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# Loss of HLA class I expression in Prostate Cancer and restoration using adenoviral and adeno-associated viral vectors

Francisco Javier Carretero Coca

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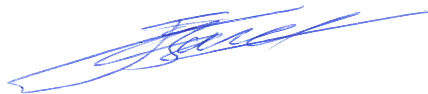

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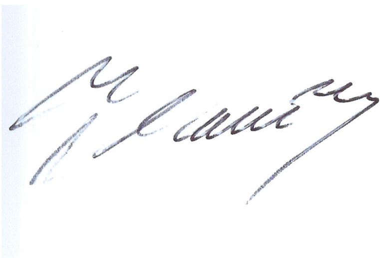

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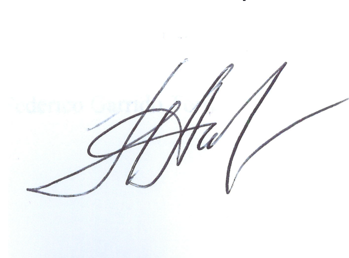

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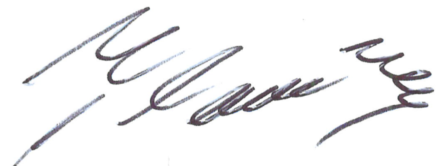

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*A mis padres*

*“Ni el más sabio conoce el fin de todos los caminos”*

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# ABSTRACT/RESUMEN

## ABSTRACT

Anti-tumour immune response and the success of cancer immunotherapy depend on the recognition of cancer cells by cytotoxic T lymphocytes (CTLs), which, in turn, depends on the proper HLA class I expression on the cell surface of malignant cells. Tumour associated antigens (TAA) and tumour specific antigens (TSA) derived from the intracellular processing of aberrant proteins as a result of somatic mutations, bind to HLA class I molecules and induce activation of CTLs after direct interaction with T-cell receptors (TCR). Tumour cells frequently develop escape mechanisms to avoid recognition by the immune system which creates an advantage for tumor growth and dissemination.

One of the most widely studied immune escape mechanisms is the loss of HLA-I expression, which is found with high frequency in tumours of different histological origin and often correlates with poor prognosis and resistance to therapy. Although HLA-I aberrations have been widely reported in cancers of different histotype, some types of malignancies, including prostate cancer, are still poorly studied with limited information regarding frequency of HLA-I loss and the implicated molecular mechanisms.

HLA-I alterations can be classified into distinct phenotypes ranging from total loss of expression to partial loss of locus or allele. Based on the nature of the associated molecular defects, they can be also grouped into two groups: reversible regulatory ('soft') alterations, and irreversible structural ('hard') alterations. Clinical implications of "hard" lesions are considered more serious due to they can not be recovered by the use of cytokines or immunomodulatory treatments. In such cases, gene therapy, with the introduction of a wild type HLA gene using viral vectors becomes an attractive strategy

for HLA recovery. In this context, selection of an appropriate vector is becoming increasingly important. Since HLA-A2 is the most common HLA-I allele in Caucasian population with widest tumor-associated peptide repertoire, and the loss of A2 allele caused by the loss of HLA-I haplotype has been linked to cancer progression and resistance to immunotherapy, we decided to develop a gene therapy approach aimed at recovery/upregulation of tumor HLA-A2 expression using viral vectors.

In the present study, we analyzed HLA-I expression in 42 cryopreserved prostate cancer samples, as well as the molecular mechanisms implicated in the loss of its expression. Immunohistochemical analysis demonstrated that 88% of the studied tumours have at least one type of HLA alteration. 50% of the samples showed complete loss of HLA-I expression either with heterogeneous or totally negative pattern, with a strong positive correlation with tumour relapse, perineural invasion and high D`Amico risk. The rest of the studied tumours demonstrated locus and allelic losses in 26% and 12% respectively. Molecular analysis showed loss of heterozygosity (LOH) at chromosome 6 (where HLA genes are located) in 32% of the studied tumours, while only one tumour showed LOH in chromosome 15. mRNA expression analysis of microdissected samples revealed that HLA-I negative tumours have significantly reduced expression of *Beta-2-microglobulin (β2m)*, antigen processing machinery (APM) components *TAP2* and *tapasin*, and of the transcriptional regulator *NLRC5*, and an apparent downregulation of *LMP2*, *LMP7* and the Interferon Regulatory Factor (*IRF1*), as compared to a control group of prostate benign hyperplasia (BH). These data point to a coordinated downregulation of HLA and APM expression as the main mechanism responsible for HLA loss, although large proportion of the studied tumors also showed structural losses in chromosome 6.

Moreover, we analyzed twelve previously unreported cell lines derived from prostate tissue of patients with prostate cancer. FACS analysis revealed different types of HLA-I aberrations, ranging from locus and/or allelic downregulation to a total absence of HLA-I expression caused by a 480 pb deletion in one copy of β2m gene and LOH in chromosome 15 harboring another copy. This is the first report of such β2m defect in human prostate cancer. Use of cancer cell lines facilitates the study of HLA-I alterations, as well as allow the manipulation of different factors implied in its expression, modulating or even recovering HLA-I expression.

In the second part of the thesis, we analyzed the transduction efficacy of different serotypes of adeno-associated vectors (AAV) and an adenovirus carrying the green fluorescent protein (GFP) gene in several cancer cell lines of different histological origin. The obtained results indicate that adenoviral vector gives the highest trasgene expression, followed by AAV serotype 2 (AAV2). In addition, we constructed two new recombinant vectors, adenoviral and AAV2, both carrying *HLA-A2* gene, both of which demonstrated high efficacy in an *in vitro* A2 gene transfer into human tumor cells of different histotype. Using these vectors we were able to: 1) recover endogenous A2 allele in a cell line which had lost A2 expression due to a haplotype loss; 2) upregulate HLA-A2 expression in cells with low baseline A2 level; and 3) introduce an additional A2 allele into tumour cells that do not have it in their genotype. Moreover, adenovirus mediated co-transfection of *HLA-A2* and *β2m* demonstrated that the *de novo* expressed proteins associate to form HLA-I/β2m complex on the cell surface, which gives an option of correcting simultaneously multiple defect causing HLA-I loss

The high incidence of HLA-I loss observed in prostate cancer, caused by both regulatory and structural defects, are associated with disease progression and may pose a real threat to patient health by increasing cancer progression and resistance to T-cell-

based immunotherapy. We could speculate that structural genetic aberrations are likely to be continuously accumulating during cancer progression generating tumor escape variants with lower immunogenicity. Our findings suggest that an effective immunotherapy migh benefit from a complementary treatment using gene therapy to bypass the selection and outgrowth of HLA-I-negative tumor cells.

The recovery of the endogenous missing HLA-I specificity may be beneficial in the restoration of the natural HLA-I expression responsible for antigen-presentation leading to increased tumour rejection by CTLs. Our results indicate that viral vectors coding for HLA molecules represent a useful tool in upregulation of tumor HLA class I expression which can be used to increase rejection of tumors harboring structural genetic defects responsible for HLA class I loss. Tumor HLA-I upregulation may enable cancer biologists to better understand how to specifically target CTL-mediated immune responses and further improve therapeutic efficacy of cancer immunotherapy.

# RESUMEN

La respuesta inmunológica antitumoral y el éxito de la inmunoterapia dependen del reconocimiento de la célula tumoral por parte de los linfocitos T citotóxicos (CTLs), que a su vez depende de la correcta expresión de las moléculas HLA de clase I (HLA-I) en la superficie celular de las células tumorales. Los antígenos asociados a tumor (TAA) o antígenos específicos de tumor (TSA), derivados del procesamiento intracelular de proteínas anómalas resultado de mutaciones somáticas, se unen al complejo HLA-I e inducen la activación de CTLs tras el reconocimiento con el receptor de la célula T (TCR). Sin embargo, con frecuencia las células tumorales desarrollan mecanismos de escape que evitan que sean reconocidas por el sistema inmunitario, aportando una ventaja para el crecimiento y diseminación tumoral.

Uno de los mecanismos de escape más ampliamente estudiados es la pérdida de la expresión de moléculas HLA-I, que se encuentra frecuentemente en cánceres de distinto origen histológico, y ha sido asociada con la progresión tumoral y la falta de respuesta al tratamiento. Aunque las alteraciones en la expresión de HLA han sido estudiadas en diversos tipos de cáncer, hay algunos tumores, entre los que se incluye el cáncer de próstata, en los que todavía existe controversia en cuanto a la frecuencia de pérdida de HLA-I y en los mecanismos moleculares implicados en ella.

Las alteraciones de la expresión de HLA-I suele dividirse en diferentes fenotipos según el tipo de pérdida, que van desde pérdidas totales de expresión, a alteraciones parciales como la pérdida de un locus o un alelo HLA. A su vez, teniendo en cuenta la naturaleza del mecanismo molecular del que se deriva la alteración, las pérdidas de HLA pueden agruparse en lesiones reversibles/regulatorias (“soft”), o lesiones irreversibles/estructurales (“hard”). Las implicaciones clínicas de los defectos

estructurales se consideran más serias debido a que no pueden recuperarse mediante el uso de citoquinas o tratamientos inmuno-moduladores. En estos casos, la terapia génica, con la introducción del gen silvestre sano mediante vectores virales, se convierte en una estrategia interesante para la recuperación de HLA. En este contexto, la selección de un vector apropiado es cada vez más importante. Siendo HLA-A2 el alelo HLA-I más extendido entre la población caucásica, teniendo uno de los repertorios más amplios de péptidos asociados a tumor presentados por dicho alelo y habiéndose descrito su pérdida de expresión asociada a la progresión tumoral y a la resistencia a la inmunoterapia, decidimos desarrollar una estrategia basada en la terapia génica con el objetivo de incrementar o recuperar la expresión de HLA-A2 tumoral usando vectores virales.

