

Chapter 2

HLA Class I Expression in Human Cancer

Altered HLA Class I Expression in Malignant Cells

Loss or down-regulation of HLA class I antigens in tumor cells has been frequently observed in a variety of human malignancies and it represents an important cancer immune escape mechanism (Garrido et al. 1997a; Marincola et al. 2000; Campoli et al. 2002; Chang et al. 2005; Aptsiauri et al. 2007). Viruses use similar mechanism to avoid recognition and elimination by the immune system (Ploegh 1998). The first description of MHC class I loss was done in a mouse model (Gardener lymphoma) in Dr. Festenstein laboratory in 1976 (Garrido et al. 1976a, b). The production and characterization of monoclonal antibodies against HLA molecules (Barnstable et al. 1978) made possible to analyze HLA expression in human cell lines and solid tumors. At first, the reported percentage of HLA class I loss was low (10–30 %) (Garrido et al. 1993), since only monomorphic monoclonal antibodies (recognizing an epitope common to all HLA class I molecules) were available at that time. These studies were able to detect only total loss of tumor HLA class I expression and due to low incidence these findings did not attract much attention and were not considered to be significant. Later on, with the appearance of more specific monoclonal antibodies (anti-locus-A, -B and anti-allele-specific antibodies), which recognize polymorphic portions of these molecules, the incidence of HLA altered expression in cancer has been found to be much higher, increasing the relevance of these defects in the immune response against tumor. Using a broad panel of monoclonal antibodies on cryostat tumor tissue sections these alterations have been found in 60–90 % of tumors depending on the histological type of cancer (Blades et al. 1995; Cabrera et al. 1996, 1998, 2000; Koopman et al. 2000; Kageshita et al. 2005). Unfortunately, the number of available allele-specific monoclonal antibodies is still limited. Therefore, the true percentage of HLA class I defects, especially allelic losses, may perhaps be much higher in different types of malignancy.

Thus, early studies using immunohistological analysis of different tumors showed a very low frequency of allelic loss. However, with the arrival of other techniques, such as the study of microsatellites to detect loss of heterozygosity

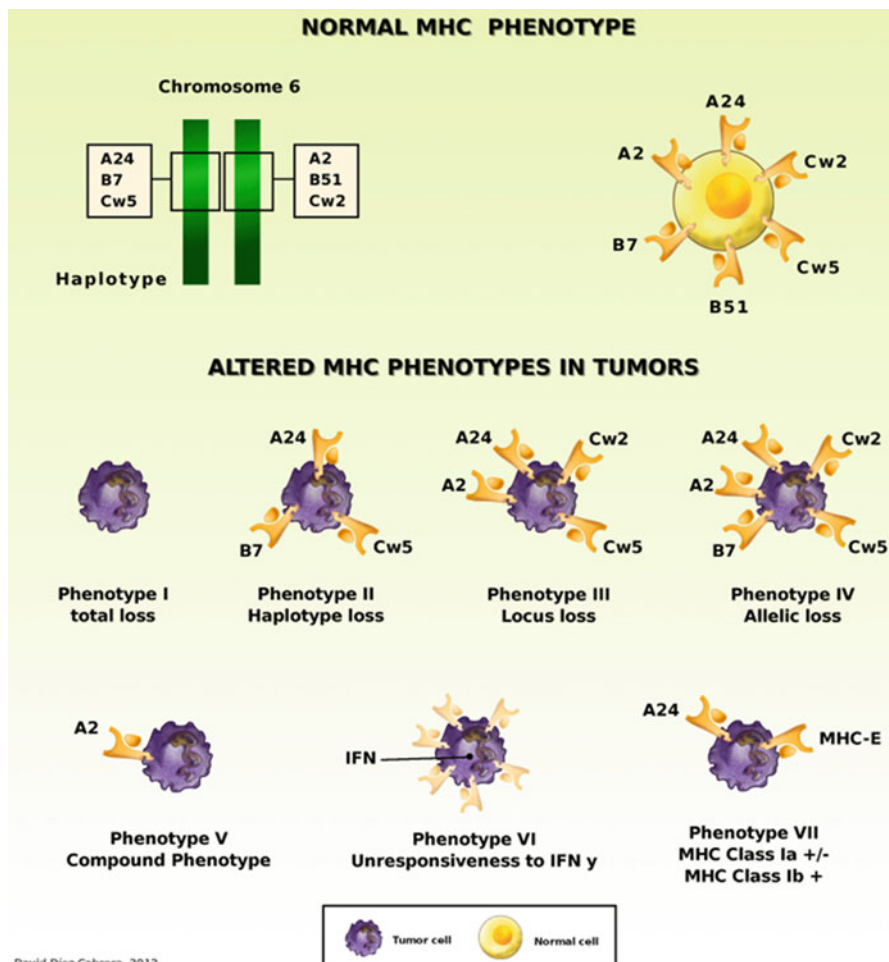


Fig. 2.1 Altered MHC class I phenotypes found in human tumors. Two copies of the genes coding for HLA class I heavy chain are located in chromosome 6. Each cell normally expresses six HLA class I alleles (two HLA-A, two HLA-B, and two HLA-C). HLA class I molecules can be totally or partially absent from tumor cells (Phenotype I to Phenotype V). In some cases tumor cells do not change class I expression after treatment with IFN- γ (Phenotype VI). Some tumor cells express aberrant HLA-E molecules together with low expression of HLA-A, -B, or -C classical class I antigens (Phenotype VII)

