Distinct Molecular Mechanisms of Altered HLA Class II Expression in Malignant Melanoma

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Simple Summary: There exists limited knowledge about the underlying molecular processes controlling the expression of HLA class II APM components and their prognostic significance in melanoma. Therefore, this study analyzed the basal and regulated expression of HLA class II antigens and components in melanoma cell lines and patients’ lesions in conjunction to T-cell infiltration. The heterogeneous constitutive HLA class II APM expression was caused by distinct molecular mechanisms and was partially linked to immune cell infiltration and clinical parameters. These results contribute not only to a better understanding of the regulation of HLA class II expression in melanoma, but might have an impact on the design of novel (immuno)therapies for the treatment of this disease.

Abstract: Background: The human leukocyte antigen (HLA) class II molecules are constitutively expressed in some melanoma, but the underlying molecular mechanisms have not yet been characterized. Methods: The expression of HLA class II antigen processing machinery (APM) components was determined in melanoma samples by qPCR, Western blot, flow cytometry and immunohistochemistry. Immunohistochemical and TCGA datasets were used for correlation of HLA class II expression to tumor grading, T-cell infiltration and patients’ survival. Results: The heterogeneous HLA class II expression in melanoma samples allowed us to characterize four distinct phenotypes. Phenotype I totally lacks constitutive HLA class II surface expression, which is inducible by interferon-gamma (IFN-γ); phenotype II expresses low basal surface HLA class II that is further upregulated by IFN-γ; phenotype III lacks constitutive and IFN-γ controlled HLA class II expression, but could be induced by epigenetic drugs; and in phenotype IV, lack of HLA class II expression is not recovered by any drug tested. High levels of HLA class II APM component expression were associated with an increased intra-tumoral CD4+ T-cell density and increased patients’ survival. Conclusions: The heterogeneous basal expression of HLA class II antigens and/or APM components in melanoma cells is caused by distinct molecular mechanisms and has clinical relevance.

Keywords: HLA class II; CIITA; IFN; methylation; signal transduction

1. Introduction

The implementation of high-throughput technologies led to the identification of a large series of mutations, which appeared to be involved in the development, maintenance and progression of malignant melanoma (MM), but might also serve as suitable targets...
for T-cell-based immunotherapies, due to the creation of neo-antigens [1–3]. Despite that tumor-associated antigens (TAA) can be recognized by CD8+ cytotoxic T lymphocytes (CTL) in the context of HLA class I antigens, T-cell-based immunotherapies of melanoma might exhibit a lower efficacy than expected [4], and patients often develop resistances to these treatments [5]. This impaired response of MM patients is often associated with a downregulation or loss of HLA class I antigens and/or components of the HLA class I antigen-processing machinery (APM), leading to evasion from immune surveillance [6–8], disease progression and/or poor patients’ outcome [9,10], but their expression could be frequently upregulated by interferon (IFN)-α and IFN-γ [11,12]. There exists increasing evidence that HLA class II molecules encoded by HLA-DP, -DQ and -DR are also important for mounting an anti-tumoral immune responses, which can influence the prognosis of patients with various solid tumors and the efficacy of immunotherapies [13–17]. Recently, HLA class II surface expression has been shown to predict responses to anti-PD-1, but not to anti-CTLA-4 immunotherapy [18], suggesting its use as a potential biomarker for the responses prediction to specific immune checkpoint inhibitors (iCPIs) [14].

HLA class II surface molecules present foreign antigens to CD4+ T cells in order to initiate, control and/or maintain adaptive immune responses [19,20]. The HLA class II APM is complex and involves a number of components including the chaperones HLA-DM and HLA-DO. The expression of HLA class II antigens is tightly controlled by various transcription factors (TF), which are known to bind to highly conserved proximal promoter sequences of the HLA class II molecules [20–22]. The non-DNA-binding class II transactivator protein (CIITA) mediates the interaction between co-factors, chromatin remodeling factors and the general transcription machinery [23–25] and is central, but not sufficient, for the transcription of HLA class II antigens, which requires the presence of the enhanceosome complex. CIITA expression is transcriptionally regulated in a cell-type-specific manner, using different promoters [26]. Furthermore, promoter hypermethylation and histone acetylation can control HLA-DRα and CIITA transcription [27–32]. The expression of CIITA and selected HLA class II APM components could be reconstituted by the treatment with demethylating agents, histone deacetylase inhibitors (HDACi) and IFN-γ [20,33]. Furthermore, gene transfer of CIITA into tumor cells resulted in a stimulation of tumor specific CD4+ T cells in vivo associated with a long-lasting protective immunity [34], as well as an increased repertoire of tumor-associated HLA class II antigens [35].

Next to its physiologic expression on antigen presenting cells (APC), constitutive HLA class II expression was also detected in malignant cells. In freshly isolated primary and metastatic melanoma, 50–60% of tumor cells expressed HLA class II antigens [36]. In some tumor entities, HLA class II expression was associated with a favorable prognosis [37–39], while it correlated with a more aggressive phenotype and a higher risk of metastases in other cancers [40–43]. Concerning MM, there exist conflicting results regarding the role of HLA class II antigens in disease outcome and therapy response. Furthermore, a distinct expression pattern of HLA class II antigens was found during melanoma progression, suggesting a dynamic role of HLA class II function [44].

Based on these data, an increased knowledge concerning the underlying molecular processes controlling the expression of HLA class II APM components and their prognostic significance in MM is required. These might range from mutations in HLA class II regulatory genes [45] to transcriptional, posttranscriptional and epigenetic control [28,46–51]. Furthermore, the immune-cell repertoire might influence the HLA class II expression, since HLA-class-II-pathway component expression has been shown to be associated with B and T cell infiltration [52]. Therefore, this study analyzed the basal expression of HLA class II antigens and selected components of the HLA class II APM in MM cell lines and lesions, its regulation by IFN-γ and epigenetic drugs and its correlation to T-cell infiltration, in order to delineate the processes leading to the heterogeneous HLA class II expression in this disease. The clinical relevance of these results was demonstrated by correlation of the HLA class II APM expression and immune cell infiltration to tumor grading and to the patients’ survival by analysis of a melanoma dataset from The Cancer Genome Atlas (TCGA).
2. Materials and Methods

2.1. Melanoma Cell Lines, Cell Culture and Treatment

The melanoma cell lines used in this study were either provided by Dr. Soldano Ferrone (Harvard University, Boston, MA, USA) or obtained from ESTDAB cell bank (now transferred to the European Collection of authenticated cell cultures, https://www.phe-culturecollections.org.uk/products/celllines/generalcell/browse.jsp, accessed on 12 May 2021, and their characteristics have been described elsewhere [53,54]). The melanocytes were purchased from Lonza (Pharma&Biotech, Basel, Switzerland) and cultured in melanocytes growth medium (MGM-4 Bullet Kit; Lonza Biosciences, Basel, Switzerland). All melanoma cell lines (n = 47) were maintained in RPMI1640 medium supplemented with 1% 100 mM L-glutamine, 10% fetal calf serum (FCS) and respective antibiotics.

For determination of the IFN inducibility of HLA class II expression, melanoma cell lines were either left untreated or treated for 24 and/or 48 h with 400 U/mL recombinant IFN-α (Prospec, Rehovot, Israel) or IFN-γ (PAN Chemicals, Sofia, Bulgaria) respectively. For epigenetic studies, melanoma cell lines were daily treated with fresh medium containing the demethylating agent 5′-aza-2′-desoxycytidine (AZA; Sigma, Saint Louis, MO, USA; 1–10 µM) or the histone deacetylase inhibitors (HDACi) entinostat (ENT; Selleck Chemicals, Munich, Germany; 1–5 µM) or trichostatin A (TSA; 200 ng/mL; Sigma, Taukirchen, Germany), respectively, for the indicated time points alone and/or in combination with IFN-γ.