En la presente tesis se ha analizado la expresión de HLA de clase I en 42 muestras crio-preservadas de cáncer de próstata, así como los mecanismos moleculares implicados en la pérdida de su expresión. El análisis inmunohistoquímico reveló que cerca de un 90% de los tumores estudiados mostraba algún tipo de alteración en la expresión de HLA-I. Un 50% de las muestras presentaba una pérdida total de expresión de HLA-I, ya fuera con un patrón heterogéneo o un patrón totalmente negativo, mostrando una asociación con el incremento en la incidencia de recidiva tumoral, invasión perineural y alto riesgo D´Amico. El resto de tumores mostró perdidas de locus y alélicas en un 26% y un 12% respectivamente. Los análisis moleculares mostraron que la pérdida de heterocigosidad (LOH) en el cromosoma 6 (donde se localizan los genes HLA) era frecuente, detectándose en un 32% del total de muestras analizadas, mientras que sólo un tumor mostró LOH en el cromosoma 15. Los análisis de la expresión de mRNA de las muestras microdisectadas revelaron que en los tumores HLA negativos existía una reducción significativa en los niveles de *Beta-2-microglobulina (β2m)*, los componentes de la maquinaria de procesamiento antigénico *TAP2* y *tapasina*,

y el regulador *NLRC5*, y una reducción aparente de *LMP2*, *LMP7*, y el factor regulador del IFN (*IRF1*), en comparación con un grupo control de muestras benignas de próstata Estos resultados apuntan a una baja-regulación coordinada de la expresión de genes HLA y APM como el principal mecanismo responsable de la pérdida de HLA, aunque una amplia proporción de los tumores estudiados también presentaba defectos estructurales en el cromosoma 6.

Además, se analizaron doce líneas celulares de próstata obtenidas de pacientes que sufrían cáncer prostático, que no habían sido estudiadas previamente. El análisis por citometría de flujo reveló diferentes tipos de aberraciones en la expresión de HLA-I, que van desde pérdidas alélicas o de locus, a una pérdida total de expresión en una de las líneas causada por una deleción de 480 pb en una de las copias del gen *β2m,* junto a una LOH en el cromosoma 15 en la otra copia, siendo esta la primera mutación en *β2m* descrita en cáncer de próstata. El uso de líneas celulares cancerígenas facilita el estudio de las alteraciones de HLA, así como permite la manipulación de diferentes factores implicados en su expresión, modulando e incluso recuperando la expresión de HLA-I.

En la segunda parte de la tesis, analizamos la eficiencia de transfección de diferentes serotipos de vectores adeno-asociados (AAV) y un adenovirus portando el gen de la proteína verde fluorescente (*GFP*) en diversas líneas celulares cancerosas de diferente origen histológico. Los resultados obtenidos confirmaron que el vector adenoviral ofrece una mayor transducción del trasgén, seguido por el AAV serotipo 2 (AAV2). Además, se construyeron dos nuevos vectores recombinantes: un vector adenoviral y un AAV2 portando ambos el gen HLA-A2, con los que conseguimos transferir el gen de forma eficiente en células tumorales. Usando estos vectores fuimos capaces de: 1) Recuperar la expresión de HLA-A2 en una línea celular que había perdido previamente A2 debido a una pérdida de haplotipo; 2) Incrementar la expresión

de HLA-A2 en células con bajo nivel de expresión; 3) Introducir HLA-A2 como un alelo adicional en células con un genotipo no HLA-A2. Además, la co-transfección de *HLA-A2* y *β2m* usando vectores adenovirales en líneas totalmente negativas para HLA-I, demostró la expresión completa del complejo HLA-A2/β2m en la superficie celular, dando opción a corregir simultáneamente múltiples alteraciones estructurales que causen pérdida de la expresión de HLA-I.

La alta incidencia de pérdidas de HLA-I observada en cáncer de próstata, causadas tanto por defectos regulatorios como por defectos estructurales, parece estar asociados con la progresión de la enfermedad y puede resultar un impedimento para la mejora de la salud del paciente, favoreciendo la progresión tumoral y la resistencia a las inmunoterapias basadas en CTLs. Podríamos especular que las aberraciones genéticas/estructurales se acumulan de forma continua durante la progresión tumoral, generando variantes con baja inmunogenicidad que escapan del control inmunológico. Nuestros resultados sugieren que una inmunoterapia eficaz se beneficiaría de un tratamiento complementario basado en la terapia génica para eludir la selección y el crecimiento de células tumorales HLA-I negativas.

La recuperación de una especificidad endógena de HLA-I perdida podría ser beneficiosa en la restauración de la expresión natural de HLA-I responsable de la presentación del antígeno, y así incrementar el rechazo tumoral por parte de los CTLs. Nuestros hallazgos indican que los vectores virales que codifican moléculas HLA pueden representar una herramienta conveniente para la re-expresión de HLA-I en células tumorales, incrementado el rechazo de tumores que albergan defectos genéticos/estructurales responsables de la pérdida de HLA-I. La regulación positiva de HLA-I podría permitirnos entender mejor como desarrollar una respuesta inmunitaria

específica mediada por CTLs y mejorar la respuesta terapéutica de la inmunoterapia frente al cáncer.

# INTRODUCTION

# 1.- TUMOR IMMUNOLOGY

## 1.1 Immune recognition of cancer

It is well known that the innate and adaptive immune system protects the host from foreign pathogens, and is generally tolerant towards self-antigens. In the context of a growing tumor, the immune system is likely exposed to previously unseen antigens arising from genetic abnormalities, so the immune system is able to recognize and eliminate some tumors early in their development. Although it was already in 1909 when Paul Ehrlich proposed that the immune system in some way can identify and remove nascent tumor cells in the organism (Ehrlich, 1909), it was not until 50 years later when this early idea was revisited by Burnet and Thomas, proposing the concept of "immunological surveillance of cancer", describing that the immune system of the host recognizes antigens of arising tumors and eliminates them before they become clinically evident. Burnet proposed that tumor cell specific neo-antigens could provoke an effective immunologic reaction that would eliminate developing cancers (Burnet, 1970; Burnet, 1957, 1964). Alternately, Thomas theorized that complex long-lived organisms must possess mechanisms to protect against neoplastic disease similar to those mediating homograft rejections (Thomas, 1959). In the last years, many studies have provided evidences supporting the theory of immunosurveillance. The evidence against immunosurveillance was derived largely from an inability to detect remarkable differences in cancer occurrence in athymic nude mice compared to their normal wild-type equivalents (Stutman, 1974), and because cancer incidence in long-term immunosuppressed organ transplant recipients was thought either not to be increased or to be limited to cancers with a viral etiology.

Since the athymic mice have a low population of αβ T cells, a normal population of γδ T cells, and a higher population of natural killer (NK) cells (Hayday, 2000; Maleckar and Sherman, 1987; Pardoll, 2003) it is easy to think why several studies failed to prove the immunosurveillance hypothesis when testing the prevalence of tumor development in immunocompromised mice, only finding a higher rate of tumors produced by virus when compared with controls (Grant and Miller, 1965; Sanford et al., 1973; Stutman, 1974). The development of new murine models, like the Severe Combined Immune Deficiency (SCID) mice, allow to perform new studies which prove that the immune system can control also non-viral tumors, showing how SCID mice develop more tumors than controls (Engel et al., 1997). Dighe and coworkers showed that fibrosarchoma grows faster in mice treated with antibodies against interferon-γ (IFN-γ) (Dighe et al., 1994), while Shankaran demonstrated that mice without perforin, a main component of the cytolitic granules of citotoxic T cells, are more susceptible to develop tumors (Shankaran et al., 2001). Proving the hypothesis in human was likewise difficult, but still there are several evidences. First of all, both primary and acquired immunodeficiencies are associated with higher cancer risk. Epidemiology studies show that severe primary immunodeficiencies are associated with increased risk of malignancy (Gatti and Good, 1971; Kinlen et al., 1985; Salavoura et al., 2008; van der Meer et al., 1993), although is certain that most of them have a viral etiology. Nevertheless, other immunosuppressed patients, like those infected by the HIV-1, show elevated risk for cancer linked to oncogenic viruses, such as Kaposi sarcoma, Hodgkin's and non-Hodgkin's lymphoma, cervical cancer, and liver cancer. These patients also have higher risk of cancers that are not linked to oncogenic viruses, like lung cancer and multiple myeloma (Clifford et al., 2005; Shiels et al., 2009). Moreover, cancer incidence in HIV-infected patients was found to be inversely related to CD4+ T cell

counts in blood, which supports the association between immunosuppression and increased cancer risk (Guiguet et al., 2009). Other immunosuppressed patients, those who have been administered with immunosuppressive drugs (cyclosporine A) after organ transplantation, are also more prone to cancer development, in particular lymphoma (Calne et al., 1978; Opelz and Döhler, 2004), but also cancers of colon, lung, prostate, bladder, larynx and testis (Birkeland et al., 1995). Notably, most of the lymphomas were associated with Epstein Barr virus infection (EBV); however, lymphomas not associated with EBV infection have been also reported after transplantation (Leblond et al., 1998). Thus, life-long treatment of organ transplant recipients with immunosuppressive treatment leads to an increased risk for developing different malignancies, some related to known infections, and other unrelated (lung cancer and melanoma). In addition, quantity and quality of the immune cell infiltrate represent an independent prognostic factor in human primary tumors (Fridman et al., 2012).

