Multiple Structural and Epigenetic Defects in the Human Leukocyte Antigen Class I Antigen Presentation Pathway in a Recurrent Metastatic Melanoma Following Immunotherapy

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Background: Understanding the mechanism of tumor immune resistance can help design effective cancer immunotherapy. Scant information is available about the molecular basis of multiple HLA class I antigen-processing machinery defects in malignant cells, although this information contributes to our understanding of the molecular immunoescape mechanisms utilized by tumor cells and may suggest strategies to counteract them. In the present study we reveal a combination of IFN-γ-dependent structural and epigenetic defects in HLA class I antigen-processing machinery in a recurrent melanoma metastasis after immunotherapy. These defects include loss of tapasin and one HLA haplotype as well as selective silencing of HLA-A3 gene responsiveness to IFN-γ. Tapasin loss is caused by a germ-line frameshift mutation in exon 3 (TAPBP<sup>som</sup>delA<sup>p</sup>) along with a somatic loss of the other gene copy. Selective silencing of HLA-A3 gene and its IFN-γ responsiveness is associated with promoter CpG methylation nearby site-α and TATA box, reversible after DNA methyltransferase 1 depletion. This treatment combined with tapasin reconstitution and IFN-γ stimulation restored the highest level of HLA class I expression and its ability to elicit cytotoxic T cell responses. These results represent a novel tumor immune evasion mechanism through impairing multiple components at various levels in the HLA class I antigen presentation pathway. These findings may suggest a rational design of combinatorial cancer immunotherapy harnessing DNA demethylation and IFN-γ response.

Immunotherapy with checkpoint inhibitory molecule-specific monoclonal antibodies (mAb) has been convincingly shown to be effective in the treatment of various types of malignancies including melanoma (1–3). Growing evidence indicates that the clinical responses observed in the treated patients reflect the induction or enhancement of T cell-mediated immune responses to mutated peptides presented by HLA class I–β<sub>2</sub>m<sup>2</sup> complexes (4, 5). The assembly and transport of HLA class I–β<sub>2</sub>m–peptide trimolecular complex to the plasma membrane of tumor cells requires the coordination of various antigen-processing machinery components including peptide transporter TAP1/2 heterodimer, chaperones calnexin, ERp57, and calreticulin, and bridging factor tapasin (6). The role of HLA class I antigen-processing machinery components in the response to immunotherapy with checkpoint blockade has rekindled interest in the characterization of the HLA class I antigen-processing machinery in malignant cells, as defects in this machinery in tumor cells may provide them with an escape mechanism from host’s immune system.

Distinct molecular mechanisms have been found to underlie HLA class I antigen-processing machinery component abnormalities in tumor cells characterized to date (7, 8). Although HLA class I heavy chain and β<sub>2</sub>m subunits are frequently lost because of irreversible mutations, such as loss-of-heterozygosity (LOH), deletion, and missense/frameshift mutations, other antigen-processing machinery components are mostly deregulated; the latter abnormality can often be corrected by IFN-γ. Irreversible antigen-processing machinery defects in tumors caused by mutations are rare and have thus far been described in TAP1 in two tumor cell lines (9, 10) and TAP2 in one colorectal tumor lesion (11), whereas IFN-γ-uncorrectable tapasin

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2 The abbreviations used are: β<sub>2</sub>m, β<sub>2</sub>-microglobulin; LOH, loss of heterozygosity; 5AdC, 5-aza-2′-deoxycytidine; CTL, cytotoxic T lymphocyte; DNMT, DNA methyltransferase; MFI, mean fluorescence intensity; MSP, methyla-

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loss with unknown underlying mechanisms has been identified in one tumor cell line (12). Emerging evidence indicates the presence of multiple defects in HLA class I antigen-processing machinery components within a single tumor cell population (13, 14); some of them are acquired sequentially (14). Given the role of immunoselection in the outgrowth in a patient with a malignant disease of tumor cell populations with HLA class I antigen-processing machinery component defects (15), the presence of multiple HLA class I antigen-processing machinery defects in a tumor cell may reflect multiple rounds of immune selection by cytotoxic T lymphocytes (CTLs) recognizing tumor antigen (TA) epitopes with decreasing degrees of immunodominance as genetic and/or epigenetic alterations accumulate in a tumor cell population.

The therapeutic application of immune checkpoint blockade is likely to increase the frequency of multiple HLA class I antigen-processing machinery defects in tumor cells, as unleashing of TA-specific T cells from blockade will increase the selective pressure imposed on tumor cell populations. In a clinical setting such a combination of defects could pose a serious challenge as immunotherapeutic interventions would ultimately fail because of the exhaustion of appropriate targets. Information about the combination of multiple defects present in malignant cells and the underlying molecular mechanisms may contribute to our understanding of tumor-host interactions and to the optimization of the rational design of effective immunotherapeutic strategies for the treatment of patients with cancer. Therefore, in the present study we set out to characterize the combination of novel structural and epigenetic HLA class I antigen-processing machinery defects we identified in a recurrent metastatic melanoma selected from a panel of 57 melanomas because of a diminished HLA class I expression level. We provide the first evidence for an irreversible tapasin mutation, associated with a HLA haplotype loss and selective epigenetic IFN-γ unresponsiveness of a HLA-A allospecificity.