2.2. qPCR Analysis

Total cellular RNA from melanoma cell lines and melanocytes was prepared and reverse transcribed into cDNA, as recently described [54]. Then qPCR was performed on a Rotorgene 6.000 system (Corbett Research, Sydney, Australia), employing the platinum SYBR Green qPCR Supermix-UPG (Invitrogen, Carlsbad, CA, USA) and respective primers listed in Table S1, using standard protocols, as recently described [55]. The mRNA levels were normalized to the expression of glyceratealdehyde-3-phosphate dehydrogenase (GAPDH). Data were analyzed with a comparative quantification mode of the Rotor gene 6.000 software version 1.7. The results were normalized to melanocytes. All qPCR analyses were performed with RNA from at least 3 independent experiments. The scoring of the mRNA expression levels was based on cycles and categorized as “1” (>26 cycles), “2” (22–26 cycles), “3” (18–21 cycles), “4” (14–17 cycles) and “5” (10–13 cycles).

2.3. Monoclonal Antibodies

For flow cytometry and/or immunohistochemistry (IHC), following monoclonal antibodies (mAb) for staining of HLA class II APM components, we used anti-pan-HLA class II, anti-HLA-DR, anti-HLA-DP, anti-HLA-DQ, anti-CIITA, anti-HLA-DM, anti-HLA-DO and anti-Ii (Table S2). Staining with an anti-HLA class I mAb (Table S2) was performed to determine the IFN responsiveness.

2.4. Flow Cytometry

For flow cytometry, 5 × 10^5 cells either left untreated or treated for the indicated time points with IFN-γ and/or DAC, TSA or ENT, respectively, were incubated with a fluorescence-labeled anti-human pan-HLA class II; anti-human HLA-DR, -DP and -DQ mAbs; or isotype controls for 1 h. After washing, HLA class II surface expression was measured on a NAVIOS flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed by using the Kaluza Software. The data were expressed as x-fold increase in mean fluorescence intensity (MFI) of the total population over the isotype control. A representative gating strategy is shown in Figure S1. The x-fold MFI of 1 was scored negative; MFI > 50 was scored high; and MFI was between 10 and 50, was scored low (<20% positive cells) or was medium (>20% positive cells).
2.5. Analysis of CIITA Methylation Pattern

For determination of the methylation status of the CIITA promoter, combined bisulfite restriction analysis (COBRA) and direct sequencing of bisulfite-treated DNA was performed, as recently described [54]. For COBRA, a nested PCR was performed with primers (Table S1B), and PCR products were digested with the restriction enzymes BstUI, Taq I, RsaI or Hpy188I (New England Biolabs, Frankfurt, Germany), recognizing CpG-specific sequences. The PCR products were then separated on 3% agarose gels. For determination of the methylation pattern, the degree of cleavage compared to the corresponding uncleaved control was categorized into different groups, as previously described [54].

2.6. Immunohistochemistry of Tissue Microarrays (TMAs)

The expression of HLA class II APM pathway components was determined in a melanoma-specific TMA consisting of 368 primary malignant melanoma lesions with available pT status for 362 samples, 39 metastases and 62 benign nevi, using conventional IHC [55–57]. Paraffin-embedded tissue blocks were stained with the respective primary antibodies, overnight, at 4 °C, followed by immunostaining with the UltraView Universal Alkaline Phosphatase Red Detection Kit (Ventana, Tucson, AZ, USA), as recently described [56]. Slides were counterstained with hematoxylin, dehydrated and mounted. The immune reactivity was defined according to the following scoring system: 0 = negative, 1 = 1–20%, 2 = 20–50%, 3 = 50–70% and 4 = 70–100%.

2.7. Statistical Analysis

Statistical analysis was performed with SigmaPlot Version 11 (Inpixon HQ, Palo Alto, CA, USA) using the Student’s t-test; p-values of < 0.05 were considered as significant and are indicated in the figures. Experiments were performed 2 or 3 times. For contingency table analysis, two-sided chi-square and Fisher’s exact test were employed for statistical correlation between clinicopathological and immunohistochemical parameters of the tumor samples. The tumor stage was correlated to the expression of HLA class II APM pathway components, CD4+ and CD8+ T cell infiltration, using the statistic software RV 4.0.2. Kruskal–Wallis tests were applied to compare the expression of individual components between different pT stages. Bonferroni corrections were applied to adjust for multiple testing, and adjusted values of < 0.05 were considered significant.

2.8. Bioinformatics Evaluation of Clinical Relevance

For the correlation of HLA class II APM component expression with overall survival (OS) of melanoma patients, the r2 database (https://hgserver1.amc.nl, accessed on 20 April 2021 with the TCGA melanoma Tumor Skin Cutaneous Melanoma (SKCM) study was used. A total of 470 samples from melanoma metastasis were separately included in the analysis. A p-value of < 0.05 was considered as significant. The following settings were chosen: Kaplan–Meier by gene expression; cutoff modus, median; and follow-up time, 370 months. Inclusion criteria: number of samples in subset, n = 468; no subset selected.

3. Results

3.1. HLA Class II Expression in Melanoma Cells

Since the role of HLA class II antigens in melanoma is controversially discussed, a TMA consisting of 368 melanoma lesions was stained with an anti-pan-HLA class II-specific mAb antibody. Overall, 326/368 melanoma lesions gave informative IHC results, demonstrating a heterogeneous intratumoral staining. Representative stainings of melanoma lesions with a distinct HLA class II status (HLA class II high, HLA class II medium and HLA class II low) are shown in Figure 1. Overall, 126/326 melanoma lesions were defined as negative, 95/326 as low expressors, 68/326 as medium expressors and 37/326 as high expressors, with a staining mainly localized at the cell surface (Table 1). In contrast, no HLA class II expression was found in all benign nevi analyzed.
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![Figure 1. Representative immunohistochemical analysis of HLA class II antigens on melanoma lesions. IHC analysis was performed as described in Materials and Methods. Tissues were stained with the anti-HLA class II antibody LGII-612.14. Shown are representative stainings of a HLA class II negative (spot 150), HLA class II medium (spot 163) and HLA class II high (spots 7 and 259) expressing lesions. Scale bar: 100 µm.](image)

Table 1. Distinct protein expression pattern of HLA class II antigens and/or APM components in melanoma lesions and melanoma cell lines.

| Samples                | HLA-II APM       | n 1   | High     | Medium   | Low      | Negative |
|------------------------|------------------|-------|----------|----------|----------|----------|
| Melanoma lesions       | HLA class II     | 326   | 37 (11.33%) | 68 (20.86%) | 95 (29.14%) | 126 (38.65%) |
| HLA-DO                 | 294              | 0     | 13 (4.42%)  | 26 (8.84%)  | 255 (86.73%) |
| CIITA tumor            | 314              | 0     | 1 (0.32%)   | 23 (7.32%)   | 284 (90.45%) |
| CIITA lymph.           | 314              | 12 (3.82%) | 55 (17.52%) | 139 (44.27%) | 108 (34.39%) |
| Melanoma cell lines    | HLA class II     | 47    | 4 (8.51%)   | 13 (27.66%) | 5 (10.64%) | 25 (53.19%) |

1 Number of samples analyzed.

The heterogeneous HLA class II antigen expression in situ was further confirmed in melanoma cell lines (n = 47), using melanocytes as a control. Moreover, 45% of melanoma...
cell lines (22/47) constitutively expressed HLA class II surface antigens, which varied from low/medium to high expression levels, while 25/47 melanoma cell lines lacked HLA class II surface expression. This led to the categorization of melanoma cell lines into negative, low/medium and high expressors (Tables 1 and 2).