A fundamental point of cancer immunology is how the Immune system can recognize cancer cells and distinguish them from normal cells. The analysis of tumor recognition by the immune system led to the discovery of new antigens presented by tumor cells. Cancer cells harbor mutations in protein-coding genes that are specifically recognized by the immune system, which are known as tumor specific antigens (TSA) (Mumberg et al., 1996), but malignant cells also express tumor associated antigens (TAA) (van der Bruggen et al., 1991), antigens that are expressed in high quantities by cancer cells but can also be found in healthy cells. New TSA are continuously been discovered in different tumors as melanoma (Wang et al., 1999; Wölfel et al., 1995), colorectal cancer (Wölfel et al., 1995), non-small-cell lung cancer (Echchakir et al., 2001) or chronic myeloid leukemia (Clark et al., 2001).

Major histocompatibility complex class I (MHC-I) molecules plays a crucial role in the interaction of tumor cells with the host immune system as they present TAAs or TSAs to cytotoxic T lymphocytes (CTLs). MHC-I molecules are expressed in the surface of target cells, and is recognized by the T cell receptor (TCR) of CD8+ T-cells (Aptsiauri et al., 2007a) (fig. 1). When this recognition occurs in conjunction with co-stimulatory molecules, such as the one establish between CD28 and CD80, the CTLs become activated and lead to cancer cells destruction.

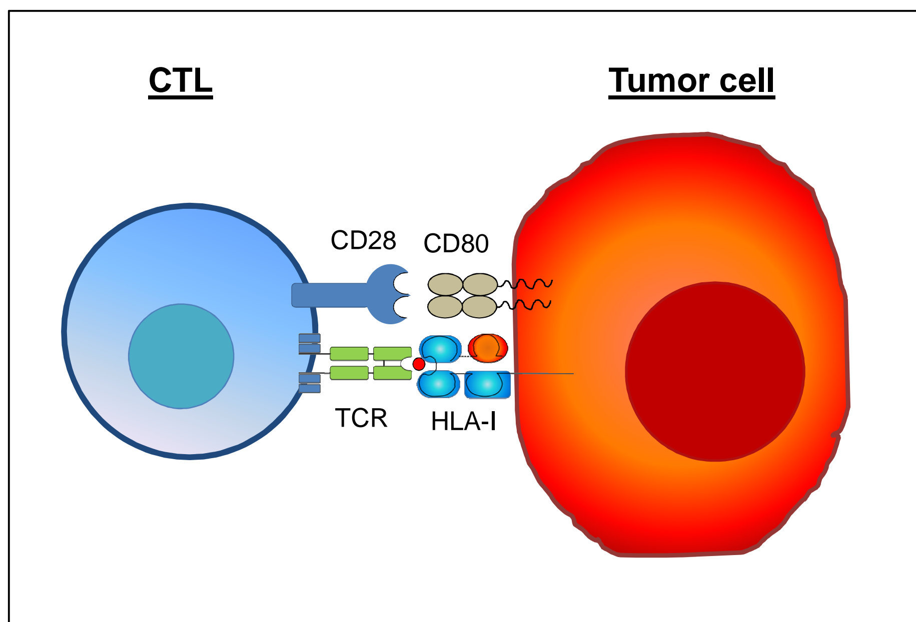



Figure 1. Tumor cells recognition by CTL. CTLs interact with cancer cells trough TCR which recognizes TAA bound to MHC-I complex in the tumor cell surface. This interaction needs the participation of adhesion molecules and co-stimulatory signals provided by different molecules as the CD28-CD80 interaction.

Not only the adaptive immune system can recognize and kill cancer cells. NK cells are innate lymphocytes that can eliminate infected and malignant cells. NK cells express NKG2D molecule on cell surface, an activating receptor which recognizes several molecules, known as NKG2D ligands, that are upregulated in cells after stress situation that could generate a damage in DNA (Gasser et al., 2005). This receptor-ligand interaction helps in detecting and elimination of transformed cells (Bauer et al., 1999). NKG2 ligands in human include MICA, MICB and six different ULBP proteins

(Groh et al., 1996), that were found to be expressed by many freshly isolated carcinomas of lung, breast, kidney, ovary, prostate and liver (Groh et al., 1999; Jinushi et al., 2003).

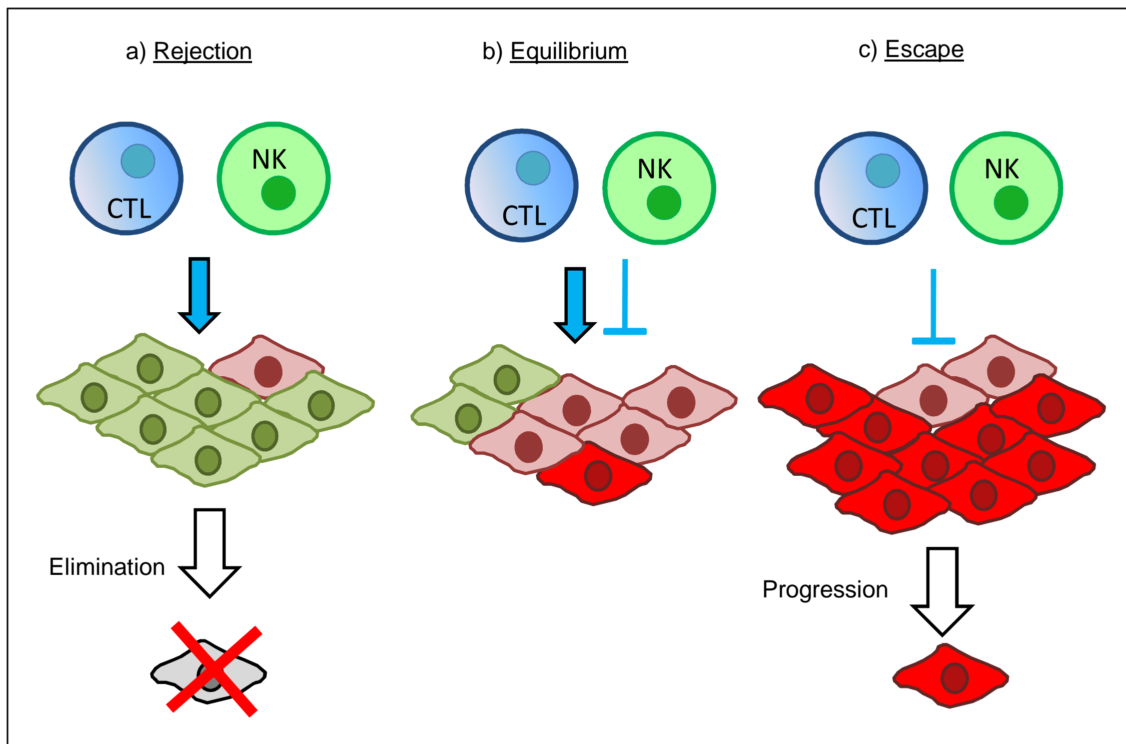



Figure 2. Tumor-host interaction in cancer evolution. Even at early stages of tumorigenesis, malignant cells may express distinct tumor-specific markers and generate pro-inflammatory signals that initiate the cancer rejection process (a). In the first phase of elimination, cells and molecules of innate and adaptive immunity may remove the developing tumor and protect the host from tumor. However, if this process is not successful, the tumor cells may enter in an equilibrium state (b), where they may be either maintained chronically or immunologically edited by immune system and select new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and escape immunosurveillance (c).

All these works show experimental evidences of the immunosurveillance concept proposed originally by Thomas and Burnet. However, tumors arise and develop in the presence of a healthy immune system, so the immunosurveillance concept is insufficient to describe the complex interactions between tumor cells and the host´s immunological system during the tumor development. With accumulation of new knowledge on tumor-host interaction, the theory of cancer immunosurveillance evolved to the concept of “cancer immunoediting” (Dunn et al., 2002) which establishes that the immune system is able to edit tumor trough its continuous interaction, killing those

malignant cells which are recognizable by effectors cells, but selecting and leading to the emergence of low immunogenic variants. In this way, immune system has a dual nature, helping to prevent tumor progression in early stages, but also promoting the selection of less immunogenic tumor variants.

Immunoediting process has three dynamic phases (Schreiber et al., 2011). First of them is the elimination stage, which corresponds with the original concept of immunosurveillance, where innate and adaptive immunity detect cancer cells and remove them before the tumor becomes clinically apparent (Smyth et al., 2006; Vesely et al., 2011). If tumors cells are completely destroyed, the elimination phase represents the endpoint of cancer immunoedition (Fig. 2a). At the same time, tumor cells that are not eliminated are kept in the "equilibrium phase", where immune system is able to control tumor growth, but without removing completely all cancer cells, which persist in a period of latency (Fig. 2b). Here, the constant interaction between tumor and immunological system promote tumor edition, causing a selection of phenotypes with low immunogenicity, which are better suited for survival in the immunocompetent host and become resistant to immune effector cells (Koebel et al., 2007; Teng et al., 2008). Tumor variants selected in previous stage can grow in the host immunologic environment and progress, reaching the third stage of immunoedition, the "escape" phase (Restifo et al., 2002) (Fig. 2c). Numerous experimental data have shown that tumors which manage to escape can subvert the immune system through direct or indirect mechanisms, favoring its growth. The escape phase represents the failure of the immune system to control or to eliminate transformed cells; moreover, the immune system contributes tumor progression by selecting more aggressive tumor variants, suppressing the antitumor immune response or promoting tumor cell proliferation (Dunn et al., 2004).

### 1.2 Tumor immune escape mechanisms

In the last two decades, many studies have focused on defining the molecular basis of tumor escape mechanisms. We know that tumors can, direct or indirectly, prevent the development of anti-tumor response trough liberation of immunosuppressive cytokines or the intervention of cells with suppressive activity (Khong and Restifo, 2002; Sakaguchi et al., 2001). Tumor escape can also be a consequence of intratumoral changes, which affect tumor recognition by immune effector cells, such as the loss of expression of tumor antigens, loss of major histocompatibility complex class I (MHC-I) components (Marincola et al., 2000), loss of NKG2D ligands (Groh et al., 2002), loss of response to IFN-γ (Kaplan et al., 1998), and alterations in the expression of the apoptosis and death receptors and corresponding signaling pathways (Takeda et al., 2002) (Catlett-Falcone et al., 1999). Classification and a summary of tumor immune-escape mechanisms are depicted in table 1.