**Experimental Procedures**

**Cell Lines**—The melanoma cell line COPA-159 was established from an axillary lymph node metastasis removed from a patient with a progressive disease despite prior post-surgery (left arm primary site) vaccination with C-Vax/BCG (16) followed by chemotherapy combined with high-dose IL-2. C-Vax is an antigen-rich, allogeneic whole-cell vaccine preparation containing >20 defined immunogenic melanoma-associated and tumor-associated antigens. Administered intradermally in conjunction with adjuvant BCG, the C-Vax/BCG vaccine has been shown to elicit effective T cell and antibody responses in the treated patients. COPA-159 cells were maintained in ISCOVE’s medium (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated FCS (ICN, Costa Mesa, CA). The melanocytic strain FOM-101–1 was kindly provided by Dr. M. Herlyn (The Wistar Institute, Philadelphia, PA) and maintained in MCDB-153 medium (Sigma) supplemented with 10% chelated FCS, 20 pmol cholera toxin (Sigma), 250 nM bovine FGF (a gift of Dr. M. Herlyn), 100 nM endothelin 3 (Bachem, Torrance, CA), and 10 ng/ml stem cell factor (R & D Systems, Minneapolis, MN). The lymphoblastoid cell line LG-2 was maintained in RPMI 1640 medium (Thermo Scientific Hyclone, Logan, UT) supplemented with 10% heat-inactivated FCS. Cells were grown in a humidified 5% CO₂ atmosphere at 37 °C. Patients’ peripheral blood mononuclear cells (PBMC) were isolated by a Ficoll gradient with Ficoll-Paque Plus (GE Healthcare) according to the manufacturer’s instructions. HLA typing, performed by PCR-SSP using the PCR-SSP Kit (Dynal, Smestad, Oslo, Norway), identified the HLA phenotype A*03/24, B*49/56, Cw*01/07, DRB1*1101/04, and DQB1*0301/04 in patients’ PBMC but only the HLA haplotype A*03, B*56, Cw*01, DRB1*1101, and DPB1*0401 in the cell line COPA-159 and in the tumor tissue from which the cell line COPA-159 had been derived.

**Monoclonal and Polyclonal Antibodies**—The mAb W6/32, which recognizes β₂m-associated HLA-A, -B, -C, -E, and -G heavy chains (17, 18), mAb LGIII-147.4.1, which recognizes β₂m-associated HLA-A heavy chains, excluding -A23, -A24, -A25, -A32 (19), mAb B23.1, which recognizes β₂m-associated HLA-B and -C heavy chains (20), mAb HCA2, which recognizes β₂m-free HLA-A (excluding -A24), -B7301, and -G heavy chains (21, 22), mAb HC-10, which recognizes β₂m-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, and -B (excluding -B5702, -B5804, and -B73) heavy chains (21–23), β₂m-specific mAb L368 (24), TAP1-specific mAb NOB-1 (25), TAP2-specific mAb NOB-2 (26), calnexin-specific mAb TO-5 (27), calreticulin-specific mAb TO-11 (27), ERp57-specific mAb TO-2 (27), and tapasin-specific mAb TO-3 (27) were developed and characterized as described. All of the above-mentioned mouse mAb are IgG1 except mAb W6/32 and HC-10, which are both IgG₂a. The anti-idiotypic mAb MK2-23, IgG₁ (28) and F3-C25, IgG₂a (29), which were both used as isotype-matched irrelevant controls, were developed and characterized as described. Actin-specific mAb was from EMD Milipore (Billerica, MA). DNMT1-specific rabbit polyclonal antibodies (NB100–264) were from Novus Biologicals (Littleton, CO). R-phycocerythrin-conjugated F(ab’)₂ fragments of goat anti-mouse Fc antibodies and horseradish peroxidase-conjugated goat anti-mouse Fc antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**IFN-γ, 5-Aza-2’-deoxycytidine (5AdC), Trichostatin A (TSA), Synthetic Oligonucleotides**—The human IFN-γ was from PeproTech (Rocky Hill, NJ). 5-AdC and TSA were from Sigma. The oligonucleotide primers (Table 1) were from Integrated DNA Technologies, Inc. (Coralville, IA) or Mission Biotech, Inc. (Taipei, Taiwan).

**FACS Analysis**—Surface and intracellular staining of cells were performed as described (13). Results are expressed as -fold increase in mean fluorescence intensity (-fold MFI).