Table 2. Characterization of the HLA class II APM component expression in melanoma cell lines.

| Cell Line  | CIITA | HLA-DRα | HLA-pan li | Cathepsin | HLA-DMα | HLA-DMβ | HLA-DOA | HLA-DOB | CLIP | % Positive |
|------------|-------|---------|------------|-----------|---------|---------|---------|---------|-----|------------|
| melanocytes| 2     | 2       | 3          | 3         | 3       | 3       | 2       | 1       | 2   | 2%         |
| HaCaT      | 2     | 2       | 3          | 4         | 3       | 3       | 2       | 1       | 2   | 2%         |
| BUF586     | 1     | 1       | 2          | 3         | 3       | 3       | 2       | 1       | 1   | 1%         |
| Mel624     | 1     | 1       | 2          | 3         | 3       | 3       | 1       | 1       | 1   | 1%         |
| BUF1495    | 1     | 1       | 2          | 3         | 3       | 3       | 2       | 1       | 1   | 1%         |
| BUF1286    | 1     | 1       | 2          | 3         | 3       | 3       | 2       | 1       | 1   | 1%         |
| BUF1182    | 1     | 2       | 3          | 1         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF5536    | 1     | 1       | 1          | 3         | 3       | 3       | 2       | 1       | 1   | 1%         |
| FM6        | 1     | 1       | 1          | 3         | 3       | 3       | 2       | 1       | 1   | 1%         |
| BUF1330    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 2       | 3   | 2%         |
| BUF501ATC  | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 2%         |
| BUF1011    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1195    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1383    | 1     | 1       | 1          | 3         | 2       | 1       | 1       | 1       | 1   | 1%         |
| FM81       | 1     | 1       | 1          | 3         | 2       | 1       | 1       | 1       | 1   | 1%         |
| BUF1402    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1287    | 1     | 1       | 1          | 3         | 3       | 2       | 1       | 1       | 1   | 1%         |
| J727/23    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| SkMel2911  | 1     | 1       | 1          | 3         | 2       | 2       | 1       | 1       | 1   | 1%         |
| BUF1280    | 1     | 1       | 1          | 3         | 2       | 2       | 1       | 1       | 1   | 1%         |
| Brooks 86  | 1     | 1       | 1          | 3         | 3       | 2       | 1       | 1       | 1   | 1%         |
| UKRVMeli1a | 1     | 1       | 1          | 3         | 3       | 2       | 1       | 1       | 1   | 1%         |
| BUF1379    | 1     | 1       | 1          | 3         | 3       | 2       | 1       | 1       | 1   | 1%         |
| COL0857    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| FM39       | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1520    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF624     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| WM1362     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| MZMel3     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| M17        | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| FM82       | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1088    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| FM39       | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF357     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| FM28       | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| FM34       | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| FM3        | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| MKR        | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| WM1352c    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| 2058 Brooks | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF526     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1317    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| Gk-M       | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| Me1359     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| MelU50     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| COLO794    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| MZMel2     | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| ZKR        | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |
| BUF1102    | 1     | 1       | 1          | 2         | 2       | 3       | 1       | 1       | 1   | 1%         |

1 Heat map based on the takeoff: “1” is after 26 cycles, “2” between 22 and 26, “3” between 18 and 21, “4” between 14 and 17, and “5” between 10 and 13; 2 antibody staining.
3.2. Correlation of Heterogeneous HLA Class II Surface Antigen Expression with Altered APM Component Expression

Since the heterogeneous basal HLA class II surface expression detected in melanoma lesions and cell lines might be due to altered mRNA transcription, levels of the major HLA class II APM components were determined by qPCR. As shown in the heat map in Table 2 and summarized in Table 3, highly variable mRNA levels were detected for CIITA, HLA-DR, -DM and -DO; CLIP; the invariant chain (li); and cathepsin S in the melanoma cell lines analyzed, which were directly associated with HLA class II surface expression levels determined by flow cytometry (Table 2). Melanoma cell lines lacking HLA class II surface antigens expressed low-to-marginal transcript levels of some major HLA class II APM component, while high HLA class II expressors exerted high mRNA expression levels of most major HLA class II components analyzed (Table 2). Interestingly, the frequency of HLA class II component expression highly varied between the molecules (Table 3).

Table 3. Heterogeneous mRNA levels of HLA class II APM components in melanoma cell lines.

| HLA Component | Melanoma Cells |
|---------------|----------------|
|               | High | Medium | Negative |
| CIITA         | 3    | 21     | 23       |
| CLIP          | 16   | 13     | 18       |
| pan-li        | 16   | 23     | 8        |
| HLA-DRα       | 15   | 11     | 21       |
| HLA-DOα       | 5    | 12     | 30       |
| HLA-DOβ       | 0    | 19     | 28       |
| cathepsin S   | 1    | 40     | 6        |
| HLA-DMα       | 11   | 36     | 0        |
| HLA-DMβ       | 6    | 36     | 5        |

Furthermore, the immunohistochemical staining of the TMA demonstrated a heterogeneous expression pattern of HLA-DO and CIITA proteins in the melanoma lesions. Overall, 255/294 informative cases lacked HLA-DO expression, and 26/294 cases expressed low HLA-DO levels, while 13/294 cases analyzed expressed medium HLA-DO levels. In addition, a heterogeneous, but also a distinct expression pattern of CIITA was found between lymphocytes and tumor cells. In tumor cells, 284/314 cases lacked CIITA expression, 23/314 expressed low and 1/314 lesions medium levels of CIITA. In contrast, only 108/314 cases were negative for CIITA staining in immune cells, 139/314 cases showed a low, 55/314 cases a medium and 12/314 cases a high CIITA expression (Table 1).

3.3. Distinct Responsiveness of Melanoma Cells to IFN-γ

IFN-γ is a strong inducer of HLA class II APM expression in professional APC, but also in non-APC, including tumor cells [58]. Therefore, HLA-class-II-negative (16/25) and selected constitutively HLA-class-II-expressing (9/22) melanoma cell lines were treated with IFN-γ for 24 and 48 h prior to the analyses of HLA class II surface expression, using flow cytometry. Treatment of melanoma cells with IFN-α served as control. IFN-γ, but not IFN-α, treatment (Figure S2A) significantly upregulated HLA class II surface expression in 5/11 HLA class II-negative melanoma cells, but to a distinct extend regarding its kinetics and intensity (Figure 2a). As representatively shown for three selected melanoma cell lines and HaCat as a control, the upregulation of HLA class II mRNA (Figure 3) and surface expression (Figure 2a) by IFN-γ was associated with an increased expression of some HLA class II APM components, e.g., HLA-DO, HLA-DM, CIITA, CLIP and cathepsin S (Figure 3). It is noteworthy that both IFN-γ (Figure 2b) and IFN-α (Figure S2B) were able to upregulate HLA class I surface expression in these melanoma cell lines, suggesting a functional IFN-γ signaling pathway.
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Figure 2. Heterogeneous basal and IFN-γ inducible surface expression of HLA class I and II antigens. Representative melanoma cells (n = 21) were left untreated or treated for 24 and 48 h with IFN-γ, before flow cytometric analysis of HLA class II surface expression was determined, as described in Material and Methods (a). Staining with a HLA-ABC antibody served as a control for IFN-γ responsiveness (b). The results are presented as x-fold in MFI over the isotype control.