(LOH) on chromosome 6, it has been shown that LOH (haplotype loss) is the most frequent alteration of class I expression (Feenstra et al. 2000; Koopman et al. 2000; Maleno et al. 2002, 2004a, 2006). This alteration is caused by various defects in the HLA genomic region (short arm of chromosome 6, 6p21), including chromosomal dysfunction, mitotic recombination, and genetic conversion.

Many years of analysis of HLA expression in human tumors and tumor cell lines permitted us to classify HLA class I alterations in seven phenotypes according to the cell surface expression pattern (Garcia-Lora et al. 2003) (see Fig. 2.1):

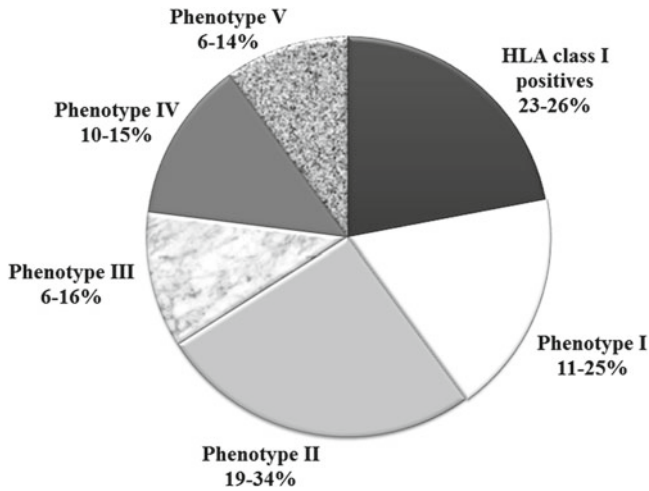


Fig. 2.2 Distribution of HLA class I phenotypes among different types of cancer

Phenotype I: Total loss of HLA class I molecules

Phenotype II: Loss of an HLA class I haplotype

Phenotype III: Loss of an HLA class I locus

Phenotype IV: HLA class I allelic loss

Phenotype V: Compound phenotype

Phenotype VI: Failure to respond to interferon (IFN)

Phenotype VII: Low expression (down-regulation) of classical HLA molecules (Ia) with aberrant expression of non-classical HLA molecules (Ib)

Based on the results of tumor immunohistochemistry and molecular analysis many groups have investigated the frequency of various HLA class I altered phenotypes in different types of cancer. This information is important for better understanding of tissue-specific factors that influence HLA alterations during malignant transformation (Fig. 2.2).

Difficulties to Define HLA Class I Altered Phenotypes in Tumor Tissue

Immunohistochemistry remains to be the key method to analyze the expression of HLA molecules on tumor cells. We believe that accurate detection of HLA complex on tumor cell surface is fundamental, since it predetermines tumor cell recognition by both CTL and NK cells. However, in solid tumors this process has both technical difficulties and problems with the result interpretation, such as the following:

- Pathology laboratories routinely work with formalin-fixed paraffin-embedded tissues. However, the structure of HLA complex is very sensitive to this type of

tissue processing and loses specific epitopes that are recognized by anti-HLA antibodies. Therefore, frozen tissues sections are the best choice for HLA analysis.

- Most antibodies that work in paraffin recognize only intra-cytoplasmic epitopes but not cell surface HLA complex, like HC-10 antibody which is directed against free intracellular heavy chain molecule. It is not unusual to see a positive immunolabeling of frozen tumor sections with HC-10 antibody and negative when W6/32 antibody (recognizes cell-surface HLA complex) is applied. This result usually indicates that there is a defect either in the assembly of HLA heavy chain with $\beta 2m$ or in the transport of the HLA complex to the cell surface (Cabrera et al. 2003b). In some cases we observed controversial results when we find HC-10-negative tumor nests in W6/32-positive tissue samples indicating tumor heterogeneity (unpublished data).
- Currently only limited number of monoclonal antibodies against HLA alleles is available (Garrido et al. 1997b). Monoclonal antibodies that recognize HLA alleles must be screened on frozen tissue sections, because some antibodies that are highly specific in cytotoxic assays do not react or display abnormal reactions in immunohistological techniques. To determine whether these antibodies are suitable, HLA typing of autologous patient PBMC should be done.
- Difficulties in the defining HLA class I expression phenotypes and in the interpretation of the intensity of immunohistochemical reactions analysis of HLA class I expression in tumor samples was discussed at the HLA expression & Cancer exercise of the 15th International Histocompatibility and Immunogenetics Workshop. Several participating laboratories were provided with the same tumor tissue material and antibodies. Bases on their final reports it was established that the intensity of immunohistochemical reactions was difficult to define since it varied significantly between the laboratories. Examples of immunohistological techniques with differences in the intensity of tumor cell staining are shown in Fig. 2.3.
- At the Workshop it was also discussed that partial HLA class I losses (haplotype loss, allelic loss, etc.) do not always correlate with the expected intensity of the immunolabeling. For instance, one might expect that tumors with LOH should show weaker staining due to the loss of the half of HLA genetic material. However, most of the workshop participants reported strongly positive W6/32 and $\beta 2m$ labeling of frozen section of tumor samples known to have LOH (unpublished data).