**Western Blot Analysis**—Western blotting was performed as described (13).

**Immunohistochemical Staining**—Formalin-fixed, paraffin-embedded melanoma tissue sections were stained with mAbs utilizing the EnVision system (DAKO, Carpinteria, CA) following the manufacturer’s directions. Formalin-fixed, paraffin-embedded tonsil sections (Novus Biologicals, Littleton, CO) were used as controls.
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TABLE 1

| Gene      | Sequence (5’–3’) | Application |
|-----------|------------------|-------------|
| TAPBP     |                  |             |
| Sense primers |                  |             |
| TPS-5’UT  | AGAAGTCCAGCCGCGGCGTAAAA | RT-PCR/PCR  |
| TPS-E3F   | CTTCCTACACTGACGCCCCTGGA | RT-PCR/PCR  |
| TAPA1-A   | GCCAGACTCCAGAAGCCAGAAGA | RT-PCR      |
| Antisense primers |                  |             |
| TPS-E3R   | CCAATGGATGAGAACAGCTCTAC GC | RT-PCR      |
| TPS-E4R   | GTTACACAGCAAGCCTCAGAGG | RT-PCR      |
| TAPA1-B   | CAATCCACCTCGACCAGGCCCAGAAGA | RT-PCR      |
| TPS-R     | CTCGGTCTCTTCTTTGGATATCCGGCA | RT-PCR      |

HLAG-A*0301 |                  |             |
HLAG-A*3-F   | AGTGGCCGTCAGGCGCCGGACGC | RT-PCR      |
HLAG-A*3-R   | TCCCTCCAGACCTTTGAAGTGAAGG | RT-PCR      |
HLAG-A*RT-F  | GTTACCTAGGAGACGTTCTGTT | RT-qPCR     |
HLAG-A*RT-R  | GTTACAGCCAGGAGTTTAACAC | RT-qPCR     |
HLAG-A*3M-sense |                  |             |
| TAPBP |                  |             |
| The full-length wild-type human TAPBP was cloned into pcDNA3.1 (Invitrogen) following the manufacturer’s instructions with TAPBP-specific primers (Table 1). Transfection and establishment of stable clones were performed as described (13). |

RT-PCR and Nucleotide Sequence Analysis—Total RNA isolation, synthesis of first-strand cDNA, RT-PCR, and sequence analysis were carried out as described (13). RT-qPCR was performed as follows. For one reaction, 2 µl of cDNA, 10 µl of 2X Power SYBR Green PCR Master Mix reagents, 2 µl of primer pairs, and 6 µl of distilled water were mixed. PCR was performed by the thermal cycler of the ABI 7500 system (Applied Biosystems, Foster City, CA). Relative mRNA expression was calculated using the difference between cycle threshold (Ct) values of genes of interest and Ct value of housekeeping gene (GAPDH) using the formula ΔCt. The difference between the ΔCt values of an experimental sample and those of a control sample was calculated and expressed as -fold change in mRNA expression using the formula $2^{-\Delta\Delta\text{Ct}}$. 

LOH Analysis—The heterozygosity status of MHC was analyzed as reported (30) with primers listed in Table 1. LOH is defined as a LOH index (intensity of tumor allele one/intensity of tumor allele two)/(intensity of normal allele one/intensity of normal allele two) lower than 50%.

Construction of Tapasin-expressing Plasmid and Transfection—The full-length wild-type human TAPBP cDNA was PCR cloned into pcDNA3.1 (Invitrogen) following the manufacturer’s instructions with TAPBP-specific primers (Table 1). Transfection and establishment of stable clones were performed as described (13).

DNA Demethylation and Histone Acetylation Treatment—Cells (~40% confluence) in the exponential phase were incubated with 5-AdC (2 µM), TSA (100 nM), or their combinations
and allowed to grow for 72 h at 37 °C. The viability of cells incubated with the indicated doses of 5-AdC and TSA was >97%, as determined by trypan blue exclusion (Sigma).

Methylation-specific PCR (MSP) and Pyrosequencing—Primers for MSP were designed for proximal region of the COPA-159 HLA-A3 promoter (−244 to +12) and HLA-B56 promoter (−202 to −73) using Methyl Primer Express Software Version 1.0 (Applied Biosystems). PCR was carried out with 100 ng of sodium bisulfite-modified genomic DNA (Qiagen, Valencia, CA) with an annealing temperature 61 °C and 58 °C for methylated primers and unmethylated primers, respectively. Pyrosequencing of bisulfite-converted DNA was performed using PCR products amplified with primers (PyroMark Assay Design Software 2.0) using PyroMark PCR Kit (Qiagen) with the following conditions: 1 cycle at 94 °C for 15 min, 45 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Biotin-labeled PCR products were captured by streptavidin-Sepharose HP (Amersham Biosciences). PCR products bound to the beads were purified and made single-stranded using a Pyrosequencing Vacuum Prep Tool. The sequencing primers were annealed to the single-stranded PCR products bound to the beads and sequenced using the PyroMark Q24 system (Qiagen). The methylation level at each CpG site was quantified by percent target cell cytotoxicity was calculated by the formula: % specific lysis = [(experimental release − spontaneous release)/(total release − experimental release)] × 100.