3.4. Upregulation of HLA Class II Surface Expression upon Treatment of Melanoma Cells with Epigenetic Drugs

The lack of basal and IFN-γ inducible HLA class II expression was detected in 11/16 melanoma cell lines, which might be due to epigenetic silencing mediated by methylation or altered histone acetylation [47,59]. To study whether both processes are responsible for the lack of HLA class II surface expression, different melanoma cell lines were treated with AZA, HDACi or a combination of both for 5 days, followed by mRNA analysis of major HLA class II APM components or flow cytometric analysis of HLA class II surface antigens. Moreover, 2/11 HLA class-II-negative and IFN-γ resistant melanoma cell lines induced HLA class II mRNA expression (Figure 4a), but not HLA class II surface expression (data not shown), upon AZA treatment. In addition, AZA treatment had also no effect on HLA class II surface expression of constitutive HLA class II expressing melanoma cell lines. Treatment with TSA in combination with AZA had additive effects on the mRNA HLA class II expression (Figure 4a), suggesting that both methylation and histone acetylation are underlying molecular mechanisms impairing HLA class II mRNA expression in some melanoma cells [60]. The AZA- and TSA-mediated upregulation of HLA class II mRNA expression was accompanied by an increased expression of HLA class II APM components, but this significantly differed between the HLA class II APM components, as well as between the distinct cell lines analyzed. This is representatively shown for HLA-DR, CLIP and CIITA in Figure 4a,b. Similar results were obtained by using ENT as HDACi.

3.5. CIITA Expression as a Major Regulator of the HLA Class II Surface Expression

Since the HLA class II transactivator CIITA has been shown to be involved in chromatin remodeling [61] and the HLA class II surface antigens could be induced in some melanoma cell lines, it was analyzed whether the lack of HLA class II surface antigen is due to the methylation of the CIITA promoter, as already shown for gastric and colorectal cancer, for example [62]. Therefore, the CIITA methylation status of melanoma cells (19/47)
was investigated by COBRA analysis [63], demonstrating a total or partial methylation (25–75%) of the CpG islands in the CIITA promoter. CIITA was frequently methylated in HLA class II \textit{low/neg} melanoma cells, which did not respond to IFN-\(\gamma\) despite a functional IFN-\(\gamma\) pathway (Figure 4c), but could be reverted by the AZA treatment.

**Figure 3.** Link of IFN-\(\gamma\)-mediated upregulation of HLA class II surface expression with HLA class II APM components. The expression of HLA class II APM components of selected untreated and IFN-\(\gamma\) treated (24 h) melanoma cell lines was determined by qPCR. The data were normalized to \(\beta\)-actin expression, and relative mRNA expression was presented as bar charts by setting mRNA transcript levels of untreated cells to 1.
induced HLA class II mRNA expression (Figure 4a), but not HLA class II surface expres-
sion (data not shown), upon AZA treatment. In addition, AZA treatment had also no effect
on HLA class II surface expression of constitutive HLA class II expressing melanoma cell
lines. Treatment with TSA in combination with AZA had additive effects on the mRNA
HLA class II expression (Figure 4a), suggesting that both methylation and histone acety-
lation are underlying molecular mechanisms impairing HLA class II mRNA expression in
some melanoma cells [60]. The AZA- and TSA-mediated upregulation of HLA class II
mRNA expression was accompanied by an increased expression of HLA class II APM
components, but this significantly differed between the HLA class II APM components,
as well as between the distinct cell lines analyzed. This is representatively shown for HLA-
DR, CLIP and CIITA in Figure 4a,b. Similar results were obtained by using ENT as
HDACi.

Figure 4. Epigenetic control of HLA class II APM components. Melanoma cells were left untreated or treated for 48 h with
AZA (10 µM), TSA (200 ng/mL) and/or a combination of both, before mRNA expression of HLA-DR I (a) and HLA class II
APM components (b) was determined by qPCR. The data are expressed as relative mRNA levels by setting untreated cells
as 1. The data are shown as mean ± SE from two different experiments. (c) The methylation status of CIITA was analyzed
in HLA class II negative melanoma cell lines, as described in Materials and Methods. The results are presented as total,
partial (25–75%) and no methylation.
3.6. Correlation of HLA Class II APM Components and Immune Cell Infiltration with Clinical Relevance

In order to determine the clinical relevance of HLA class II expression, the basal HLA class II and/or CIITA expression of melanoma lesions, as well as the level of immune cell infiltration, was correlated to tumor staging. As shown in Figure 5, the distinct HLA class II expression was not correlated to tumor grading ($p = 0.2975$). In contrast, CIITA expression in lymphocytes, but not in tumor cells, correlated to tumor staging ($p = 0.0029$ and $p = 1$, respectively). The tumor stage was further associated with the frequency of CD4+ T-cell infiltration, which was the highest in pT1 melanoma and the lowest in pT4 tumors ($p = 0.0027$). However, no statistically significant correlation exists between tumor stage, IFN-$\gamma$ and HLA-DO (Figure S3). In summary, tumor grading is correlated with CD4+ T-cell infiltration and CIITA expression.

![Figure 5](image_url)

**Figure 5.** Correlation of HLA class II APM expression and immune cell infiltration with tumor staging. The staining of the TMA was performed as described in Materials and Methods. Expression of CIITA in lymphocytes and CD4+ T-cell infiltration correlated to pT stages of tumor samples.
Furthermore, for comparison of the prognostic relevance of HLA class II APM component expression, in silico analyses of TCGA data were performed by using datasets from 234<sup>high</sup> and 234<sup>low</sup> HLA class II APM component expressors. As shown in Figure 6, the HLA class II APM<sup>high</sup> patient group has a significantly increased overall survival (OS) compared to patients with HLA class II APM<sup>low</sup> expression.

![Kaplan Curves](image)

**Figure 6.** Correlation of HLA class II APM expression with the survival of melanoma patients, using TCGA data. For analysis of the OS of melanoma patients, the TCGA data were analyzed as described in Material and Methods.

4. Discussion

Despite the fact that HLA class II expression is mainly found on APC, basal expression of HLA class II antigens has been also detected on distinct tumor types—particularly in hematopoietic malignancies, but also solid tumors—while others totally lack HLA class II expression. The constitutive HLA class II expression on tumor cells results in their recognition by tumor-antigen-specific CD4<sup>+</sup> T cells, generating a Th1 response [64]. Since the role of HLA class II molecules in MM has not yet been characterized in detail, this study...
determined the frequency of basal HLA class II surface expression in a large cohort of melanoma lesions by staining different TMAs. These data were correlated to the immune cell infiltration and to tumor staging. In addition, a large number of melanoma cell lines (n = 47) were analyzed regarding their constitutive and inducible HLA class II surface antigen expression in order to determine the underlying molecular mechanisms of deficient HLA class II expression in MM. A distinct HLA class II surface expression pattern was found on both melanoma cell lines and melanoma lesions, with a relatively high frequency when compared to other solid tumors entities [15,65,66]. Low levels of HLA class II surface antigens in melanoma samples were associated with a high tumor grading, while high levels of HLA class II antigens were found in pT1 melanoma. These data suggested a clinical relevance of HLA class II antigen expression. In order to get further insights into the clinical impact of HLA class II expression the HLA class II staining pattern should have been correlated to the OS of patients. Unfortunately, survival data were not available for our TMA cohort. Therefore, we used TCGA data as surrogate analysis data, confirming the association of HLA class II and tumor staging and further extended these results to patients’ survival.