Table 1: Various types of cancer immune escape mechanisms. Modified from (Aptsiauri et al., 2013).

| Mechanisms affecting tumor cells directly | |
|---|---|
| **Loss of tumor antigen expression** | (Berset et al., 2001; Jäger et al., 1996; Khong et al., 2004) |
| **Defects in antigen-processing and presentation pathways**:<br>- Loss or downregulation of HLA-I molecules and/or β2m.<br>- Loss or downregulation of APM molecules | (Aptsiauri et al., 2007a; Garrido et al., 2010a; Marincola et al., 2000; Meissner et al., 2005; Restifo et al., 1996; Seliger et al., 2001) |
| **Resistance to IFN-γ or IFN-α**/β through mutation or epigenetic silencing genes encoding IFN-γ receptor signaling components (IFNGR1, IFNGR2, JAK1, JAK2, and STAT1) | (Dunn et al., 2005; Kaplan et al., 1998; Respa et al., 2011; Rodríguez et al., 2007) |
| **Mechanisms that provide tumors with the ability to escape immune destruction**<br>- Upregulating inhibitors of apoptosis (Bcl-XL, FLIP) | (Catlett-Falcone et al., 1999; Dong et al., 2002; Hinz et al., 2000; Kataoka et al., 1998; Shin et al., |

| | |
|---|---|
| - Expressing inhibitory cell surface molecules that kill CTLs (PDL1, FasL)<br>- Release of pro-apoptotic factors that inhibit local antitumor T cell response (TRAIL receptor, DR5, Fas B7-H1) | 2001; Takahashi et al., 2006; Zou et al., 2007) |
| **Mechanisms that prevent tumor cell recognition by NK cells or CTLs:**<br>- Loss of NK ligands as NKG2D)<br>- Tumor cell expression of HLA-E or HLA-G<br>- Lack of expression of adhesion or co-stimulatory molecules (ICAM-1, CD40)<br>- Induction of T-cell anergy, expression of CTLA-4 ligands (B7-1 and B7-2) or PDL1 | (Carosella et al., 2003; Derré et al., 2006; Fujihara et al., 1999; Groh et al., 2002; Schultze and Nadler, 2003; Tripathi and Agrawal, 2006) (Chambers et al., 2001; Dong et al., 2002; von Leoprechting et al., 1999) |
| Tumor-induced modifications in immune cells | |
| **Tumor derived immunosuppresive factors that inhibit effector immune cell function:**<br>- Transformating growth factor-β (TGF-β), IL-10<br>- Vascular endothelial growth factor (VEGF)<br>- Metabolic enzymes such as IDO and arginase<br>- Gangliosides, soluble MICA<br>- Factors that recruit regulatory cells: IL-4, IL-13, GM-CSF, IL-1β, VEGF, or PGE2<br>- Soluble forms of adhesion molecules such as CD54, CD58, or soluble FAS ligand | (Aruga et al., 1997; Gabrilovich et al., 1998; Grothey et al., 1998; Khong and Restifo, 2002; Terabe and Berzofsky, 2004; Uyttenhove et al., 2003; Villablanca et al., 2010) Herber 2012 (Bergmann-Leitner and Abrams, 2001) |
| **Accumulation of regulatory cells that decrease antitumor response through:**<br>- Release of immunosuppressive cytokines (IL-10 and TGF-β)<br>- Altering the nutrient content of the microenvironment<br>- IL-2 consumption | (Mantovani et al., 2006; Sakaguchi et al., 2001; Terabe and Berzofsky, 2004; Vesely et al., 2011) |
| **Recruitment and polarization of MDSCs** from myeloid precursors that can block T cell function by expressing TGF-β, ARG1, iNOS, and IDO | (Vesely et al., 2011) |
| **Reduction of co- stimulatory molecules by tumor infiltrating lymphocytes** | (Chaux et al., 1996) |

## 1.3 Cancer Immunotherapy

The involvement of immune system in tumor recognition and rejection has prompted the development of immune based cancer treatment. Cancer immunotherapy, or the manipulation of the immune response to elicit clearance of cancer, is based on the premise that cancer cells express TAA that can be recognized by antibodies or CTLs. Tumor immunotherapy can generally be classified in two major approaches: passive (or adaptive), and active immunotherapy.

### 1.3.1. Active immunotherapy

a) Non-specific immunotherapy.

The administration of adjuvant directly into solid tumors to stimulate inflammation and recruit immune effector cells was one of the first strategies to enhance immune response against cancer. Already in 1890, the first successful immunotherapy was reported by William Coley, after inoculating Streptococcus pyogenes and *Serratia marcescens* into an operable sarcoma (which became known as "Coley´s toxins (Coley, 1891)). Nonspecific stimulator of the immune system is still in use for cancer treatment, such as the treatment of superficial bladder cancer with Bacilus Calmette-Guerin (BCG) (Kresowik and Griffith, 2009).

Activating the immune system against cancer cells has been also tried by cytokine therapy aimed to enhance the expression and presentation of tumor antigens to T-cells. For example, IL-2 has been extensively used for the treatment of metastatic renal cell cancer and melanoma (Beyer, 2012), or the use of IFN-α for treatment of patients with high risk locally advanced melanoma (Garbe et al., 2008).

b) Specific immunotherapy

Specific cancer immunotherapy uses TAA from different sources, including lysates of tumor cells, specific peptide epitopes or mRNA/DNA coding for relevant antigens, in order to break tumor tolerance and generate specific anti-cancer CTLs. Among the specific peptides used for immunotherapy stands out the melanoma-associated antigen MAGE-3, which developed the first observed clinical response to peptide-based immunotherapy in melanoma patients (Marchand et al., 1995), or a modified gp100 peptide used in patients with metastatic melanoma (Schwartzentruber et al., 2011). Other promising approach is based in the use of dendritic cells (Vacchelli et al., 2013) pulsed with specific peptides, such as the previously mentioned MAGE-3 (Godelaine et al., 2003). Sipuleucel-T (Provenge®) has been approved by the U.S Food and Drug administration (FDA) for castration-resistant prostate cancer. It consists of autologous peripheral-blood mononuclear cells (PBMCs), including dendritic cells that have been activated with the prostatic acid phosphatase antigen (Kantoff et al., 2010a).

### 1.3.2. Passive immunotherapy

a) Adoptive immunotherapy

It is based on the *ex-vivo* selection of patient´s antitumor cells, mostly T lymphocytes (although this approach has also been carried out using DC´s and NKs), which are isolated and expanded *in vitro*, and transferred again into patients (Rosenberg, 2011; Rosenberg and Restifo, 2015). This approach together with IL-2 and chemo-radiotherapy has been applied largely for melanoma treatment (Dudley et al., 2008). Tumor-infiltrating lymphocytes have also been used in adoptive transfer, which are selected for TCR specificity, expanded with TAAs or used with engineered T cells (Curran et al., 2012; Sadelain et al., 2003).

b) Immunomodulating antibodies

Monoclonal and chimeric antibodies featuring tumor cell specificity have been approved and successfully used against different cancers (King et al., 2008). Monoclonal antibodies (mAb) exert their effects via various mechanisms that include blockade of growth factor receptors, triggering apoptosis, activating cellular cytotoxicity, and the activation of complement. Currently, different antibodies are approved or being tested, such as *rituximab* against anti-CD20 (McLaughlin et al., 1998), *trastuzumab* targeting human epidermal growth factor receptor 2 (HER2) (Brenner and Adams, 1999), *bevacizumab* against vascular endothelial growth factor (VEGF) (Ferrara et al., 2004), *cetuximab*, which inhibits epidermal growth factor receptor (EGFR) (Wong, 2005); and immunostimulatory antibodies as *ipilimumab* targeting CTLA-4 (Margolin, 2012), or different mAb directed against PDL1. These mAbs have been tested in melanoma, non-small-lung cancer, renal cancer, ovarian cancer, colorectal cancer, pancreatic cancer, breast cancer and gastric cancer (Brahmer et al., 2012).

## 2.- HLA CLASS I ALTERATIONS AND CANCER

CTLs are thought to be major effectors of anticancer immunity, but they need to recognize TAA through tumor cell surface major histocompatibility complex. Loss of tumor MHC antigens would, therefore, prevent their recognition and lysis by CTLs. Hence it is extremely important to know why these losses occur and what mechanisms are implied.

### 2.1 HLA structure and function

The major histocompatibility complex is a family of genes which products are involved in antigen presentation to T-lymphocytes, and in immunological distinguishing between the body´s own cells, recognized as "self", and foreign cells and structures, or "non-self". In humans, the MHC complex is also known as human leukocyte antigens (HLA).

There are two main groups of HLA molecules: HLA class I (HLA-I), mostly represented in human as HLA-A, HLA-B and HLA-C, and HLA-class II (HLA-II), that include HLA-DP, HLA-DQ and HLA-DR. HLA-I molecules are expressed in all nuclear cells in vertebrates, except spermatozoids; meanwhile HLA-II molecules are usually restricted to specialized antigen presenting cells and activated cells. HLA-I antigens are constituted by the classic molecules or class Ia, HLA-A, HLA-B and HLA-C (Bjorkman and Parham, 1990) and non classic or class Ib: HLA-E, HLA-F and HLA-G (Shawar et al., 1994) which have a high homology with classic HLA molecules, but posses different functions. HLA-I is a heterodimer formed by one polymorphic heavy chain or alpha (α) of 45 KD (coded by genes localized in the MHC region located in chromosome 6) bound to a non polymorphic light chain named β2-microglobulin (β2m) of 12 KD ( gene is located in chromosome 15). HLA-I is associated with a 8 to 10

aminoacid long peptide (Cresswell et al., 2005) that also provides stability to the complex (Townsend et al., 1990).