Statistical Analysis—Data are expressed as the mean ± S.D. Differences in means between control and experimental groups were evaluated with Student’s t test. p < 0.05 was considered statistically significant.

Results

Marked HLA Class I Molecule Down-regulation and Antigen-processing Machinery Component Defects in a Recurrent Melanoma Metastasis—FACS analysis with pan-HLA class I-specific mAb W6/32 of COPA-159 melanoma cells revealed their markedly down-regulated HLA class I expression compared with primary, non-transformed melanocytes FOM-101–1 and cultured B lymphoid LG-2 cells (Fig. 1A). Furthermore, FACS analysis with HLA-A-specific mAb LGIII-147.4.1 and HLA-B and -C-specific mAb B1.23.1 detected a similarly low level of expression of the gene products of HLA class I loci on them (Fig. 1A). These results were corroborated by the detection of HLA-A, -B, and -C heavy chain down-regulation utilizing intracellular staining followed by FACS or Western blotting analysis (Fig. 1, B and C).

After a 72-h incubation with IFN-γ (100 units/ml) at 37 °C, HLA-B and -C molecules, but not HLA-A molecules, were up-regulated on COPA-159 cells (Fig. 1A). Two prominent additional defects, namely, IFN-γ-correctable TAP1 down-regulation and tapasin loss, were also identified (Fig. 1, B and C). For the former defect, TAP1 expression was undetected under basal conditions but could be induced by IFN-γ to a level above that detected in untreated melanocytes (1-fold MFI 12.0 versus 8.5) (Fig. 1B). For the latter defect, tapasin expression remained undetected even after IFN-γ stimulation. The collection of defects identified in COPA-159 cells are not an in vitro artifact, as immunohistochemical staining with mAb did not detect or barely detected HLA-A, -B, and -C heavy chains, TAP1, and tapasin in the metastasis (Fig. 1D) from which COPA-159 cells originated.

Tapasin Loss Caused by a Frameshift Mutation in Exon 3 of TAPBP along with LOH at TAPBP—The steady-state TAPBP mRNA was detected by RT-PCR with combinations of primers (Fig. 2, A and B). Nucleotide sequencing analysis revealed a single adenosine (A) deletion at nucleotide position 684 in exon 3 of TAPBP (TAPBP684delA) (Fig. 2, C and D); this deletion caused a reading frameshift with subsequent introduction of five missense codons followed by the premature stop codon TGA (Fig. 2, D and E). However, the resultant putative 88-residue tapasin-truncated fragment was not detected, likely due to its rapid degradation rather than the lack of expression of the tapasin-specific mAb TO-3-defined epitope, as the latter is located within residues 29–42. The identical TAPBP mutation was also found in the genomic DNA extracted from the corresponding tumor tissue (Fig. 2D, bottom panel) and is present in ~50% of the patient’s PBMC genome with a copy number similar to that of the wild type (Fig. 2D, third panel from top, double peaks, and data not shown). These results indicate that TAPBP684delA exists as a heterozygous germ-line mutation carried by this patient.
LOH analysis revealed homozygosity at three chromosome 6p21.3 short tandem repeat sites (D6S291, D6S265, and D6S276) and one 6q short tandem repeat site (D6S311) in COPA-159 cells and the corresponding tumor tissue compared with patient’s PBMC (Fig. 3, A and B). These results suggest an extensive truncation in chromosome 6, leading to loss of one HLA haplotype including the loss of the wild-type TAPBP allele. This finding, together with TAPBP<sup>684delA</sup>, provides the genetic basis of tapasin loss in COPA-159 cells and the metastasis from which these melanoma cells originated.

**HLA Class I Up-regulation on COPA-159 Cells after Transfection with a Wild-type TAPBP cDNA**—To examine the functional impact of tapasin loss in COPA-159 cells, we reconstituted wild-type tapasin expression in these melanoma cells and tested whether their HLA class I molecule expression could be restored. Stable transfectants expressing different levels of
tapasin were established to investigate whether the extent of HLA class I up-regulation depended on the tapasin levels (Fig. 4A). FACS analysis of these transfectants stained with HLA class I framework- and locus-specific mAbs showed that total HLA class I molecule expression was not enhanced on COPA-159.TPNLow cells under basal conditions, but it was elevated, although only 1.4-fold (-fold MFI 54.6–76.4) after a 72-h incubation with IFN-γ/H9253 (100 units/ml) at 37 °C as compared with mock-transfected COPA-159 cells (COPA-159.neo) (Fig. 4A).

In contrast, more pronounced HLA class I molecule up-regulation was observed on COPA-159.TPNHigh cells, a 2.6-fold (-fold MFI 1.8–4.8) and a 4.8-fold (-fold MFI 54.6–260.4) increase under basal conditions and after incubation with IFN-γ (100 units/ml, 37 °C), respectively (Fig. 4A). These results suggest that in COPA-159 cells, when their TAP1 expression is restored by IFN-γ/H9253, the extent of their HLA class I molecule up-regulation is dependent on the tapasin level, whereas under basal conditions where their TAP1 expression is below the detection threshold, HLA class I molecule expression can be up-regulated slightly only when tapasin is overexpressed (Fig. 4B, left panel).