It has been reported by various groups that the underlying mechanisms responsible for the highly variable HLA class II expression in tumors are broad, but they have been mainly characterized in hematological disorders [67]. These include different genomic alterations of HLA class II molecules, which have been identified in Non-Hodgkin lymphoma, such as deletions, mutations and chromosomal rearrangements, leading to an impaired HLA class II expression and resistance to IFN-γ treatment [68,69]. Loss of HLA class II expression and transcriptional silencing of HLA class II molecules frequently occur in leukemia relapses after human-stem-cell transplantation [70]. The deficient HLA class II expression could be reverted in some cases by IFN-γ treatment due to IFN-responsive elements in the promoters of some HLA class II APM components. In addition, epigenetic control including methylation and histone deacetylation is often involved in the lack of basal, as well as IFN-γ-induced expression of HLA class II surface antigens, and could be reverted by demethylating agents and HDAC [47,71]. Using melanoma cell lines as models, a classification of MM into four distinct phenotypes was established: phenotype I exhibits basal, but heterogeneous HLA class II expression. The phenotype II in MM lacks HLA class II surface expression, which is IFN-γ inducible and also accompanied by an upregulation of some major HLA class II APM components. Furthermore, IFN-γ not only influences HLA class II expression directly through the EnhA, ISRE and CRE elements, but also by upregulation of CIITA [72]. This appears to be associated with the level of T-cell infiltration due to IFN-γ secretion by immune cells. However, some melanoma cells lack not only basal, but also an IFN-γ mediated upregulation of HLA class II antigens despite a functional IFN-γ signaling pathway [73]. In phenotype III, the reduced or missing HLA class II expression is due to the epigenetic control, since treatment of melanoma cells with the demethylation agent AZA and/or the HDACi TSA results in an upregulation of HLA class II expression. Thus, DNA methylation or altered histone acetylation of HLA class II antigens plays an important role in the modulation of HLA class II APM component expression, which is also of clinical relevance. Combination of AZA and ENT significantly reduced tumor growth and increased patient-derived HLA class II expression in xenografts [74].

One key molecule involved in the regulation of basal HLA class II expression is CIITA, which is of pathologic relevance in rare, but severe immune disorders [75]. Furthermore, loss-of-function mutations in CIITA resulted in the lack of HLA class II expression, which was found in various tumor types [76], while a substitution of A to G in the 5′ flanking region of the CIITA promoter was associated with a higher expression [73]. Our data suggested that the methylation of the CIITA promoter in HLA class II negative, IFN-γ-resistant MM cell lines frequently occurred, but the methylation status between the MM cell lines analyzed was highly variable from total methylation to partial (25–75%) methylation. This is in line with reports demonstrating a frequent promoter methylation of CIITA in different cancer types that was associated with an impaired HLA class II expression and
could not be reverted by IFN-γ, but by demethylating agents [77], as shown for ovarian cancer [78], diffuse large B-cell lymphoma [79] and breast cancer [80]. However, the lack of basal or downregulated CIITA expression might be due to other mechanisms, such as an upregulation of microRNAs (miRNAs) targeting the 3′ UTR of CIITA [51].

The highly variable expression of HLA class II antigens and APM components is in line with TCGA RNA sequencing data, demonstrating an association of higher expression levels of HLA class II genes—in particular, of HLA-DP and -DR—with a better survival of melanoma patients [81]. The HLA class II expression of human tumor cells may contribute to an enhanced tumor immunity, since it can induce HLA class II-restricted CD4+ T-cell responses. Pathway analyses of melanoma cell lines expressing HLA class II antigens under basal or IFN-γ stimulated conditions demonstrated signatures for PD-L1 signaling, allograft rejection and T-cell-receptor signaling. Furthermore, HLA class II antigen expression was associated with an increased therapeutic response and improved patients’ outcome [14].

In previous publications, highly variable basal HLA class II expression levels were described in primary melanoma lesions and melanoma cell lines. These were also associated with an altered frequency of tumor infiltrating CD4+ and CD8+ T cells. However, controversial results regarding the role of HLA class II antigen expression for prognosis and OS of MM patients, and in particular for those treated with immunotherapies, exist [14,16,65–67]. Thus, there is an urgent need to further characterize the role of HLA class II antigens in the context of immune cell infiltration and determine the HLA class II expression in large cohorts of melanoma patients responding and non-responding to immunotherapy.

5. Conclusions

In this study, the expression of HLA class II antigen and APM component was evaluated on multiple melanoma cell lines, as well as in patients’ specimen. Different patterns of constitutive and inducible expression of HLA class II molecules were found, which also correlated with the patients’ clinical outcome. These results contribute not only to a better understanding of the regulation of HLA class II expression in melanoma, but might have an impact on the design of novel (immuno)therapies for the treatment of this disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13153907/s1. Figure S1: Gating strategy for melanoma cell lines. Figure S2: Lack of IFN-α inducibility of HLA class II, but not of HLA class I surface expression. Figure S3: Correlation of the expression of IFN-γ, CD8, CIITA (tu) and HLA-DOB to pT stages of melanoma samples and/or immune cells. Table S1: Primers used for PCR analysis. Table S2: Antibodies used for flow cytometric analysis of protein expression.