HLA heavy chain contains 5 domains in three regions: extracellular, transmembrane and intracytoplasmatic. Extracellular domain is divided in three domains (α1, α2 and α3) with 90 amino acids each. Heavy chain is highly polymorphic, especially in apha1 and alpha2 domains, which form a hypervariable region forming a peptide binding groove (Fig. 3). This region is recognized by the T-cell receptor (TCR), while alpha3 domain binds to CD8 molecule of the CTL. HLA system is highly polymorphic due to the multiple alleles present in each of its known genes. It has been determined the existence of approximately 150 different alleles, and, with the development of new genomic typing techniques, new specificities that are constantly being discovered.

Although HLA-I molecules are able to bind different peptides, there are restrictions for peptide length and sequence (Stern and Wiley, 1994). HLA-I molecules only can bind 8 to 10 amino acid long peptides. These peptides come from proteins degraded by the proteasome, and are carried in the β2m-Heavy chain complex. This complex is expressed lately in the cell surface, where it is recognized by TCR complex of CD8+ lymphocytes, inducing cell proliferation, cytokine production, and target cell lysis (Johnsen et al., 1999; Seliger et al., 2000b). HLA-I molecules are also implied in the activity of NK cell, which are able to eliminate those cells that have lost HLA-I expression. Although NK cells do not express TCR receptor, they recognize its target using different kind of receptor: KIR (Killer Immunoglobulin-like receptor) and type C lectines. These receptors have different specificities and can be classified in two groups of receptors depending on the activating or inhibiting profile (López-Botet et al., 2000; Moretta et al., 1996).

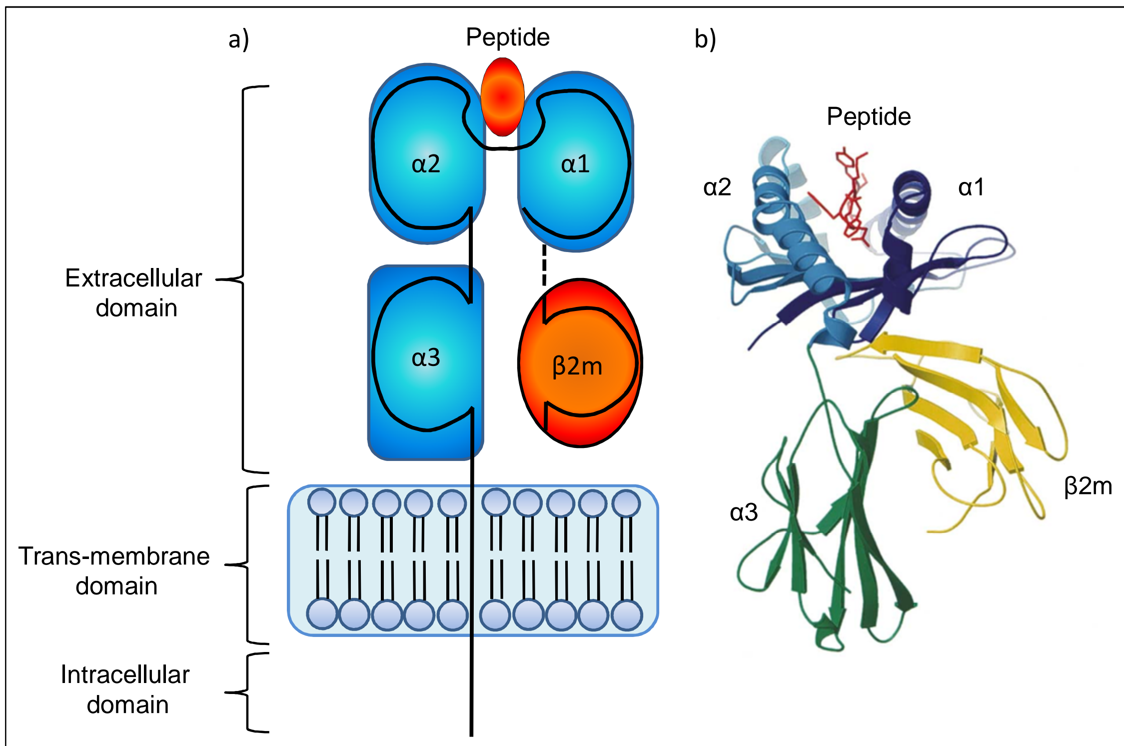



Figure 3. Structure of HLA-I molecule. a) Schematic representation of HLA-I molecule structure. The HLA molecule is composed of three domain α1, α2 and α3 which form the heavy chain, bind to a light chain (β2m). α1 and α2 forms a gap where the peptide is accommodated. b) Crystal structure of HLA-I molecule. Modified from (Yang, 2003)

HLA-II molecules are formed by two chains, denominated α and β, which form a dimer on cell surface. They are bound to the peptide adopting a tridimensional structure similar to that found in HLA-I, with the difference that HLA-II molecules bind 14 to 18 amino acid long peptides. HLA-II molecules are commonly expressed in antigen presentation cells (APC), including B lymphocytes, monocytes/ macrophages, dendritic cells, Langerhans cells, endothelial cells, thymic cells and activated T lymphocytes.

Function of HLA-II molecules is to present antigenic peptides derived from extracellular proteins and expressed on APCs to CD4+ T lymphocytes (Cresswell, 1994). Extracellular pathogens are degraded to peptides, and each peptide is loaded into the α-β dimer. This complex is finally expressed on cell surface, where it is recognized by the TCR of CD4+ T lymphocytes. If these lymphocytes recognize the peptide as "non-self" s, they will induce proliferation and activation of macrophages and the clonal

proliferation of B lymphocytes, which will secrete specific antibodies against the peptide.

## 2.2 Processing, transport, folding and presentation of antigenic peptides

The HLA-I surface antigen expression required for the presentation of antigens to CTLs is mediated by different molecular processes and implicates different molecules (Cresswell et al., 2005) (Figure 4). Shortly after or during synthesis in the endoplasmic reticulum (ER), HLA heavy chain is attached with the thiol oxidoreductase ERp57 and calnexin (David et al., 1993), a chaperone which stabilizes and preserves it from degradation (Williams, 1995). Upon binding of β2m, calnexin is released and replaced by calreticulin (Sadasivan et al., 1995) creating a multimeric complex called the peptide loading complex (PLC), which enhances the supply of peptides into the ER, and promotes their binding onto HLA-I molecules. Seven proteins conform the PLC structure: two peptide transporters associated with antigen processing (TAP1 and TAP2), which translocate class I peptide ligands from the cytosol into the ER (Androlewicz et al., 1993; Neefjes et al., 1993), the class I-specific accessory molecule tapasin, the chaperone calreticulin (Sadasivan et al., 1996), ERp57 (Morrice and Powis, 1998), and the heavy chain/ β2m dimmer.

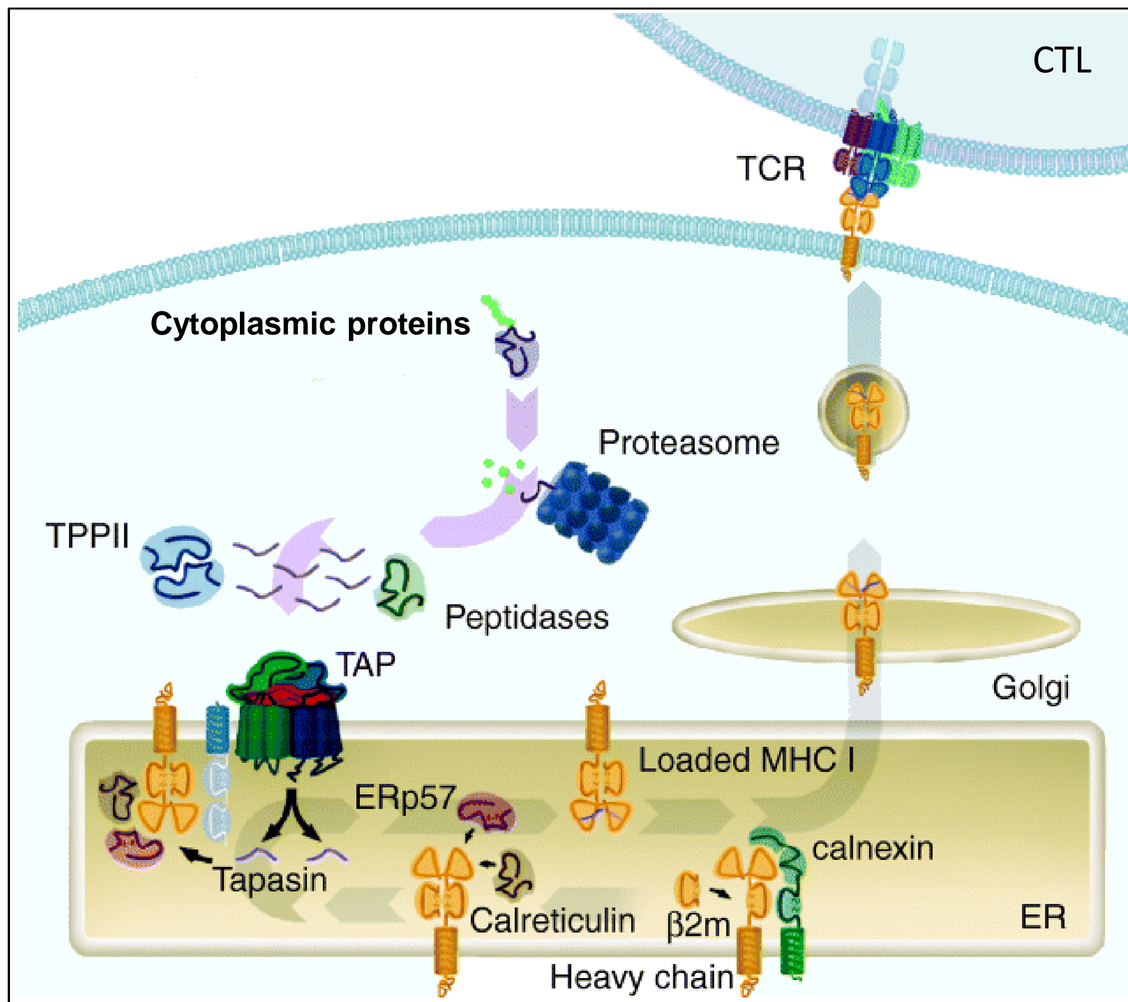



Figure 4: HLA-I antigen processing and presentation to CTL. Intracellular proteins are degraded by the proteosome and peptidases into peptides which are translocated into the endoplasmic reticulum (ER) by TAP1 and TAP2. In the ER, peptides are further trimmed by aminopeptidases and bind to HLA-I/B2M complex with the help of the chaperone tapasin. The HLA-I/B2M complex formation in the ER is done with chaperons ERp57, calnexin and calreticulin. The stable HLA-I/B2M/peptide complex is subsequently transported via the Golgi system to the cell surface, where they are recognized by CTLs. Modified from Groothuis et al 2005 (Groothuis et al., 2005).