The level of overexpression here is ~3-fold higher than normal comparing COPA-159.TPNHigh (Fig. 4A) with untreated melanocytes (Fig. 1B). To test whether the observed HLA class I molecule up-regulation by tapasin overexpression under basal conditions reflects TAP1 stabilization by tapasin, we analyzed the steady-state TAP1 levels among mock-transfected, COPA-159.TPNLow, and COPA-159.TPNHigh cells along with the analysis of their TAP2 levels. Fig. 4C shows that TAP1 remains undetected in all of the three cell lines, with no change in their TAP2 levels. These results suggest that TAP1 stabilization by tapasin may not fully account for the elevated HLA class I levels detected on COPA-159.TPNHigh cells under basal conditions.

Regardless of the observed HLA class I molecule up-regulation, HLA-A allospecificity remains undetected even on IFN-γ-treated COPA-159.TPNHigh cells (Fig. 4A and B), suggesting the existence of a gene-selective mechanism that prevents HLA-A re-expression in COPA-159 cells.

Epigenetic Inactivation of HLA-A3 Antigen Expression—No mutation was detected in the coding sequence of HLA-A3 (data not shown), the remaining HLA-A allele in COPA-159 cells because of haplotype loss. To investigate whether the proposed gene-selective mechanism operates at the epigenetic level, we treated these melanoma cells with DNA methyltransferase 1 (DNMT1) inhibitor 5AdC (2 μM) and histone deacetylase inhibitor TSA (100 nM) individually or in combination with IFN-γ (100 units/ml) for 72 h and then analyzed their HLA class I molecule expression by FACS (Fig. 5A). The results show that HLA-A protein expression was increased ~2-fold (-fold MFI 1.0–2.3) and ~8-fold (-fold MFI 1.0–7.9) by 5AdC alone and by...
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its combination with IFN-γ, respectively, an effect not seen with TSA (Fig. 5A, first row). TSA even blocked HLA-A re-expression induced by 5AdC and IFN-γ. Similarly, at the transcriptional level, HLA-A3 mRNA expression was not induced by IFN-γ alone but was up-regulated several hundred fold after treatment with 5AdC, but not with TSA alone or in combination with IFN-γ (Fig. 5B). TSA markedly suppressed the effect by 5AdC (Fig. 5B, far right two bars). In contrast, HLA-B and -C proteins (Fig. 5A, second row) and HLA-B56 mRNA (Fig. 5C) were markedly up-regulated by IFN-γ alone; this up-regulation was not enhanced by 5AdC and was suppressed by TSA. The TSA-mediated suppression was found to be associated with down-regulation of IRF-1 mRNA expression (Fig. 5D), implying insufficient IRF-1-mediated transactivation of ISRE after treatment with IFN-γ in the presence of TSA. Collectively, these results raise the possibility that DNA methylation contributes to HLA-A3 gene silencing, in particular to its IFN-γ unresponsiveness in COPA-159 cells.

To test this possibility, we analyzed the CpG methylation status of HLA-A3 and HLA-B56 gene promoters before and after treatment with 5AdC (2 μM, 72 h). Twenty-one and 19 CpG sites were identified in the HLA-A3 and HLA-B56 proximal promoter regions, respectively (Fig. 6, A and B). MSP analysis revealed a high level of general methylation in the HLA-A3 promoter under basal conditions and after stimulation with IFN-γ (100 units/ml, 72 h) but detected mainly unmethylated signals in the HLA-B56 promoter (Fig. 6C), suggesting locus-specific methylation. To our surprise, after treatment with 5AdC (2 μM, 72 h), the HLA-A3 promoter remained highly methylated (Fig. 6C) despite nearly complete DNMT1 depletion (Fig. 6D) (33). To investigate whether this finding reflects localized CpG demethylation, we performed pyrosequencing to quantify the level of methylation at each CpG site. The results show that all CpG sites in the HLA-A3 promoter were highly methylated (90%) without treatment with 5AdC, and all of them remained substantially methylated post-treatment (Fig. 6E, left panel). Although CpG sites nearby site-a and the TATA box displayed a greater extent of demethylation, they still showed >50% of residual methylation (Fig. 6E, left panel). On the contrary, all CpG sites in the HLA-B56 promoter were hypomethylated without treatment except those with slightly elevated levels close to enhancer B and CAAT box; these sites were demethylated post-treatment (Fig. 6E, right panel). Importantly, the described promoter CpG methylation profiles in mock-treated COPA-159 cells resembled those in the metastatic lesion from which COPA-159 cells originated and were in sharp contrast to those detected in the patient’s PBMC (Fig. 6F). These results support the in vivo relevance of our findings with COPA-159 cells.
Given the critical role of site-α in IFN-γ-mediated, IRF-1-dependent HLA class I gene transactivation and the role of TATA box in the binding of general transcription factors (TFIID, TFIIA, and TFIIB) to recruit RNA polymerase II for transcription initiation, our results provide evidence that selective HLA-A3 gene silencing and its IFN-γ unresponsiveness in COPA-159 cells are attributable to promoter CpG methylation close to elements for IFN-γ responsiveness and basal transcription.