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References
1. Delamarre, L.; Mellman, I.; Yadav, M. Neo approaches to cancer vaccines. Science 2015, 348, 760–761. [CrossRef]
2. Kalaora, S.; Barnea, E.; Merhavi-Shoham, E.; Qutob, N.; Teer, J.K.; Shimony, N.; Schachter, J.; Rosenberg, S.A.; Besser, M.J.; Admon, A.; et al. Use of HLA peptidomics and whole exome sequencing to identify human immunogenic neo-antigens. Oncotarget 2016, 7, 5110–5117. [CrossRef]
3. Weizman, E.; Cohen, C.J. Engineering T-Cell Specificity Genetically to Generate Anti-melanoma Reactivity. *Methods Mol. Biol.* 2015. [CrossRef]  
4. Valpione, S.; Campana, L. Immunotherapy for advanced melanoma: Future directions. *Immunotherapy* 2016, 8, 199–209. [CrossRef] [PubMed]  
5. Kelderman, S.; Schumacher, T.; Haanen, J.B. Acquired and intrinsic resistance in cancer immunotherapy. *Mol. Oncol.* 2014, 8, 1132–1139. [CrossRef] [PubMed]  
6. Aptsiauri, N.; Carretero, R.; Garcia-Lora, A.; Real, L.M.; Cabrera, T.; Garrido, F. Regressing and progressing metastatic lesions: Resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations. *Cancer Immunol. Immunother.* 2008, 57, 1727–1733. [CrossRef] [PubMed]  
7. Carretero, R.; Wang, E.; Rodriguez, A.J.; Reinboth, J.; Ascierto, M.L.; Engle, A.M.; Liu, H.; Camacho, F.M.; Marincola, F.M.; Garrido, F.; et al. Regression of melanoma metastases after immunotherapy is associated with activation of antigen presentation and interferon-mediated rejection genes. *Int. J. Cancer* 2011, 131, 387–395. [CrossRef] [PubMed]  
8. Tjin, E.P.M.; Konijnemberg, D.; Krebbers, G.; Drijfhout, J.W.; Franken, K.L.M.C.; Van Der Horst, C.M.A.M.; Bos, J.D.; Nieweg, O.E.; Kroon, B.B.R.; et al. T-Cell Immune Function in Tumor, Skin, and Peripheral Blood of Advanced Stage Melanoma Patients: Implications for Immunotherapy. *Clin. Cancer Res.* 2011, 17, 5736–5747. [CrossRef]  
9. Del Campo, A.B.; Kyte, J.A.; Carretero, J.; Zinchencso, S.; Méndez, R.; González-Aseguinolaza, G.; Ruiz-Cabello, F.; Aamdal, S.; Gaudernack, G.; Garrido, F.; et al. Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma. *Int. J. Cancer* 2015, 134, 102–113. [CrossRef]  
10. Seliger, B.; Maaeuer, M.; Ferrone, S. Antigen-processing machinery breakdown and tumor growth. *Immunol. Today* 2000, 21, 453–464. [CrossRef]  
11. Abuzahra, F.; Heise, R.; Joussen, S.; Dreuw, A.; Merk, H.; Zwadlo-Klarwasser, G.; Baron, J.M. Adjuvant interferon alfa treatment for patients with malignant melanoma stimulates transporter proteins associated with antigen processing and proteasome activator 28. *Lancet Oncol.* 2004, 5, 250. [CrossRef]  
12. Seliger, B.; Ritz, U.; Abele, R.; Bock, M.; Tampé, R.; Sutter, G.; Drexler, I.; Huber, C.; Ferrone, S. Immune escape of melanoma: First evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway. *Cancer Res.* 2001, 61, 8647–8650. [CrossRef]  
13. Donia, M.; Andersen, R.; Kjeldsen, J.W.; Fagone, P.; Munir, S.; Nicoletti, F.; Andersen, M.H.; Straten, P.T.; Svane, I.M. Aberrant Expression of MHC Class II in Melanoma Attracts Inflammatory Tumor-Specific CD4+ T-Cells, Which Dopamine CD8+ T-cell Antitumor Reactivity. *Cancer Res.* 2015, 75, 3747–3759. [CrossRef]  
14. Johnson, D.B.; Estrada, M.V.; Salgado, R.; Sanchez, V.; Doxie, D.; Opalenik, S.R.; Vilgelm, A.; Feld, E.; Johnson, A.S.; Greenplate, A.R.; et al. Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy. *Nat. Commun.* 2016, 7, 10582. [CrossRef]  
15. Seliger, B.; Kloor, M.; Ferrone, S. HLA class II antigen-processing pathway in tumors: Molecular defects and clinical relevance. *Oncoimmunology* 2017, 6, e1171447. [CrossRef] [PubMed]  
16. Dunne, M.R.; Phelan, J.J.; Michielsen, A.J.; Maguire, A.A.; Dunne, C.; Martin, P.; Noonan, S.; Tosetto, M.; Geraghty, R.; Fennelly, D.; et al. Characterising the prognostic potential of HLA-DR during colorectal cancer development. *Cancer Immunol. Immunother.* 2020, 69, 1577–1588. [CrossRef] [PubMed]  
17. Stewart, R.L.; Matynia, A.P.; Factor, R.E.; Varley, K.E. Spatially-resolved quantification of proteins in triple negative breast cancers reveals differences in the immune microenvironment associated with prognosis. *Sci. Rep.* 2020, 10, 1–8. [CrossRef]  
18. Rodig, S.J.; Gusenleitner, D.; Jackson, D.G.; Gjibi, E.; Giobbie-Hurder, A.; Jin, C.; Chang, H.; Lovitch, S.B.; Horak, C.; Weber, J.S.; et al. MHC proteins confer differential sensitivity to CTLA-4 and PD-1 blockade in untreated metastatic melanoma. *Sci. Transl. Med.* 2018, 10, eaar3342. [CrossRef]  
19. Hirschberg, H.; Braathen, L.R.; Thorby, E. Antigen Presentation by Vascular Endothelial Cells and Epidermal Langerhans Cells: The Role of HLA-DR. *Immunol. Rev.* 1982, 66, 57–77. [CrossRef]  
20. Morris, A.C.; Beresford, G.W.; Mooney, M.R.; Boss, J.M. Kinetics of a Gamma Interferon Response: Expression and Assembly of CIITA Promoter IV and Inhibition by Methylation. *Mol. Cell. Biol.* 2002, 22, 4781–4791. [CrossRef]  
21. Boss, J.M.; Jensen, P.E. Transcriptional regulation of the MHC class II antigen presentation pathway. *Curr. Opin. Immunol.* 2003, 15, 105–111. [CrossRef]  
22. Elsen, P.J.V.D.; Holling, T.M.; Kuipers, H.F.; van der Stoep, N. Transcriptional regulation of antigen presentation. *Curr. Opin. Immunol.* 2004, 16, 67–75. [CrossRef] [PubMed]  
23. Beresford, G.W.; Boss, J.M. CIITA coordinates multiple histone acetylation modifications at the HLA-DRA promoter. *Nat. Immunol.* 2001, 2, 652–657. [CrossRef] [PubMed]  
24. Boss, J.M. Regulation of transcription of MHC class II genes. *Curr. Opin. Immunol.* 1997, 9, 107–113. [CrossRef]  
25. Mühlethaler-Mottet, A.; Villard, J.; Zufferey, M.; Steimle, V.; Reith, W. CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhancerosome complex. *Genome Res.* 2000, 14, 1156–1166. [CrossRef]  
26. Mühlethaler-Mottet, A.; Otten, L.A.; Steimle, V.; Mach, B. Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *EMBO J.* 1997, 16, 2851–2860. [CrossRef]
27. Choi, N.M.; Boss, J.M. Multiple Histone Methyl and Acetyltransferase Complex Components Bind the HLA-DRA Gene. PLoS ONE 2012, 7, e37554. [CrossRef]

28. Majumder, P.; Boss, J.M. DNA methylation dysregulates and silences the HLA-DQ locus by altering chromatin architecture. Genes Immun. 2011, 12, 291–299. [CrossRef]

29. Mehta, N.T.; Truax, A.D.; Boyd, N.H.; Greer, S.F. Early epigenetic events regulate the adaptive immune response gene CIITA. Epigenetics 2011, 6, 516–525. [CrossRef]

30. Serrano, A.; Castro-Vega, I.; Redondo, M. Role of Gene Methylation in Antitumor Immune Response: Implication for Tumor Progression. Cancers 2011, 3, 1672–1690. [CrossRef]

31. Truax, A.D.; Koues, O.I.; Mentel, M.K.; Greer, S.F. The 19S ATPase S6a (S6′/TBP1) Regulates the Transcription Initiation of Class II Transactivator. J. Mol. Biol. 2010, 395, 254–269. [CrossRef]

32. Wright, K.L.; Ting, J.P.-Y. Epigenetic regulation of MHC-II and CIITA genes. Trends Immunol. 2006, 27, 405–412. [CrossRef]

33. Piskurich, J.F.; Linhoff, M.W.; Wang, Y.; Ting, J.P.-Y. Two Distinct Gamma Interferon-Inducible Promoters of the Major Histocompatibility Complex Class II Transactivator Gene Are Differentially Regulated by STAT1, Interferon Regulatory Factor 1, and Transforming Growth Factor β. Mol. Cell. Biol. 1999, 19, 431–440. [CrossRef] [PubMed]

34. Forlani, G.; Shallak, M.; Celesti, F.; Accolla, R.S. Unveiling the Hidden Treasury: CIITA-Driven MHC Class II Expression in Tumor Cells to Dig up the Relevant Repertoire of Tumor Antigens for Optimal Stimulation of Tumor Specific CD4+ T Helper Cells. Cancers 2020, 12, 3181. [CrossRef] [PubMed]

35. Forlani, G.; Michaux, J.; Pak, H.; Huber, F.; Joseph, E.L.M.; Ramia, E.; Stevenson, B.J.; Linnebacher, M.; Accolla, R.S.; Bassani-Sternberg, M. CIITA-Transduced Glioblastoma Cells Uncover a Rich Repertoire of Clinically Relevant Tumor-Associated HLA-II Antigens. Mol. Cell. Proteom. 2021, 20, 100032. [CrossRef]

