the NKp46 antibody.

3.3. Immunohistochemical staining

Immunohistochemical staining of tissue sections of formalin-fixed, paraffin-embedded tumor samples was performed utilizing standard methodology. Briefly, after deparaffination, sections were treated with 3% H_2O_2 in methanol to block endogenous peroxidases, followed by antigen retrieval by heating at 98 ◦C for 40 min in citrate buffer (pH 6.0). After incubation with protein blocking solution for 10 min at room temperature, primary antibodies were applied overnight at 4 ◦C.

3.4. Evaluation of the immune reactions

Study I

In the case of labeling with anti-HLA class I mAbs, the percentage of the area displaying stained melanoma cells was determined in the metastases. In the case of intratumoral density of CD8+ and NKp46+ lymphocytes the number of labeled cells was counted within the metastases in at least 10 randomly chosen fields per sample, using a graticule of 10×10 squares designating an area of 0.0625 mm^2 at ×400 magnification. For patients with more than one metastasis available the average values were also calculated for each marker, separately for pre- and post-treatment samples.

Study II

HLA class I staining was scored as 0, 1, and 2 when the percentage of stained melanoma cells was <25%, 25–75%, and >75%, respectively, based on the criteria established by the 12th International Histocompatibility Workshop (1996). Expression on normal cells in the samples (e.g., immune cells, cells of the vasculature) served as positive control. In the case of HLA-DR,DQ,DP, the percentage of the area displaying positive tumor cell staining was determined in the metastases. We also calculated a combined score of HLA class I and class II expression (HLA I/II score) based on the number of anti-HLA-I antibodies showing higher positivity than the cutoff level, combined with HLA-II positivity in a given sample (score of 1 in the case of high labeling with at least 3 anti-HLA-I antibodies, and/or HLA-II expression ≥3%; score of 0 when neither of the above criteria are met). For patients with more than one metastasis available, the mean of the scores obtained for the different metastases were calculated for each marker.

3.5. Statistical analysis

Study I

The statistical significance of the differences between pre- and post-treatment samples was determined using the Mann-Whitney U test.

Study II

For the statistical analysis of differences in HLA expression levels between different patient groups, we applied the Mann–Whitney U test, while Fisher's exact test was used to compare the proportions of cases in different groups. Correlations were analyzed by Pearson's nonparametric correlation method.

Survival analysis was carried out using the Kaplan–Meier method and log-rank test.

4. Results

Study I

Responses to therapy were evaluated based on immune-related response criteria (irRC). One patient (Pt1) was scored as complete response (CR). Pt2 achieved stable disease (SD) lasting for 10 months, while Pt 3 showed short-term SD lasting for 4 months; the other three patients exhibited progressive disease (PD). Pt1 and Pt2 were classified as responders while the other four patients as nonresponders in the analysis.

Comparing pre-treatment and post-treatment samples of all patients evaluated together, the expression of HLA class I subunits, as measured by the % of stained melanoma cells, was significantly lower in post-treatment metastases compared to pre-treatment ones. The medians and ranges of the percentage values of melanoma cells stained by HLA-A heavy chain-specific mAb HCA-2, by HLA B,C heavy chain-specific mAb HC10 and by anti-B2M mAb NAMB-1 were 94.0 (5.1–100), 91.0 (4.5–100) and 90.5 (62.2–100) in the pre-treatment metastases, and 63.5 (0–83.6), 25.0 (0–84.2) and 57.6 (0–93.1) in the post-treatment metastases, respectively. The percentage of melanoma cells stained by all three mAbs tested was higher than 80 in the majority of the 18 pre-treatment metastases analyzed, compared to only 1 of the 11 post-treatment metastases.

Comparison of the HLA class I subunit expression levels in pre- and post-treatment metastases removed from each individual patient revealed HLA-I downregulation mainly in the case of progressing lesions of nonresponding patients; in contrast, minimal or no change was found in responding patients (Pt1 and Pt2). Interestingly, in the case of Pt1 exhibiting the best overall response and long-term survival, the baseline HLA class I subunit expression was high in the pre-treatment metastases and remained high in the post-treatment (residual) metastases. In contrast, HLA class I subunit downregulation was maximal in the metastases from Pt5 and Pt6 exhibiting the shortest PFS and OS.