New techniques are currently used to study HLA class I alterations in tumor samples, including RT-PCR, microsatellite analysis for detection of LOH, DNA sequencing, and others. But none of them alone can give us a complete picture of HLA class I alterations in a tumor. For example, LOH tells us about a haplotype loss, but this tumor may also have other alterations, like a total loss of expression. Furthermore, in several tumors using tissue microdissection and real-time PCR we observed high levels of HLA-B locus-specific transcription, however, on the protein level we did not see cell surface expression of locus B (Fig. 2.4) (Carretero et al.

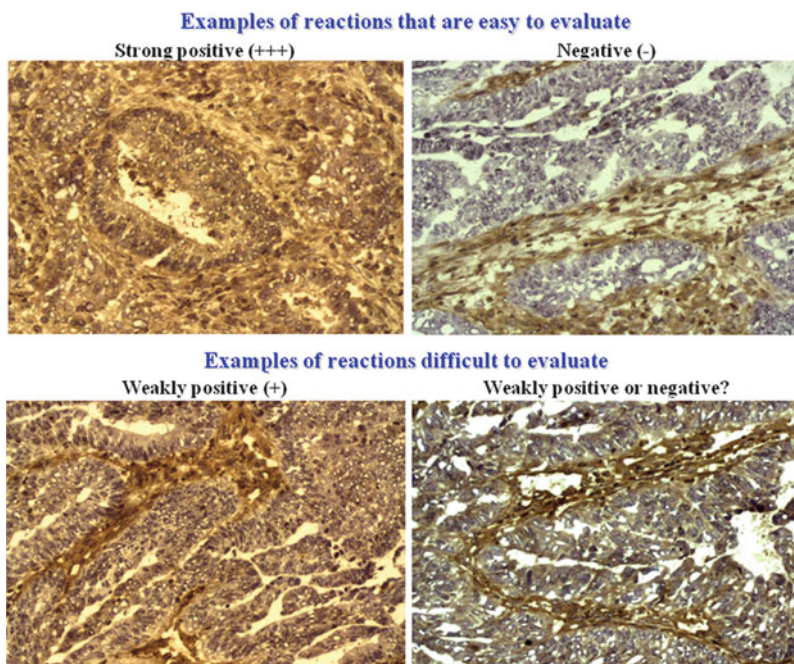


Fig. 2.3 Difficulties in the interpretation of the variations in the intensity of tumor HLA class I immunolabeling

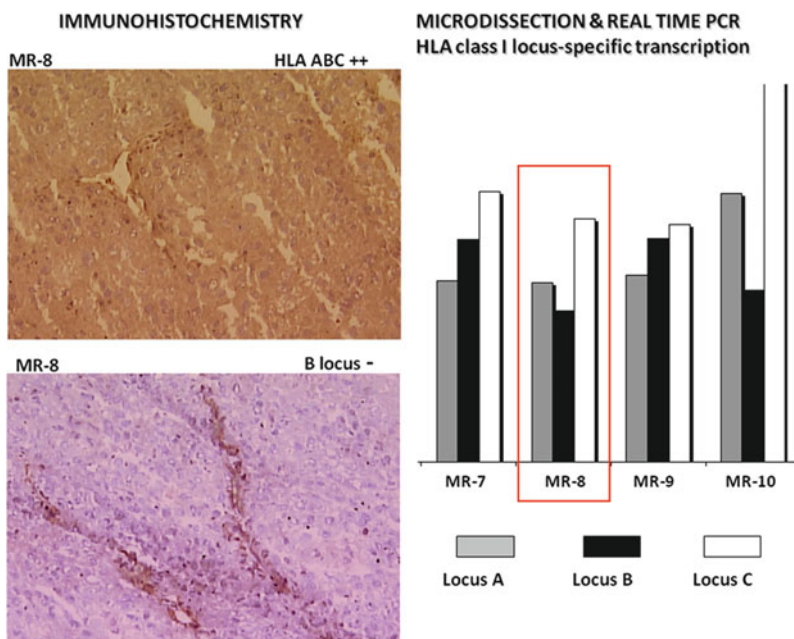


Fig. 2.4 High levels of HLA-B locus-specific transcription (*right panel*) and absence of tumor cell surface expression of locus B as shown by immunohistochemistry (*left panel*)

2008). In our experience, this is a good example demonstrating the frequent discrepancy between gene expression and cell surface protein expression of HLA class I molecules in tumor cells. We believe that in order to define HLA altered phenotypes it is essential to use a combination of all the existing techniques.