The majority of antigenic peptides are derived from intracellular proteins that are degraded via the multicatalytic proteasome complex. The peptides are translocated, via TAP1 and TAP2 subunits, from the cytosol into the lumen of the ER and, if necessary, they are cut by ER associated aminopeptidases (ERAAP or ERAP1) to 8–10 amino acids (Bukur et al., 2012). The activity of the pathway could be modified by IFN-γ generating the immunoproteasome, which consists of novel active subunits as the low molecular weight proteins LMP2 and LMP7 (Gaczynska et al., 1993). β2m has been shown to be also a chaperone-like molecule for HLA-I folding. The association of

heavy and light chain is a requisite for cell-surface expression of the complex (Ploegh et al., 1979; Rein et al., 1987) and in the absence of β2m, most HLA-I molecules are not expressed efficiently on the cell surface (del Campo et al., 2009; Seong et al., 1988). Once peptides are bound, stable trimmers of heavy chain/β2m/peptide are released from the loading complex and exported to the cell surface via the trans-Golgi and displayed on the surface.

Different transcriptional factors, such as interferon regulatory factor 1 (IFR-1) and NOD-, LRR- and CARD-containing 5 (NLRC5) factor, control the expression of HLA-I-related genes (Kobayashi and van den Elsen, 2012). It has been shown that knockdown of NLRC5 in different human cell lines and primary dermal fibroblasts leads to reduced MHC class I expression, whereas introduction of NLRC5 into cell types with very low expression of MHC class I augments MHC class I expression to levels comparable to those found in lymphocytes. Moreover, expression of NLRC5 positively correlates with MHC class I expression in human tissues. Functionally, both the N-terminal effector domain of NLRC5 and its C-terminal leucine-rich repeat domain are needed for activation of MHC class I expression (Neerincx et al., 2012).

## 2.3 HLA class I loss and tumor escape

### 2.3.1 HLA class I alterations

HLA complex plays a major role in the immunological cell response against cancer (Crowley et al., 1991) due to its implication in tumor cells recognition by CTLs, as well as in the T cell specific immunotherapy used in cancer treatment (Boon et al., 1997). Therefore, HLA molecules play a key role in protecting the organism against neoplastic disease in early stages, and also are leading the selection of resistant tumor variants.

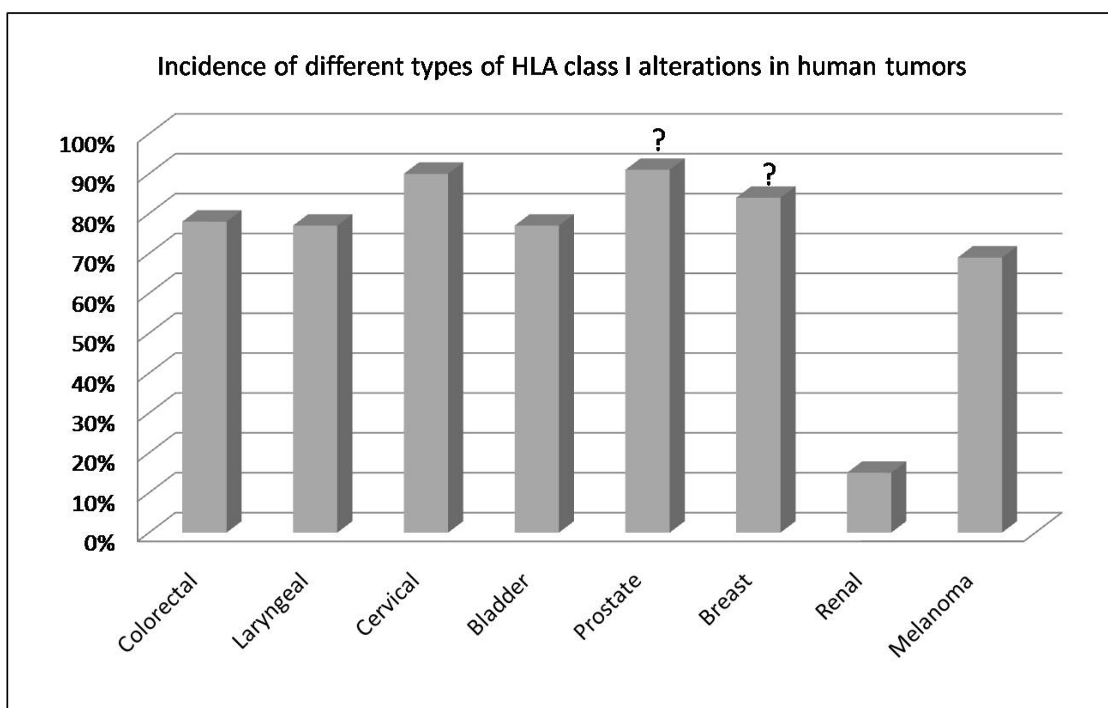



Figure 5. Incidence of HLA-I alterations in different human tumors. Available information about the frequency of alterations in prostate and breast cancer is limited and controversial.

Loss or down-regulation of HLA class I antigens in tumor cells has been frequently observed in a variety of human malignancies including melanoma, bladder, colorectal, lung, laryngeal, breast and renal carcinoma (Aptsiauri et al., 2007b; Campoli et al., 2002; Chang et al., 2005; Garrido et al., 1997; Marincola et al., 2000) resulting in tumor immunoescape from CTLs. The same mechanism to avoid recognition and elimination by the immune system is used by viruses (Ploegh, 1998). Combination of different techniques such as immunohistochemistry and microsatellites analysis to detect loss of heterozygosity helped to determine that the frequency of cancer HLA alteration varies among tumors of different histological type (Fig. 5) (Blades et al., 1995; Cabrera et al., 1996; Cabrera et al., 1998; Cabrera et al., 2000; Kageshita et al., 2005; Koopman et al., 2000). According to the cell surface expression pattern, HLA class I alterations have been classified in seven different phenotypes (Garcia-Lora et al., 2003a) (Fig. 6):

- 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)

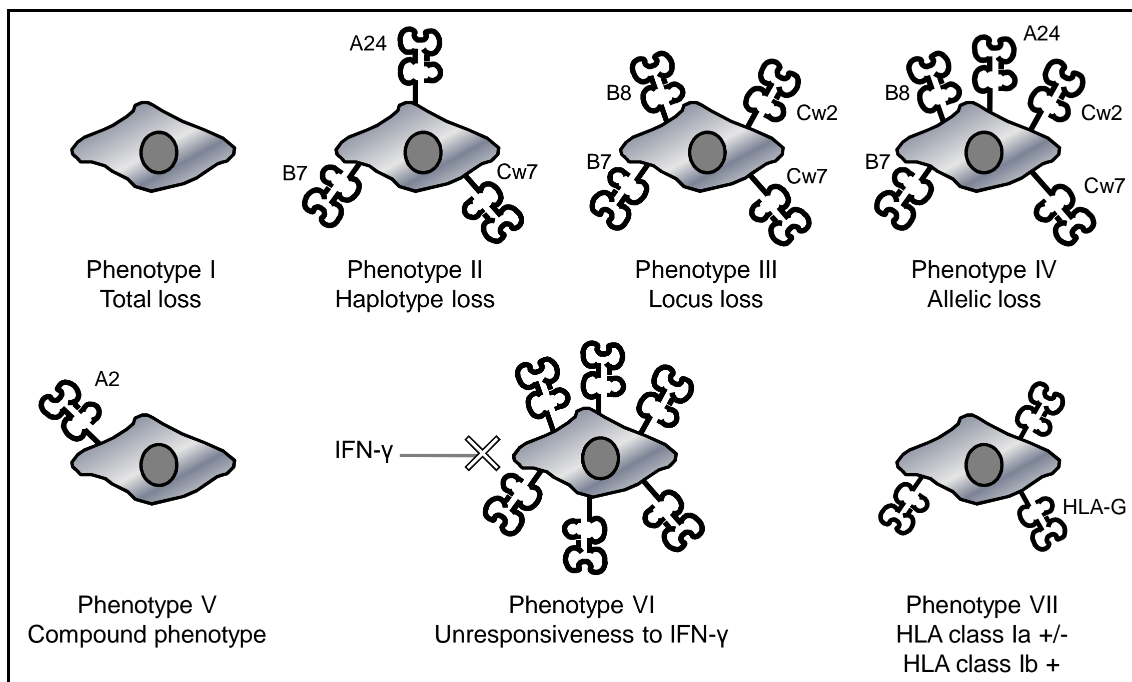



Figure 6: Classification of altered MHC class I phenotypes found in human tumors. Each cell normally expresses six HLA class I alleles (two HLA-A, two HLA-B, and two HLA-C). HLA class I molecules can be totally or partially absent from tumor cells (Phenotype I to Phenotype V). In some cases, tumor cells do not upregulate class I expression after treatment with IFN-γ (Phenotype VI). Some tumor cells express aberrant HLA-E molecules together with low expression of classical class I antigens HLA-A, -B, or -C (Phenotype VII).