Enhancement by Tapasin Transfection Combined with 5AdC and IFN-γ Treatment of HLA-A3 Re-expression on COPA-159 Cells and of Their Susceptibility to Cognate CTL Cytotoxicity—Although HLA-A3 protein expression was induced on COPA-159 cells treated with 5AdC (2 μM, 72 h) in combination with IFN-γ (100 units/ml, 72 h), its level remained markedly lower than that detected on untreated melanocytes (Fig. 5A, first row, fifth panel from left versus Fig. 1A, second row, third panel from left). In contrast, the levels of HLA-B and -C proteins on similarly treated COPA-159 cells and on untreated melanocytes were comparable (Fig. 5A, second row, fifth panel from left, versus Fig. 1A, third row, third panel from left). To test whether this difference reflects impaired transport of HLA-A3 molecules in cells lacking tapasin, COPA-159.TPN transfectants were similarly treated with IFN-γ and 5AdC and then analyzed for HLA class I molecule expression. Fig. 7A shows that 5AdC- and IFN-γ-treated COPA-159.TPN<sup>High</sup> cells express ~8.6-fold higher HLA-A3 protein levels than similarly treated COPA-159.TPN<sup>Low</sup> cells com-

FIGURE 4. HLA class I molecule up-regulation on the melanoma cells COPA-159 after reconstitution of tapasin expression. A, FACS analysis of COPA-159 cells stably transfected with empty vector (COPA-159.neo) or wild-type tapasin cDNA (COPA-159.TPN<sup>Low</sup>, -fold MFI 5.4, and COPA-159.TPN<sup>High</sup>, -fold MFI 18.4, by intracellular staining) and surface-stained with HLA class I-specific mAbs under basal conditions (continuous line) or after a 72-h treatment with IFN-γ (300 units/ml) (broken line). Numbers in plots indicate staining intensity over background (-fold MFI). B, results of three independent experiments in A were averaged and are presented as relative expression in -fold MFI increase (mean ± S.D.) compared with untreated COPA-159.neo cells *, p < 0.05, **, p < 0.01 (Student’s t test). L, low; H, high. C, Western blot analysis of tapasin, TAP1, and TAP2 expression in COPA-159.neo, COPA-159.TPN<sup>Low</sup>, and COPA-159.TPN<sup>High</sup> cells. Untreated melanocytes were used as a normal control. Actin expression was used as a loading control. Results are representative of three independent experiments.
pared with only ~2-fold up-regulation of HLA-B and -C expression on COPA-159.TPN^{High} cells versus COPA-159.TPN^{Low} cells. These results along with the comparable levels of total HLA-A3 and HLA-B and -C heavy chain expression detected by Western blotting in lysates of the mock-transfected and both tapasin transfectants (Fig. 7B) suggest a prominent role of tapasin in the transport of HLA-A3 molecules compared with that of HLA-B and -C molecules in the investigated COPA-159 cells. Therefore, the maximal level of HLA-A3 re-expression on COPA-159 cells could be achieved by a combinatorial treatment with genetic complementation (for tapasin), cytokine stimulation (IFN-γ for TAP1), and epigenetic reactivation (5AdC for HLA-A3 IFN-γ responsiveness). Importantly, this combinatorial treatment also led to the highest level of killing of COPA-159 cells by HLA-A3-restricted, gp100_{17–25}-specific CTLs (Fig. 7C, right most bars), likely contributed by cooperation between tapasin and TAP1 to facilitate the assembly and membrane transport of HLA-A3-gp100_{17–25} peptide complex (Fig. 7C).

Discussion

The present study has characterized the multiple defects in the HLA class I antigen presentation pathway found in a recurrent metastatic melanoma with disease progression after active immunotherapy with C-Vax/BCG. They occur not only at the regulatory level, which is IFN-γ-reversible, but also at multiple gene and epigenetic levels, which are IFN-γ-irreversible. The latter include one HLA haplotype loss, a mutation in TAPBP and epigenetic silencing of the IFN-γ responsiveness of a HLA class I allele. Although the presence of multiple HLA class I antigen-processing machinery component abnormalities in a melanoma cell has been previously described (13, 14), to the best of our knowledge the melanoma metastasis we have characterized here represents the
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FIGURE 6. Hypermethylation of HLA-A3 gene promoter in the melanoma cells COPA-159 and in the metastasis from which these cells originated. A and B, the HLA-A3 and HLA-B56 promoter sequence marked with identified CpG sites (numbers) and regulatory elements (gray box) in COPA-159 cells. Regions of −311−1 and of −306−1 (relative to ATG) are shown for HLA-A3 and HLA-B56, respectively. C, MSP analysis of COPA-159 HLA-A3 and HLA-B56 promoter CpG methylation status under basal conditions and after a 72-h treatment with IFN-γ (300 units/ml), 5AdC (2 μM), or their combination. U, unmethylated; M, methylated. The right panel shows the input promoter fragments (−311−1 for HLA-A3; −306−1 for HLA-B56) amplified by flanking primers. D, Western blot analysis of COPA-159 cells for DNMT1 expression under basal conditions and after a 72-h treatment with the indicated agents as in C. E and F, pyrosequencing analysis of HLA-A3 (left panels) and HLA-B56 (right panels) promoter CpG methylation status in COPA-159 cells mock-treated versus treated with the indicated agents (E) and in the corresponding tumor lesion versus the patient’s PBMC (F). Arrows indicate sites nearby regulatory elements with marked demethylation. Results are expressed as % methylation level (mean ± S.D.) at each CpG site obtained in three independent experiments. *, p < 0.05; **, p < 0.01, versus mock-treated (E) or patient’s PBMC (F) (Student’s t test).
first example of the combination of five distinct abnormalities within one melanoma cell population.