36. Taramelli, D.; Fossati, G.; Mazzocchi, A.; Delia, D.; Ferrone, S.; Parmiani, G. Classes I and II HLA and melanoma-associated antigen expression and modulation on melanoma cells isolated from primary and metastatic lesions. Cancer Res. 1986, 46, 433–439. [PubMed]

37. Garrido, F.; Cabrera, T.; Concha, A.; Glew, S.; Ruiz-Cabello, F.; Stern, P.L. Natural history of HLA expression during tumour development. Immunol. Today 1993, 14, 491–499. [CrossRef]

38. Ma, X.C.; Hattori, T.; Kushima, R.; Terata, N.; Kodama, M. Expression of HLA-Class II Antigen in Gastric Carcinomas: Its relationship to histopathological grade, lymphocyte infiltration and five-year survival rate. Acta Oncol. 1994, 33, 187–190. [PubMed]

39. Sadanaga, N.; Kuwano, H.; Watanabe, M.; Maekawa, S.; Mori, M.; Sugimachi, K. Local immune response to tumor invasion in esophageal squamous cell carcinoma: The expression of human leukocyte antigen-DR and lymphocyte infiltration. Cancer 1994, 74, 586–591. [CrossRef]

40. López-Nevo, M.A.; García, E.; Romero, C.; Oliva, M.R.; Serrano, S.; Garrido, F. Phenotypic and genetic analysis of HLA class I and HLA-DR antigen expression on human melanomas. Exp. Clin. Immunogenet. 1988, 5, 203–212. [CrossRef]

41. Mostafa, A.; Codner, D.; Hirasewa, K.; Komatsu, Y.; Young, M.N.; Steimle, V.; Drover, S. Activation of Erx Signaling Differentially Modulates IFN-γ Induced HLA-Class II Expression in Breast Cancer Cells. PLoS ONE 2014, 9, e87377. [CrossRef]

42. Van Duinen, S.G.; Ruiter, D.J.; Broecker, E.B.; Van Der Velde, E.A.; Sorg, C.; Welvaart, K.; Ferrone, S. Level of HLA antigens in malignant melanoma: Clinical course. J. Clin. Pathol. 1988, 41, 1078–1084. [CrossRef]

43. Zaloudik, J.; Moore, J.; Ghosh, A.; Mechl, Z.; Rejthar, A. DNA content and MHC class II antigen expression in malignant melanoma. Exp. Clin. Immunogenet. 2004, 29, 144–154. [CrossRef]

44. Michel, S.; Linnebacher, M.; Alcaniz, J.; Voss, M.; Wagner, R.; Dippold, W.; Becker, C.; Doeberitz, M.V.K.; Ferrone, S.; Kloor, M. Lack of HLA class II antigen expression in microsatellite unstable colorectal carcinomas is caused by mutations in HLA class II regulatory genes. Int. J. Cancer 2010, 127, 889–898. [CrossRef]

45. Izuoka, N.; Oka, M. CIITA methylation and decreased levels of HLA-DR in tumour progression. Br. J. Cancer 2004, 91, 813–815. [CrossRef] [PubMed]

46. Morimoto, Y.; Toyota, M.; Satoh, A.; Murai, M.; Mita, H.; Suzuki, H.; Takamura, Y.; Ikeda, H.; Ishida, T.; Sato, N.; et al. Inactivation of class II transactivator by DNA methylation and histone deacetylation associated with absence of HLA-DR induction by interferon-γ in haematopoietic tumour cells. Br. J. Cancer 2004, 90, 844–852. [CrossRef] [PubMed]

47. Barbieri, R.; Rimondi, A.P.; Buzzoni, D.; Luppi, L.; Nastruzzi, C.; Orlando, P.; Gambari, R. Hypomethylation of the human HLA-DR alpha gene in breast carcinomas and autologous metastases. Clin. Exp. Metastasis 1989, 7, 417–426. [CrossRef]

48. Redondo, M.; Ruiz-Cabello, F.; Concha, A.; Hortas, M.L.; Serrano, A.; Morell, M.; Garrido, F. Differential expression of MHC class II genes in lung tumour cell lines. Eur. J. Immunogenetics 1998, 25, 385–391. [CrossRef] [PubMed]

49. Truax, A.D.; Thakkar, M.; Greer, S.F. Dysregulated Recruitment of the Histone Methyltransferase EZH2 to the Class II Transactivator (CIITA) Promoter IV in Breast Cancer Cells. PLoS ONE 2012, 7, e36013. [CrossRef] [PubMed]

50. Codolo, G.; Toffoletto, M.; Chemello, F.; Coletta, S.; Teixidor, G.S.; Battaglia, G.; Munari, G.; Fassan, M.; Cagnin, S.; De Bernard, M. Helicobacter pylori Pylori Dampens HLA-II Expression on Macrophages via the Up-Regulation of miRNAs Targeting CIITA. Front. Immunol. 2020, 10, 2923. [CrossRef]
52. Forero, A.; Li, Y.; Chen, D.; Grizzle, W.E.; Updike, K.L.; Merz, N.D.; Downs-Kelly, E.; Burwell, T.C.; Vakalavas, C.; Buchsbaum, D.J.; et al. Expression of the MHC Class II Pathway in Triple-Negative Breast Cancer Tumor Cells Is Associated with a Good Prognosis and Infiltrating Lymphocytes. Cancer Immunol. Res. 2016, 4, 390–399. [CrossRef] [PubMed]

53. Rodeck, U.; Herlyn, M.; Menissen, H.D.; Furlanetto, R.W.; Koprowski, H. Metastatic but not primary melanoma cell lines grow in vitro independently of exogenous growth factors. Int. J. Cancer 1987, 40, 687–690. [CrossRef]

54. Wulfänger, J.; Biehl, K.; Tetzner, A.; Wild, P.; Ikenberg, K.; Meyer, S.; Seliger, B. Heterogeneous expression and functional relevance of the ubiquitin carboxyl-terminal hydrolase L1 in melanoma. Int. J. Cancer 2015, 133, 2522–2532. [CrossRef] [PubMed]

55. Surmann, E.-M.; Voigt, A.Y.; Michel, S.; Bauer, K.; Reuschenbach, M.; Ferrone, S.; Doeberitz, M.V.K.; Kloor, M. Association of high CD4-positive T cell infiltration with mutations in HLA class II-regulatory genes in micrositellite-unstable colorectal cancer. Cancer Immunol. Immunother. 2015, 64, 357–366. [CrossRef] [PubMed]

56. Meyer, S.; Fuchs, T.J.; Bosserhoff, A.K.; Hofsfäder, F.; Pauer, A.; Roth, V.; Buhmann, J.M.; Moll, I.; Anagnostou, N.; Brandner, J.M.; et al. A Seven-Marker Signature and Clinical Outcome in Malignant Melanoma: A Large-Scale Tissue-Microarray Study with Two Independent Patient Cohorts. PLoS ONE 2012, 7, e38222. [CrossRef]

57. Wickenhauser, C.; Bethmann, D.; Kappler, M.; Eckert, A.; Steven, A.; Bukur, J.; Fox, B.; Beer, J.; Seliger, B. Tumor Microenvironment, Two Independent Patient Cohorts. Cancer Immunol. Immunother. 2015, 64, 357–366. [CrossRef] [PubMed]