### 2.3.2 Irreversible and reversible defects underlying HLA class I altered expression

Generation of tumor HLA phenotypes can occur at any step required for the protein synthesis, assembly, transport or expression on cell surface, which, in turn, can

occur at the genetic, epigenetic, transcriptional, and posttranscriptional levels, representing either regulatory abnormalities or structural defects, such as mutations or chromosomal aberrations. Thus, HLA-I alterations can be classified into two main groups: reversible regulatory defects (also called “soft”), and irreversible structural defects (called “hard”) (Garrido et al., 2010b). Although regulatory defects on transcriptional level are more common among various types of malignancy, the HLA structural 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 immunotherapy can be successfully applied. However, peptide-based immunotherapy aimed to augment T-cell-specific tumor recognition may not be effective in case of irreversible damage of HLA genes(del Campo et al., 2014). Therefore, development of an adequate diagnostic approach for precise identification of the HLA class I expression phenotype and underlying molecular mechanisms is essential.

a) Reversible alterations.

The reversible HLA class I deficiencies involve all levels of the HLA class I-restricted antigen presentation machinery on transcriptional level. They can be repaired, at least partially *in vitro*, by cytokines (IFN-γ, TNF-α). The IFN-mediated upregulation of APM components normally leads to enhanced MHC class I surface expression and improves antitumor CTL responses (Martini et al., 2010; Seliger et al., 2000a). 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 (Rodríguez et al., 2007).

Downregulation of TAP1/2 and LMP2/7 gene has been demonstrated in different cell lines and tumor lesions (Cabrera et al., 2003; Meissner et al., 2005). LMP7 downregulation 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., 2003; 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).

b) Irreversible Alterations

Total loss of HLA class I expression is caused by various mutations and chromosomal defects involving genes encoding heavy chain or β2m. Haplotype loss is one of the most frequently described alteration affecting HLA-I expression. It is caused by various defects in the HLA genomic region (short arm of chromosome 6, 6p21), including chromosomal dysfunction, mitotic recombination, and genetic conversion, or by loss of one copy of chromosome 6, which ultimately leads to the hemizygous loss of

HLA-A, -B and -C alleles (Torres et al., 1996). This type of HLA class I alteration mechanism has been described in different types of malignancy as laryngeal tumor (Maleno et al., 2002), melanoma (Rodriguez et al., 2005), colorectal tumor (Maleno et al., 2004) or non-Hodgkin's lymphoma. 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 (β2m 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. 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 (Jiménez et al., 2001; Lehmann et al., 1995; Serrano et al., 2000). Mutations in β2m genes range from large deletions to single nucleotide deletions and mutations are distributed randomly among the genes (Benitez et al., 1998; Feenstra et al., 1999; Paschen et al., 2003; Restifo et al., 1996). A mutation "hotspot" located in the CT repeat region of exon 1 of the β2m gene has been described (Pérez et al., 1999) reflecting an increased genetic instability in this region in malignant cells. A summary of β2m mutations discovered in tumor cell lines and tumor specimens has been reviewed previously (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: β2m mutation in one copy of the β2m 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 IFN-mediated signaling pathway (Rodriguez et al., 2005; Seliger et al., 2008).

### 2.3.3 HLA Class I defects and correlation with clinico-pathological characteristics

Downregulation or low expression of MHC class I antigens has been demonstrated to have an important cancer prognostic value in various studies, while other studies have failed to show a correlation between HLA expression and patient prognosis (Chang et al., 2003; Marincola et al., 2000; Powell et al., 2012). It has been observed that down-regulation of HLA class I expression in breast cancer has a significant association with adverse prognostic factors (Morabito et al., 2009). In addition, it has been reported that patients with conserved HLA class I expression have significantly better disease free interval than those with loss of HLA class I (Kaneko et al., 2011). In the same way, 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). 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., 2013). Another study revealed down-regulation of HLA class I as an independent

factor of poor prognosis in stage I patients, but not in late-stage cancer patient (Kikuchi et al., 2007). Two studies have found that total absence of HLA class I resulted in a favorable prognosis as compared to patients with low tumor HLA expression. It has been also described that high expression of HLA class I in tumor cells is 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 (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).

Inconsistencies among these studies may be explained by various reasons (Aptsiauri et al., 2013). First, most of the works have been realized in paraffin-embedded tissue, that only allow the use of a limited group of antibodies which in many cases detect only the intracytoplasmic free heavy chain, but not HLA-I surface expression. In other cases, where cryopreserved tumors were available, the use of mAb was limited to detect only total HLA-I loss, while partial losses were not studied. In addition, there are differences in the techniques with different degree of sensitivity, as immunohistochemistry, which is often difficult to evaluate, or differences in the classification of HLA-I lesions or labeling patterns, which could lead to discrepancies in data interpretation. .

**2.4 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 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 initiated a new era of cancer immunotherapy aimed at increase tumor immunogenicity

and T-cell-mediated antitumor immunity. Unfortunately, despite the significant advances in the understanding of antitumor immunity and considerable efforts to enhance immune reactivity against malignancies, consistent and long-lasting responses to cancer vaccines remain vague, and the overall clinical efficacy cancer immunotherapy are still below expectations (Rosenberg et al., 2004; Wang et al., 2009a). 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 improved (Klebanoff et al., 2011). In addition, the understanding of the molecular mechanisms of cancer immune escape and the role of complex interactions between tumor and the host has led 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 immunosuppression, etc. (Schlom, 2012; Walter et al., 2012). Furthermore, 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).

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 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.

HLA class I expression defects, induced by different molecular mechanisms, have been well documented in a wide variety of primary tumors and metastatic lesions. As a result, different HLA altered phenotypes are generated (fig. 2) and have an important implication in cancer immunotherapy (Garrido and Algarra, 2001; Khong and Restifo, 2002). HLA class I total downregulation is usually caused by defects in transcriptional activity of heavy chain genes, dysfunction in components of the antigen presenting machinery (APM), or some epigenetic events (Garcia-Lora et al., 2003b). These regulatory effects can be corrected through IFN-gamma treatment or others cytoquines. Actual immunotherapy protocols based on peptides have the purpose of increase tumor immunogenicity and CTL response. Its success also depend of HLA-I expression in cancer cells (Boon et al., 1997). So far, most immunotherapies induce stimulation of anti-tumor cell response poorly associated with significant clinical improvement (Rosenberg et al., 2004). It is believed that immunoselection produced during tumor progression and the selective pressure caused by immunotherapy support the propagation of tumors with different escape mechanism, many of which are characterized by loss or down-regulation of HLA-I molecules. Cancer immunotherapy may induce an increasing in HLA-I regulation in some metastatic lesions that eventually regress, but not in those lesions that have a tendency to progress (Algarra et al., 2004; Cabrera et al., 2007; Festenstein, 1987; Marincola et al., 2000; Zitvogel et al., 2006). In melanoma patients under immunotherapy, the lack of response to treatment and the generation of progressing metastasis seem to be associated with irreversible defects in HLA genes (Carretero et al., 2008). In other study, recurrent tumors of patients suffering bladder cancer treated with BCG immunotherapy showed more profound alterations in HLA-I (LOH-15 or LOH-6) than patients treated with chemotherapy. Moreover, post-BCG lesions showed a change in the HLA-I expression pattern that was

not observed in chemotherapy treated patients, suggesting an immunotherapy-induced immune selection orchestrated by the HLA-I loss of expression. Therefore, the restoration of normal expression of HLA class I patients with mutations in heavy chain, β2m, haplotype loss or other structural defects in HLA genes through gene therapy, become an interesting approach in the treatment of these patients.

Figure 7 illustrates how immunotherapy treatments and cancer progression may be affected by HLA alterations.