With regard to TAPBP mutations, Cresswell and co-workers (34) first identified an exon-skipping mutation in TAPBP in the 220 variant, generated by irradiation mutagenesis in vitro of the parental lymphoid cell line C1R. Later, Yabe et al. (35) described a large homozygous deletion of exons 4–7 in TAPBP in PBMC obtained from a patient with type II bare lymphocyte syndrome. More recently, Belicha-Villanueva et al. (12) revealed a tapasin-loss phenotype by the M553 melanoma cell line, although its underlying mechanism remains unclear. We report here the first case of tapasin loss caused by a combination of genetic defects (TAPBP684delA and LOH) in a malignant cell, although TAPBP684delA appears to be cancer-unrelated as it is present as a heterozygous germ-line mutation that becomes homozygous after cancer-related LOH. This finding is strikingly similar to the results by Chen et al. (9) who showed that TAP1 loss in a human SCLC cell line was caused by a cancer-unrelated germ-line point mutation in combination with cancer-related repression of the other allele. The lack of documented cancer-related structural defects in tapasin and TAP1 as well as in other antigen-processing machinery components suggests that deregulation is the main mechanism affecting their expression. A clear example is represented by TAP1 down-regulation in COPA-159 cells described in this study and by antigen-processing machinery component down-regulation found in many other tumor types (36). In sharp contrast, mutations that cause H92522m loss are all cancer-related; no germ-line H92522m mutations have been identified thus far (7). Whether the H92522m locus is genetically less stable and/or whether antigen-processing machinery has indispensable alternative functions in malignant cells remains to be determined. Individuals carrying heterozygous germ-line antigen-processing machinery component mutations may have a higher risk to have tumors with a null genotype due to frequent LOH in malignant tumors (37). Hypermethylation of HLA class I gene promoters that silences the genes has been reported in some tumor types, including melanoma (38 – 40). Recent studies have begun to determine the level of methylation at each CpG in the HLA gene loci in a variety of tumors (41, 42). Using pyrosequencing, we quantified methylation at all the CpG sites individually in the HLA-A3 proximal promoter in COPA-159 cells and found that they were not equally susceptible to demethylation treatment; specifically, those close to site- and the TATA box were demethylated to a greater extent than other sites. Because site- and the TATA box are two crucial elements for IFN-γ-mediated
transcription (43), it is likely that CpG demethylation allows the access of IRF-1 and other trans-acting factors to ISRE and site-α as well as the access of general transcription factors to the TATA box, leading to local remodeling of the chromatin. This may explain why HLA-A3 gene IFN-γ responsiveness can be restored by 5AdC. Alternatively, a 5AdC-mediated DNA damage response by itself may also remodel the chromatin through the action of repair enzymes. Nevertheless, examining the direct role of chromatin remodeling in restoring IFN-γ responsiveness remains a challenge, as histone deacetylase inhibitor TSA impairs IRF-1 expression and IFN-γ downstream signaling. Notably, despite nearly complete DNMT1 depletion by 5AdC, >60% of the analyzed HLA-A3 promoter copies remain methylated in cultured COPA-159 cells. This finding may reflect the activity of other DNMTs such as DNMT3a/3b, which mediate de novo CpG methylation (44). In this regard the observed HLA-A3 re-expression, in particular after IFN-γ treatment, can be interpreted as the reactivation of a small portion of selected promoter regions that drive transcription in the time frame investigated. The extent of possible remethylation overtime and its impact on resilencing the HLA-A3 gene will require further investigations. On the other hand, the antagonizing action of TSA to IFN-γ and 5AdC may pose an obstacle to the therapeutic application of TSA or other histone deacetylase inhibitors with similar mechanisms of action, as its efficacy can be compromised in selected patients because of impaired IFN-γ-mediated immune responses.