Reversible and Irreversible Molecular Defects Underlying Altered Expression of HLA Class I Antigens on Tumor Cells

From experimental work it is clear that the malignant behavior of a cancer cell depends not only on the level of tumor MHC class I expression, but also on the molecular mechanisms which cause alterations in the MHC class I expression. Generation of various tumor MHC phenotypes can occur at any step required for the protein synthesis, assembly, transport or expression on cell surface. These defects can occur at the genetic, epigenetic, transcriptional, and posttranscriptional levels and represent either regulatory abnormalities that can be recovered with cytokine treatment or more severe structural defects. Thus, MHC alterations can be classified into two main groups: reversible regulatory defects, and irreversible structural defects (Garrido et al. 2010). Although regulatory defects on transcriptional level are more common among various types of malignancy, the structural MHC defects may have profound implications in the T-cell mediated rejection of tumor cells in primary or metastatic lesions and in the outcome of cancer immunotherapy. When the mechanism underlying total HLA class I loss is on transcriptional level, the expression of surface HLA class I antigens can be reversed by cytokine treatment and T-cell based therapy can be successfully applied. However, peptide-based immunotherapy aimed at augmenting T-cell-specific tumor recognition may not be effective in case of irreversible damage of HLA genes. Therefore, development of an adequate diagnostic approach for precise identification of the HLA class I expression phenotype and underlying molecular mechanisms is central.

Reversible Defects

The reversible MHC class I deficiencies involve all levels of the MHC class I-restricted antigen presentation machinery on transcriptional level. They can be repaired, at least partially and in vitro, by cytokines (IFN-gamma, TNF-alpha). The IFN-mediated upregulation of APM components normally leads to enhanced MHC class I surface expression and improves antitumor CTL responses (Seliger et al. 2000; Martini et al. 2010). Thus, it represents a valuable strategy for the treatment of patients with APM deficiencies. However, in some cases, tumors remain insensitive to IFN treatment despite the lack of structural alterations in APM components, suggesting an impaired IFN signal transduction (Rodriguez et al. 2007).

Down-regulation of TAP1/2 and LMP2/7 gene has been demonstrated in different cell lines and tumor lesions (Cabrera et al. 2003a, c; Meissner et al. 2005). LMP7 down-regulation was found in correlation with the level of MHC class I expression in various human cancer cell lines (Yoon et al. 2000). A high frequency of LMP2, LMP7, and TAP1 down-regulation or loss was observed in tumor lesions and cell lines obtained from head and neck cancer patients, which could be reversed by IFN- γ treatment (Meissner et al. 2005). Impaired expression of immunoproteasome subunits (Cabrera et al. 2003a, c; Miyagi et al. 2003) and tapasin (Cabrera et al. 2005) is involved in different types of HLA class I molecule loss in human colon cancer.

Epigenetic events associated with tumor development and cancer progression have been found to underlie changes in HLA and APM expression and activity. HLA class I gene hypermethylation leading to HLA loss has been demonstrated in various types of cancer. These alterations can be reversed *in vitro* with pharmacologic agents that induce DNA hypomethylation or inhibit histone deacetylation (Serrano et al. 2001).

Irreversible Alterations

Total loss of HLA class I expression is caused by various mutations and chromosomal defects involving genes encoding heavy chain or β 2-microglobulin. HLA haplotype loss is one of the most frequent described phenotypes. This alteration is caused by the hemizygous loss of HLA-A, -B and -C alleles or by loss of one copy of chromosome 6 (Torres et al. 1996). This type of HLA class I alteration mechanism has been described in different types of malignancy, e.g., laryngeal tumor (Maleno et al. 2002), melanoma (Rodriguez et al. 2005), colorectal tumor (Maleno et al. 2004a), non-Hodgkin's lymphoma (Drénou et al. 2004), and pancreatic cancer (Ryschich et al. 2004). Allelic loss of single HLA alleles defines another HLA phenotype that is caused by a wide array of genetic defects including point mutations, frameshifts, or deletions (Jiménez et al. 2001).

LOH in chromosome 15 (β 2m gene region) can be frequently detected in tumors (in 40 % of colon melanomas and laryngeal carcinomas and in 50 % of bladder carcinomas) (Maleno et al. 2011). This lesion in chromosome 15 may be unnoticed since tumor cells might have "normal" HLA class I pattern and it could represent one of the early events in malignant cells leading to generation of precommitted tumors to become HLA escape variants. LOH in chromosome 15 in tumors can be found more frequently than mutations in β 2m gene.