We also examined the extent of infiltration of CD8+ T cells in pretreatment vs. post-treatment tumors. The infiltration showed considerable intertumor variability and did not exhibit any consistent change between pre- and post-treatment time points.

We detected a very low number of NK cells infiltrating both pre-treatment and post-treatment tumors; furthermore, no significant difference could be found between the two sample sets.

Study II

Of the 40 stage IV melanoma patients involved in this study, 28 exhibited a complete (n=14) or partial response (n=14). Follow-up time of surviving patients was median 62.5 months (22–91). Among clinical parameters, the ECOG performance status and pretreatment LDH level showed an association with the response.

Pretreatment surgical samples were analyzed for HLA expression, evaluating it using scores between 0 and 2 for HLA class I and determining the percentage of positive tumor area in the case of HLA class II.

Considering all metastases irrespective of their source (patients, location), tumor cell HLA-I positivity levels determined by the four different antibodies (HCA2, HC10, NAMB-1, EMR8-5) showed a strong positive correlation, with the lowest correlation coefficient (0.6812) in the case of HCA2/HC10 and highest (0.8236) in the case of NAMB-1/EMR8-5 (all p values < 0.0001), while HLA-II staining showed a weaker correlation with HLA-I positivity (correlation coefficients 0.2182–0.2944). HLA class I expression scores were the highest in the case of the EMR8-5 pan-HLA-I antibody (mean ± SD, 1.4 ± 0.7) and the lowest in the case of HC10 (1.1 ± 0.8). Melanoma cells in metastases of 20 patients (50%) did not express HLA class II, while mean tumor cell staining higher than 10% was observed in 11 patients (27.5%). The tumor samples of eight patients showed 3–4% HLA-II staining, mainly in tumor cells near the inflammatory cells at the margin of metastases, consistent with locally induced HLA expression.

Among the responders, the proportion of patients showing HLA-II expression in ≥3% of melanoma cells was higher compared to non-responders (17/28 vs. 2/12, p=0.0158). Similarly, a significant difference was found in the case of two anti-HLA-I antibodies (HC10 and EMR8-5), with fewer cases showing decreased expression in responders. A combined score of HLA-I and -II expression was introduced (being positive in the case of high HLA class I and/or HLA class II expression), which proved very effective in predicting treatment response (p=0.0019).

Survival analysis demonstrated a near-significant (p = 0.0530) association of PFS with HLA class I expression detected by the HC10 antibody and a highly significant association (p = 0.0025) with HLA class II expression. The combined score of HLA class I and class II expression was also significantly linked to PFS (p = 0.0166). Overall survival analysis demonstrated significant association only with HLA class II expression (p=0.0126).

5. Conclusions

Study I

(1) In conclusion, we found a decreased HLA class I expression level by malignant cells in post-treatment progressing metastases of melanoma patients receiving ipilimumab therapy compared to pre-treatment metastatic samples. This finding was a consistent feature in our cohort of patients with progressing tumors, but was not observed in the residual metastases of a responding patient.

(2) The extent of infiltration of CD8+ T cells in pretreatment vs. post-treatment tumors did not exhibit any consistent change between pre- and post-treatment time points.

(3) We detected a very low number of NK cells infiltrating both pre-treatment and post-treatment tumors; no significant difference could be found between the two sample sets.

Study II

(1) Among the responders, the proportion of patients showing HLA-II expression in ≥3% of melanoma cells was higher compared to non-responders.

(2) Similarly, a significant difference was found in the case of two anti-HLA-I antibodies (HC10 and EMR8-5), with fewer cases showing decreased expression in responders.

(3) A combined score of HLA-I and -II expression was introduced, which proved very effective in predicting treatment response.

(4) Survival analysis demonstrated a significant association of PFS with HLA-II expression and with the combined score of HLA-I/II expression. Overall survival analysis demonstrated significant association only with HLA-II expression.

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