58. Campoli, M.; Ferrone, S. HLA antigen changes in malignant cells: Epigenetic mechanisms and biologic significance. Oncogene 2008, 27, 5869–5885. [CrossRef] [PubMed]

59. Suzuki, K.; Luo, Y. Histone Acetylation and the Regulation of Major Histocompatibility Class II Gene Expression. Adv. Protein Chem. Struct. Biol. 2017, 106, 71–111. [CrossRef]

60. van den Elsen, P.J.; van der Stoep, N.; Vietor, H.E.; Wilson, L.; van Zutphen, M.; Gobin, S.J. Lack of CIITA expression is central to the absence of antigen presentation functions of trophoblast cells and is caused by methylation of the HLA-DR inducible pro-moter (PIV) of CIITA. Hum. Immunol. 2000, 61, 80–86. [CrossRef]

61. Satoh, A.; Toyota, M.; Ikeda, H.; Morimoto, Y.; Akino, K.; Mita, H.; Suzuki, H.; Sasaki, Y.; Kanaseki, T.; Takamura, Y.; et al. Epigenetic inactivation of class II transactivator (CIITA) is associated with the absence of interferon-γ-induced HLA-DR expression in colorectal and gastric cancer cells. Oncogene 2004, 23, 8876–8886. [CrossRef]

62. van der Stoep, N.; Biesta, P.; Quinten, E.; van den Elsen, P.J. Lack of IFN-gamma-mediated induction of the class II transactivator (CIITA) through promoter methylation is predominantly found in developmental tumor cell lines. Int. J. Cancer 2002, 97, 501–507. [CrossRef] [PubMed]

63. Van der Stoep, N.; Biesta, P.; Quinten, E.; van den Elsen, P.J. Lack of IFN-gamma-mediated induction of the class II transactivator (CIITA) through promoter methylation is predominantly found in developmental tumor cell lines. Int. J. Cancer 2002, 97, 501–507. [CrossRef] [PubMed]

64. Sconocchia, G.; Eppenberger-Castori, S.; Zlobec, I.; Karamitopoulou, E.; Arriga, R.; Coppola, A.; Caratelli, S.; Spagnoli, G.C.; Lauro, D.; Lugli, A.; et al. HLA Class II Antigen Expression in Colorectal Carcinoma Tumors as a Favorable Prognostic Marker. Neoplasia 2014, 16, 31–42. [CrossRef] [PubMed]

65. Axelson, M.; Cook, R.S.; Johnson, D.B.; Ballo, J.M. Biological Consequences of MHC-II Expression by Tumor Cells in Cancer. Clin. Cancer Res. 2019, 25, 2392–2402. [CrossRef] [PubMed]

66. Wilkinson, S.T.; Fernandez, D.R.; Murphy, S.P.; Braziel, R.M.; Campo, E.; Chan, W.C.; Delabie, J.; Gascoyne, R.D.; Staedt, L.M.; Jaffe, E.S.; et al. Decreased major histocompatibility complex class II expression in diffuse large B-cell lymphoma does not correlate with Cpg methylation of class II transactivator promoters III and IV. Leuk. Lymphoma 2009, 50, 1875–1878. [CrossRef]

67. Solheim, O.; Johansen, T.F.; Cappelen, J.; Unsgård, G.; Selbekk, T. Transsellar Ultrasound in Pituitary Surgery With a Designated Probe. Oper. Neurosurg. 2015, 12, 128–134. [CrossRef]

68. Toffalori, C.; Zito, L.; Gambacorta, V.; Riba, M.; Oliveira, G.; Bucci, G.; Barcella, M.; Spinelli, O.; Greco, R.; Crucitti, L.; et al. Immune signature drives leukemia escape and relapse after hematopoietic cell transplantation. Nat. Med. 2019, 25, 603–611. [CrossRef]

69. Barbaro, A.D.L.; De Ambrosis, A.; Banelli, B.; Pira, G.L.; Aresu, O.; Romani, M.; Ferrini, S.; Accolla, R.S. Methylation of CIITA promoter IV causes loss of HLA-II inducibility by IFN-γ in promyelocytic cells. Int. Immunol. 2008, 20, 1457–1466. [CrossRef]

70. Langford, G.A.; Ainsworth, L.; Marcus, G.J.; Shrestha, J. Photoperiod Entrainment of Testosterone, Luteinizing Hormone, Follicle-Stimulating Hormone, and Prolactin Cycles in Rams in Relation to Testis Size and Semen Quality. Biol. Reprod. 1987, 37, 489–499. [CrossRef] [PubMed]

71. Rodriguez, T.; Mendez, R.; Del Campo, A.; Aptsiauri, N.; Martin, J.; Orozco, G.; Pawelec, G.; Schadendorf, D.; Ruiz-Cabello, F.; Garrido, F. Patterns of constitutive and IFN-γ inducible expression of HLA class II molecules in human melanoma cell lines. Immunogenetics 2006, 59, 123–133. [CrossRef]
74. Turner, T.B.; Meza-Perez, S.; Londoño, A.; Katre, A.; Peabody, J.E.; Smith, H.J.; Forero, A.; Norian, L.A.; Jr, J.M.S.; Buchsbaum, D.J.; et al. Epigenetic modifiers upregulate MHC II and impede ovarian cancer tumor growth. *Oncotarget* 2017, 8, 44159–44170. [CrossRef] [PubMed]

75. Steimle, V.; Otten, L.A.; Zufferey, M.; Mach, B. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). *Cell* 1993, 75, 135–146. [CrossRef]

76. Yavorski, J.M.; Blanck, G. MHC class II associated stomach cancer mutations correlate with lack of subsequent tumor development. *Mol. Clin. Oncol.* 2017, 7, 1119–1121. [CrossRef] [PubMed]

77. Ramia, E.; Chiaravalli, A.M.; Eddine, F.B.N.; Tedeschi, A.; Sessa, F.; Accolla, R.S.; Forlani, G. CIITA-related block of HLA class II expression, upregulation of HLA class I, and heterogeneous expression of immune checkpoints in hepatocarcinomas: Implications for new therapeutic approaches. *OncoImmunology* 2019, 8, 1548243. [CrossRef]

78. Watts, U.M.; Macdonald, C.; Bailey, C.L.; Meegan, J.M.; Peters, C.J.; McKee, K.T. Experimental Infection of Phlebotomus Papatasi with Sand Fly Fever Sicilian Virus. *Am. J. Trop. Med. Hyg.* 1988, 39, 611–616. [CrossRef] [PubMed]

79. Cycon, K.A.; Mulvaney, K.; Rimsza, L.M.; Persky, D.; Murphy, S.P. Histone deacetylase inhibitors activate CIITA and MHC class II antigen expression in diffuse large B-cell lymphoma. *Immunology* 2013, 140, 259–272. [CrossRef] [PubMed]

80. Shi, B.; Vinyals, A.; Alia, P.; Broceño, C.; Chen, F.; Adrover, M.; Gelpi, C.; Price, J.E.; Fabra, À. Differential expression of MHC class II molecules in highly metastatic breast cancer cells is mediated by the regulation of the CIITA transcription: Implication of CIITA in tumor and metastasis development. *Int. J. Biochem. Cell Biol.* 2006, 38, 544–562. [CrossRef] [PubMed]

81. Chen, Y.-Y.; Chang, W.-A.; Lin, E.-S.; Chen, Y.-J.; Kuo, P.-L. Expressions of HLA Class II Genes in Cutaneous Melanoma Were Associated with Clinical Outcome: Bioinformatics Approaches and Systematic Analysis of Public Microarray and RNA-Seq Datasets. *Diagnostics* 2019, 9, 59. [CrossRef] [PubMed]