HLA class I gene mutations include somatic recombination within class I genes (Browning et al. 1996), nonsense mutations (Koopman et al. 2000), missense mutations, deletions, and insertions (Lehmann et al. 1995; Serrano et al. 2000; Jiménez et al. 2001).

Mutations in $\beta 2m$ genes range from large deletions to single nucleotide deletions and mutations are distributed randomly among the genes (Restifo et al. 1996; Benitez et al. 1998; Feenstra et al. 1999; Paschen et al. 2003). A mutation hotspot located in the CT repeat region of exon 1 of the $\beta 2m$ gene has been proposed (Pérez et al. 1999) reflecting an increased genetic instability in this region in malignant cells. A summary of $\beta 2m$ mutations discovered in tumor cell lines and tumor specimens has been recently reviewed (Bernal et al. 2012). In most of the cases, two structural defects are necessary to produce the total loss of HLA class I on malignant cells: $\beta 2m$ mutation in one copy of the $\beta 2m$ gene and loss of the other copy associated with loss of heterozygosity (LOH) in chromosome 15 (Paschen et al. 2006).

Mutations in various APM components appear to be a rare event postulating that dysregulation rather than structural alterations is the major cause for aberrant APM component expression. TAP mutation associated with HLA class I loss was described in lung cancer (Chen et al. 1996) and in melanoma (Seliger et al. 2001).

Resistance to IFN- γ -mediated upregulation of HLA class I expression can be also a mechanism producing tumor escape variants. It is caused by defects in the Jak-STAT components of interferon (IFN)-mediated signaling pathway (Rodriguez et al. 2005; Seliger et al. 2008).

Correlation Between HLA Class I Defects and Cancer Progression in Humans

Despite the recent advances in the understanding of the role of HLA class I antigen expression in tumors, information regarding its prognostic value or its association with patient outcome remains controversial. There are a large number of publications describing a relationship between traditional pathologic criteria and/or patient survival and HLA class I expression, but the results are inconsistent. Down-regulation or low expression of MHC class I antigens has been demonstrated to have an important cancer prognostic value in various studies (Marincola et al. 2000; Chang et al. 2003; Powell et al. 2012). Morabito and coworkers (2009) observed that down-regulation of HLA class I expression in breast cancer has a significant association with adverse prognostic factors. Kaneko et al. (2011) reported that patients with preserved HLA class I expression have significantly better disease-free interval than those with loss of HLA class I. Down-regulation of HLA class I in rectal cancer has been associated with poor prognosis (Speetjens et al. 2008). On the other hand, loss of class I expression has been associated with good prognosis in breast carcinoma and non-small-cell lung cancer (Madjd et al. 2005; Ramnath et al. 2006).

At the same time, several studies have failed to show a correlation between HLA-expression and patient prognosis (Marincola et al. 2000; Chang et al. 2003; Powell

et al. 2012). Normal expression of HLA class I in a non-small-cell lung cancer was associated with a favorable prognosis compared with the heterogeneous expression group, but no significant difference was observed between the normal expression and decreased expression groups (Hanagiri et al. 2012). Kikuchi et al. (2007) revealed down-regulation of HLA class I as an independent factor of poor prognosis in stage I patients, but not in late-stage patient. Two studies have found that total absence of HLA class I resulted in a favourable prognosis as compared to patients with low tumor HLA expression. One study describes that high expression of HLA class I in tumor cells associated with better prognosis as compared to the partial down-regulation of HLA class I (Watson et al. 2006), while another report proved totally opposite findings (Menon et al. 2002). Partial HLA class I loss has also been significantly associated with decreased 5-years overall survival in breast cancer (Kaneko et al. 2011).

We believe that the inconsistencies among these studies may be explained by various reasons:

- Most of the studies are done on paraffin-embedded tissue using monoclonal antibodies able to detect only total loss of expression. In addition, as we have explained above, these antibodies react with intracytoplasmic HLA molecules and do not interact with cell surface epitopes.
- In other studies, even though they analyze cryopreserved tissue, the use of monomorphic antibodies limits the detection only to a total loss of HLA class I; and it is currently recognized other types of HLA loss are also important.
- Differences in the techniques with different degree of sensitivity; as we discussed earlier in this review, the intensity of the immunolabeling is often difficult to evaluate.
- Some studies report poor prognosis to be associated with an “intermediate” HLA class I expression. In other publications “partial HLA class I loss” is named as a bad prognosis factor. The difference in terminology used to describe abnormal HLA expression creates certain confusion. It is not clear whether “intermediate” and “partial loss” refer to the intensity of the immunolabeling or they describe the loss of a particular locus or allele.
- Expression of non-classical molecules should be analysed and taken into account because of their importance for NK cell inhibition (Carosella et al. 2003).

We believe that the correlation between HLA expression and clinical outcome cannot be clearly defined without identification of the exact type of tumor HLA defects (which alleles are missing) in each patient, which would predict the ability of CTLs to recognize tumor-associated peptides. Tumor cells with total HLA loss are not recognized by CTL, but NK cells should be able to target them for elimination. Tumors with partial loss may evade both NK- and T-cell-mediated immune surveillance; if the allele responsible for peptide presentation is missing, the remaining allele can inhibit NK cells (see Fig. 1.2).