In our experimental system, tapasin, when reconstituted at a high level, can slightly enhance HLA class I molecule (HLA-B and -C) expression without detectable TAP1 stabilization. This result is different from those of previous studies that established the TAP-stabilizing function of tapasin using other cell line systems (12, 45–47). Several possibilities may explain this discrepancy. First, in COPA-159 cells tapasin may stabilize TAP1 but to a degree still undetectable by the technique; therefore, the minimally increased TAP1 expression could enhance the membrane transport of HLA-B and -C molecules only to a small extent. Second, tapasin did not stabilize TAP1 but could functionally promote the formation of peptide-loading complexes and subsequent HLA class I assembly. Third, tapasin may associate with HLA class I in the endoplasmic reticulum to form less stable TAP-free loading complexes (48) capable of facilitating some degree of HLA class I membrane transport (46). To distinguish these possibilities, additional investigations would be needed, in particular those conducted under TAP1 gene-deficient conditions in COPA-159 cells. Furthermore, as only a limited number of cell models have been examined thus far in the field, it would be worthwhile to analyze more cell lines and cell types to exclude the possibility of cell-to-cell variations.

After stimulation by IFN-γ, which markedly up-regulates TAP1 expression, unlike under basal conditions, tapasin clearly promotes HLA class I membrane transport in a dose-dependent manner in COPA-159 cells. Interestingly, in this context, tapasin appears to influence membrane transport of HLA-A3 molecules more evidently than that of HLA-B and -C molecules in our experimental system. This finding suggests that HLA-A3, in addition to HLA-B*4402, whose stabilization is tapasin-assisted (49), represents a HLA class I allele with tapasin dependence. The functional significance of this dependence is supported by its ability to augment HLA-A3-restricted, gp100}_{17–25}-specific CTL responses.

The clinical relevance of our findings of multiple genetic and epigenetic defects in HLA class I antigen-processing machinery within a single tumor population is 2-fold. First, these HLA class I defects, which provide tumor cells with an effective escape mechanism(s) from CTL recognition and destruction in vitro, have been suggested to account for the association between HLA class I-associated antigen-processing machinery component down-regulation in malignant lesions and a poor clinical course of the disease found in several malignancies (36), including malignant melanoma (50). Second, the T cell-mediated selective pressure elicited naturally and/or introduced by C-Vax/BCG vaccination to the patient may act upon tumor cells sequentially, each time facilitating the outgrowth of a tumor cell population with an additional immune escape mechanism(s). This possibility is supported by the changes in CTL specificity, which have been found in the course of a malignant disease. In this regard, Lehmann et al. (51) demonstrated that the TAs recognized by the CTLs isolated from recurrent melanoma metastases were different from those recognized by the CTLs isolated from autologous primary lesions. In addition, some of us (14) have shown a shifting of CTL specificity from HLA-A2-MART-1_{27–35} complexes to HLA-A2-Tyr_{969–977} complexes in two sequential melanoma metastases with distinct HLA class I abnormalities caused by a mutant β2m protein (32). These results are consistent with the possibility that the specificity of a patient’s immune response can change in response to changes in the expression of targets by melanoma cells, which have developed immunoescape mechanisms. If so, the multiple defects found in the metastasis from which the COPA-159 cell line was originated may reflect multiple rounds of T cell-mediated immune selection, which have led to the accumulation of HLA class I antigen-processing machinery component abnormalities with the likely temporal sequence of one HLA haplotype loss/tapasinnull, TAP1 down-regulation, and epigenetic inactivation of HLA-A3 IFN-γ-responsiveness.

From a therapeutic viewpoint, patients with HLA class I antigen-processing machinery defects in their tumors similar to the ones we have characterized here may benefit from immunotherapeutic strategies that combine demethylating agents with agents stimulating T cell responses. This combinatorial therapy may induce the expression of a large number of HLA class I-β2m-peptide complexes on tumor cells after stimulation by IFN-γ secreted from activated T cells at the tumor site. Such a strategy may make tumor cells more immunogenic for effective cognate CTL recognition and destruction in the course of immunotherapy. The demethylating agents, azacitidine and decitabine (i.e. 5AdC), have been shown to be effective in the treatment of hematologic malignancies, such as myelodysplastic syndromes and acute myeloid leukemia (52), although their usefulness in solid tumors requires further evaluation due to toxicity (53). Nevertheless, Gollob et al. (54) have shown that low-dose decitabine can be safely administered in combination with high-dose IL-2 in patients with melanoma and may enhance the activity of IL-2. The major impact of immune checkpoint blockade with anti-CTLA4 (ipilimumab) and anti-

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PD-1 (pembrolizumab) mAbs on the survival of metastatic melanoma patients (1, 2) suggests the combination of these mAbs with low-dose decitabine for treatment of selected patients carrying HLA defects similar to the one described here and having poor responses to immune checkpoint blockade alone. Such patient-based therapeutic design thus highlights the importance of characterizing the molecular basis of HLA class I antigen-processing machinery component defects found in malignant tumors. Also it would be intriguing to investigate whether HLA-related epigenome status in tumors can be used to refine the molecular determinants of response to checkpoint blockade immunotherapy.

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