Role of HLA Class I Altered Expression in Resistance to Immunotherapy

Malignant transformation is characterized by accumulation of genetic alterations and by epigenetic aberrations in tumor cells leading to expression of atypical proteins called tumor-associated antigens (TAA). Recognition of TAA by HLA class I-restricted CD8+ T cells is fundamental for the detection and destruction of malignant cells (van der Bruggen et al. 1991). The discovery of TAA has changed the field of cancer treatment and introduced a new era of cancer immunotherapy aimed at increasing tumor immunogenicity and T-cell-mediated antitumor immunity. Unfortunately, while the new protocols of cancer immunotherapy increase the presence of tumor-specific T lymphocytes and/or demonstrated partial responses in patients with certain malignancies, they have not yet delivered significant clinical benefits, such as induction of tumor regression or increased disease-free survival (Rosenberg et al. 2004). The results of early clinical trials were not very promising, but with the introduction of adjuvants and implementation of more innovative monitoring and evaluation criteria (Response Evaluation Criteria in Solid Tumors, RECIST), the outcome of cancer immunotherapy protocols has improved (Klebanoff et al. 2011). In addition, our understanding of the molecular mechanisms of cancer immune escape and the role of complex interaction between tumor and the host has expanded leading to improved novel treatment approaches. In order to counteract immunosuppressive factors of tumor microenvironment novel strategies are being evaluated in both clinical and preclinical settings, including combination of immuno and chemotherapy, small-molecule targeted therapies, monoclonal antibodies used to block important immune checkpoint molecules, inhibitors of immune-suppression, etc. (Schlom 2012; Walter et al. 2012). Furthermore, initially, many vaccines were tested in patients with advanced metastatic disease treated with other types of cancer therapy. Clinical studies have shown that patients respond better to vaccines when they are treated at early disease stages with only limited previous clinical intervention (Schlom 2012).

Concurrent with US Food and Drug Administration (FDA) approval of the Sipuleucel-T vaccine (Kantoff et al. 2010), the first therapeutic cancer vaccine for the therapy of asymptomatic metastatic castrate-resistant prostate cancer, a broad spectrum of other cancer vaccines is at present being evaluated. Despite the obvious progress in cancer immunotherapy and vaccination, it is clear that, although it leads to a certain clinical improvement in some patients, no significant increase in cancer patient survival has been achieved yet.

Understanding of the possible causes of such poor clinical outcome has become very important for improvement of the existing cancer treatment modalities. In particular, the critical role of HLA class I antigens in the success of T cell based immunotherapy has led to a growing interest in investigating the expression and function of these molecules in metastatic cancer progression and, especially in response to immunotherapy.

As we discussed earlier in the review, the lack of tumor rejection is associated with multiple cancer immune escape mechanisms, including the loss or low expression of tumor HLA class I molecules. Absence of normal expression of HLA class I molecules

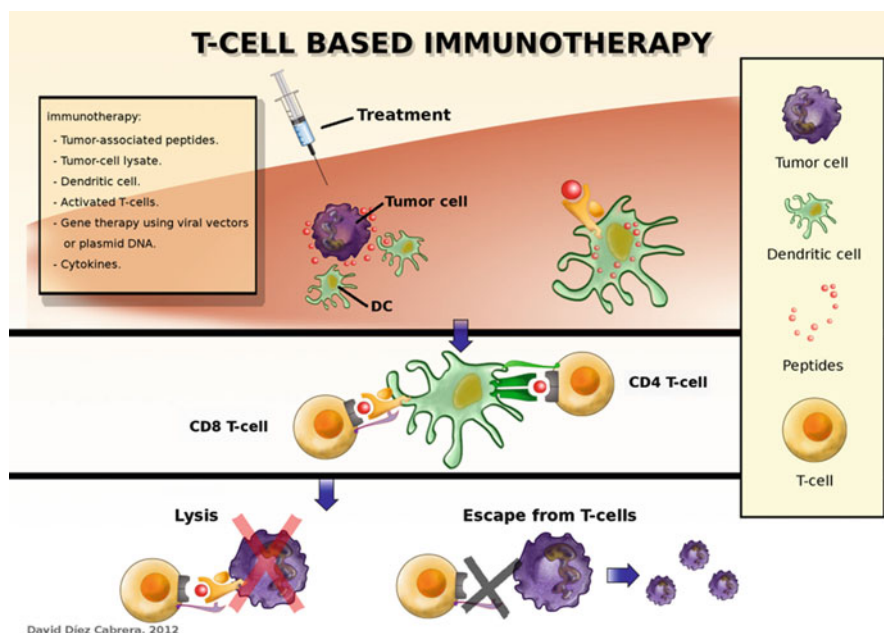


Fig. 2.5 Possible outcomes of cancer immunotherapy. The existing protocols of cancer immunotherapy are aimed at increasing the recognition of tumor cells by CD4 and CD8 T lymphocytes leading to tumor cell elimination by cytotoxic CD8 T cells. This recognition requires presentation of a tumor-associated peptide in a complex with HLA class I molecule to T cells. Therefore, absence or low expression of HLA molecules may diminish T-cell based antitumor immunity. T-cell based cancer immunotherapy induces upregulation of HLA class I in tumor cells with normal expression or with reversible HLA alterations leading to better recognition and elimination of cancer cells by CD8+ T-cell. Tumor cells escape from the immune system when HLA class I loss is caused by structural irreversible defects in HLA genes

on tumor cell surface expression obliterates TAA-peptide presentation to CTLs and leads to tumor progression. Therefore, immunotherapy aimed at increasing antitumor immune response may fail and not yield clinical benefit. Figure 2.5 shows that various types of T-cell-based cancer immunotherapy aimed are currently used in clinical setting. Each of them lead to activation of antitumor immune recognition mechanisms, starting with changes in tumor microenvironment, an increase in cytokine production, and induction of DC-mediated tumor-peptide presentation to both CD8 and CD4 T-cells. All this leads to HLA-restricted tumor cell recognition by CTLs and consequent elimination. However, if tumor cells lose normal HLA class I expression, they may escape T-cell recognition and proliferate. Therefore, the commonly observed MHC-I defects in tumors constitute a potential problem for T cell-based immunotherapy. In addition, the impact of MHC-I defects on the non-responding tumors is largely unknown and corrections of antigen presentation in these tumor types might result in much higher success rates (Lampen and van Hall 2011). Unfortunately, the majority of ongoing cancer immunotherapy clinical trials do not include tumor MHC class I expression analysis before or during the treatment, reducing the number of patient with potentially positive clinical response.

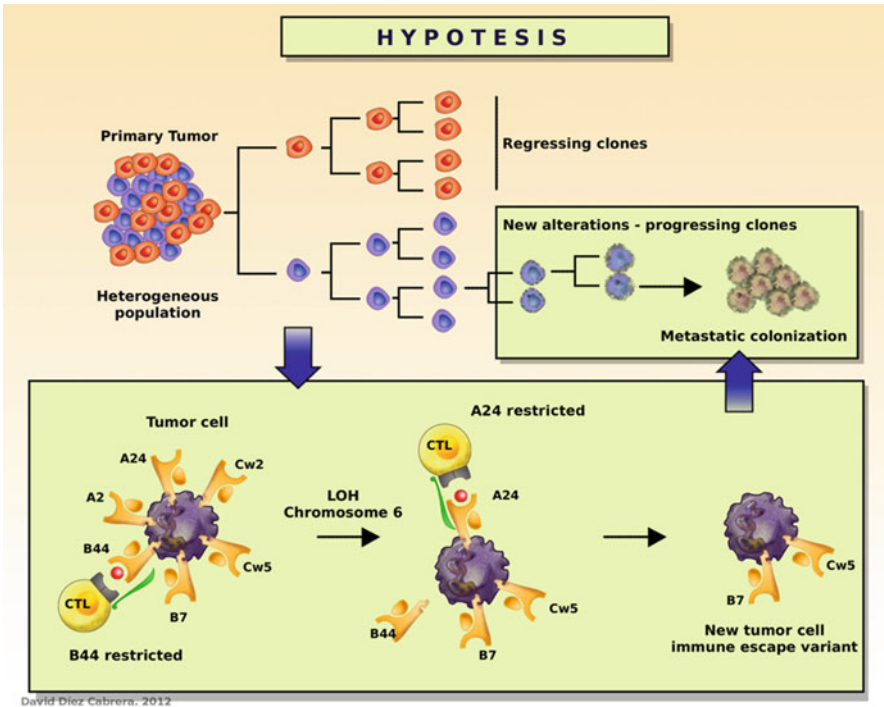


Fig. 2.6 HLA class I mediated immunoselection of tumor escape variants during cancer progression. Primary tumors consist of heterogeneous populations of cells that give rise to different cell clones undergoing immune selection. The combination of somatic evolution of genetically unstable tumor cells and immune selection during cancer development leads to the generation of tumor variants that have better survival properties. This selective pressure will lead to the expansion of new populations of cells with multiple defects capable of evading different immune responses. In this way, tumor cell with normal HLA class I expression are subjected to T-cell cytotoxic response restricted to an HLA class I allele (e.g., B44 restricted CTL reactivity). These cells are destroyed, but new HLA-negative clones appear due to additional alterations. LOH in chromosome 6 causes HLA haplotype loss and generation of B44-negative cell clones. These newly emerged clones are positive for HLA-A24 and CTL response is now A24-restricted leading to elimination of HLA-A24 positive malignant cells, but new tumor escape variants appear

The importance of monitoring tumor HLA class I expression is well illustrated by the report in which a longer overall survival in renal cell cancer was associated with immune responses to multiple tumor-associated peptides (TUMAPs) used for vaccination (Walter et al. 2012). They treated 96 HLA-A*02 RCC patients with peptides presented by HLA-A2 without previously analyzing tumor for HLA-A2 expression. We believe, that pre-selection of patients with tumors expressing HLA-A2 for this clinical trial would have improved the outcome of the therapy.

Moreover, accumulating evidence suggests that tumor cells that escape immune response during immunomodulating treatment have more dangerous metastatic phenotype due to accumulation of more profound genetic alterations (see Fig. 2.6). In this regard, the results that we have obtained recently in our laboratory support this theory.

HLA and Resistance to Immunotherapy in Melanoma and Bladder Cancer

We have studied different melanoma metastases from patients with mixed response to immunotherapy and several bladder tumors from patients treated with *Bacillus Calmette-Guerin* (BCG). We observed a strong correlation between tumor progression/recurrence and response to therapy with defects in tumor HLA class I expression and the nature of underlying mechanisms of these alterations (reversible or irreversible).

One melanoma patient developed several metastases after therapy with autologous tumor cell vaccine together and BCG (M-VAX), including three progressing and three regressing lesions. Another melanoma patient was treated first with interferon $\alpha 2b$ and later with M-VAX. We studied several progressing and regressing metastases obtained after each of the therapy modalities. All metastases showed HLA class I alterations. However, the progressing metastases developed additional and more profound defects in HLA class I expression.

All metastases from the first melanoma patient presented loss of heterozygosity (LOH) in chromosome 6. In addition, progressing metastases showed a weaker expression of HLA class I, loss of HLA-B locus, and LOH in chromosome 15 (Cabrera et al. 2007). None of the metastatic samples from the second melanoma patients showed LOH in chromosomes 6 or 15, although loss of HLA-B we detected in all the samples. Progressing metastases developed new defects in the HLA system after the therapy (Carretero et al. 2008). Quantitative expression analysis of HLA-A, B, and C genes on microdissected tumor areas demonstrated higher HLA expression in regressing than in progressing metastases (Carretero et al. 2012).

A comparative gene expression analysis of these 15 metastases (10 regressing and 5 progressing) obtained from mixed melanoma responders to different types of therapy allowed us to isolate genes differentially expressed in regressing and progressing lesions, with the majority of them being implicated in regulation of the immune response. Upregulation of antigen presentation and immune rejection pathways, including HLA-A, B, and C, antigen processing machinery (APM), interferon regulatory factor 1 (IRF-1), signal transducers and activators of transcription 1 (STAT-1), allograft inflammatory factor (AIF-1), granzymes, etc., were found in regressing metastases. In contrast, progressing metastases showed low transcription levels of genes involved in these pathways (Carretero et al. 2012). These data suggest that regressing tumors are under an acute immune rejection response. The molecular signature of tumor rejection in our case appeared to be similar to those described during allograft rejection, autoimmune disease, graft-versus-host disease and pathogen clearance.

We have also showed that BCG immunotherapy of bladder cancer induces selection of HLA class I-deficient tumor cells (Carretero et al. 2011). We performed a comparative analysis of HLA class I expression in recurrent bladder tumors in patients treated with mitomycin or BCG. HLA class I expression was studied in 18 bladder cancer patients in total. Among 13 patient treated with BCG, eight were

relapse-free, while five patients developed recurrent tumors after the therapy. Five mitomycin-treated patients were used as controls. Both primary and recurrent tumors were studied. More profound alterations in HLA class I expression were found in post-BCG recurrent tumors than in pre-BCG lesions, whereas mitomycin treatment did not change the HLA class I expression pattern. Post-BCG recurrent tumors also showed a higher incidence of structural defects underlying altered HLA class I expression: 80 and 60 % of tumors showed (LOH) in chromosomes 6 and 15, whereas only 25 % of relapse-free patients had LOH in either chromosome.

A whole genome transcriptional analysis is also being carried in 13 primary bladder tumors obtained from six relapse-free patients and seven patients with relapse after several years of follow-up. Preliminary results showed that antigen presentation and interferon pathway genes are highly expressed in tumors from relapse-free patients versus patients with recurrence, which showed higher expression level of molecules associated with Th17 lymphocytes as compared to relapse-free patients. Patients without recurrence also showed higher expression of Th1-related molecules (unpublished data).

Our results show that tumors with irreversible alterations in HLA class I can escape the immune system despite immunotherapy. We suggest that tumors with reversible alterations will better response to immunotherapy by upregulation the antigen presentation machinery, leading to tumor cell recognition and elimination by T cells. In contrast, transformed cells bearing irreversible structural defects have low probability to have a positive response to immunomodulating treatment and will continue to grow. Therefore, expression of HLA class I alterations in tumor cells is a key factor to be considered during selection of immunotherapy strategy and is a biomarker to be monitored during treatment.

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