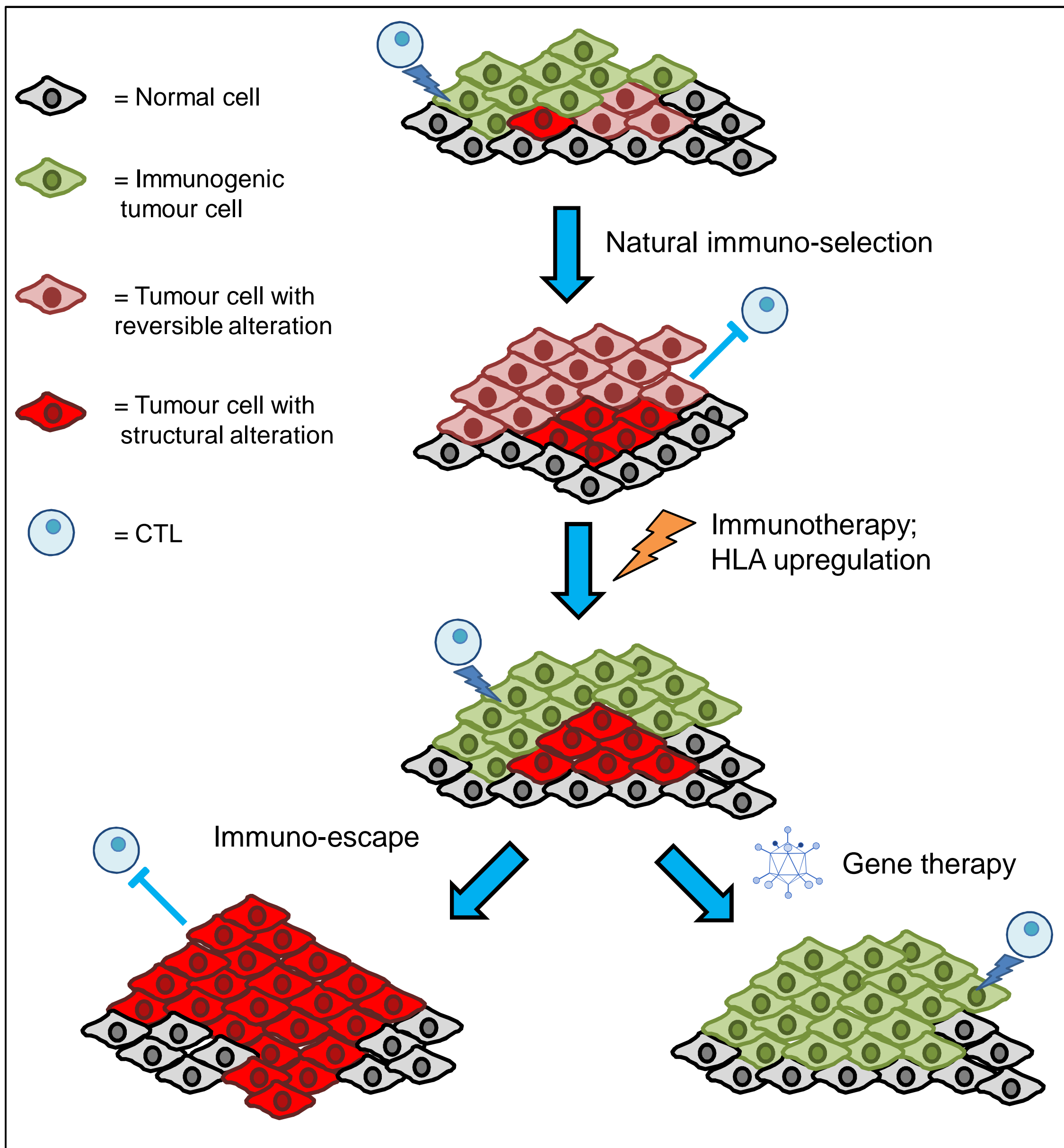



Figure 7. Tumor immune selection. In normal conditions immune system recognizes and kills cancer cells with high immunogenic profile. Low immunogenic variants can progress avoiding the immune attack, but immunotherapy treatment leads to activation of antitumor immune recognition mechanisms increasing peptide presentation to both CD8 and CD4 T-cells and promoting the response against tumor cells with reversible alterations. However, cancer cells variants with "hard" alterations can progress in this context of "selective pressure", and tumor will escape. In this case, only the recuperation of the damaged genes by gene therapy approaches may restore tumor immunogenicity and promote CTL response.

**PROSTATE CANCER**

The prostate is a compound tubule-alveolar exocrine gland of the man reproductive system, situated below the bladder and surrounding the urethra, which function is to produce the semen (along with spermatozoa and seminal vesicle). Prostate cancer (PC) is defined as the cancer that forms in tissues of prostate. Almost all the diagnosed PC are adenocarcinomas. Since other types of prostate cancer are extremely rare, when speaking of prostate cancer, we are most likely referring to adenocarcinoma. The inner part of the prostate (around the urethra) often keeps growing as men get older, which can lead to a common condition called benign prostatic hyperplasia (BH), but it is not cancer and does not develop into cancer.

According to the World Health Organization, prostate cancer is the second more common type of cancer in the world affecting men and the first in Europe, which represents 14,8% and 21,3% of total cases of cancer, respectively. Mortality from PC is around 6,6% among all cancer-related deaths in the world (9,4% in Europe), being the fifth type of cancer which causes more deaths (third in Europe) (Figure 8).

PC is a heterogeneous disease that exhibits a range of clinical behaviors, from indolent, slow-growing tumors to aggressive, fast-growing tumors with lethal progression. Although most tumors follow an indolent clinical course characterized by its slow progression, and may not cause morbidity even if left untreated, most clinically localized tumors are treated with curative intent via surgery or radiation. Even with such treatment, up to 30% of tumors relapse during long-term follow-up (Boorjian et al., 2012; Thompson et al., 2007). Although initially PC grows locally, with the advance of the disease it can spread outside the prostate gland, growing into nearby lymph nodes

first, and then it may metastasizes to the bones. Much less often the cancer will spread to the liver or other organs.

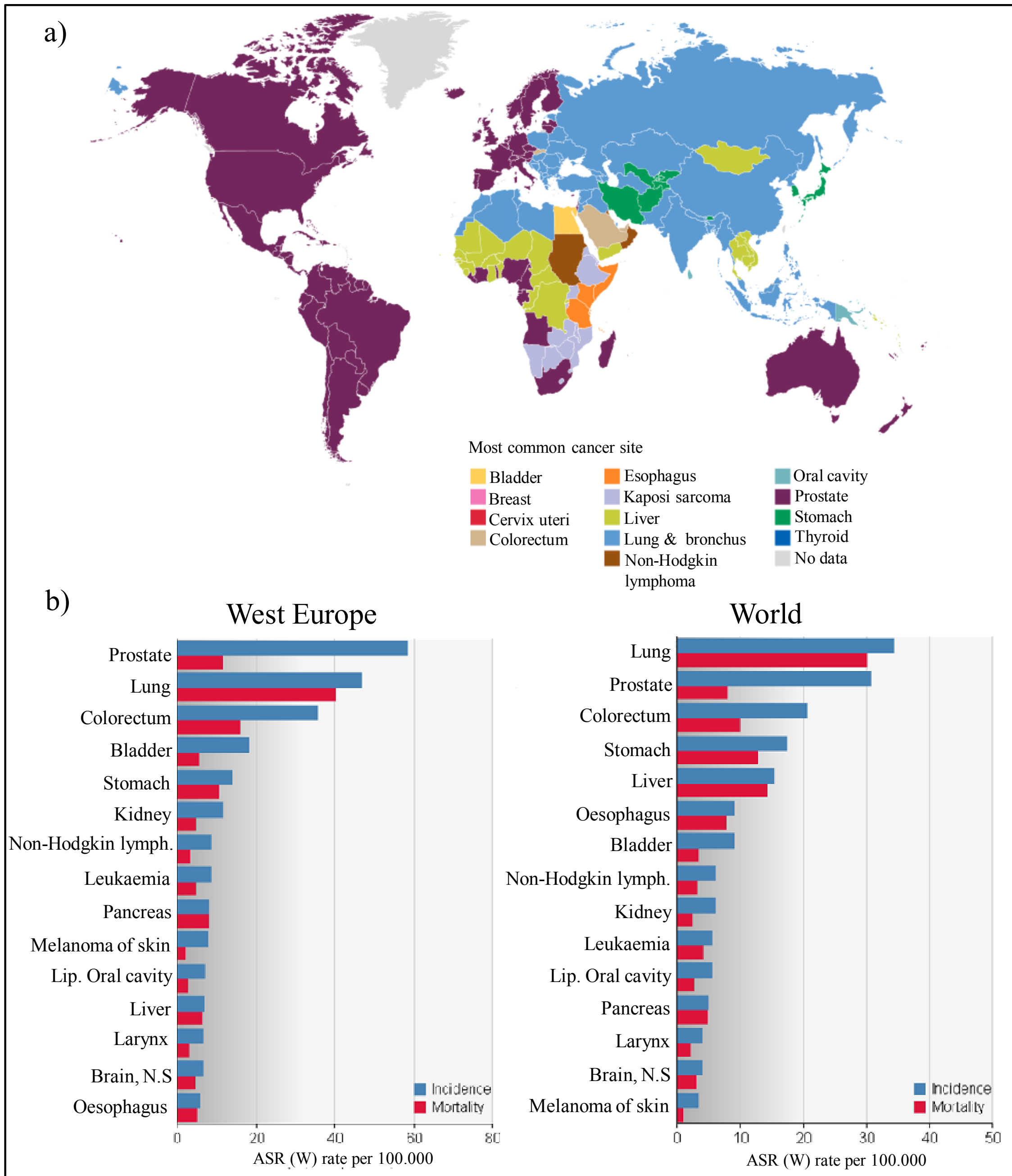



Figure 8. Incidence of Prostate Cancer. **a)** Distribution of most common cancers in men worldwide. **b)** Estimated age-standardized Cancer incidence and mortality rates in men in 2012. Reproduced from the International Agency for Research on Cancer (Globocan 2012)

Several risk factors associated with the development of PC have been identified. Most notable is age. Worldwide, about three-quarters of all PC cases occur in men aged 65 or more. A study examining data from the Health Professionals Follow-Up Study

found that smoking history, taller height, higher body mass index, family history, high caloric intakes, calcium, and alpha-linolenic acid, all significantly increased risk for fatal PC (Giovannucci et al., 2007). This study also found that higher physical activity levels are associated with lowered PC risk, which appears to apply to Caucasian but not African-American men (Singh et al., 2013). Moreover, segregation studies, twin studies, case-control, and cohort studies provide strong support for a genetic predisposition to PC (Langeberg et al., 2007; Ostrander and Stanford, 2000), with risk increasing 2- to 3-fold for men with a family history of the disease in a first-degree relative. The relative risk is further increased, 3- to 5-fold higher, if the relative was diagnosed before 65 years of age or if there are 2 or more relatives with prostate cancer. Family history is one of the indications for the initiation of prostatic specific antigen (PSA) screening at younger ages for early detection of prostate cancer (Qaseem et al., 2013). All this indicates that the mechanisms of PC pathogenesis are complex and that risk factors are still not well understood.

Importantly, if PC is detected at early stages, when it is still limited to the prostate, patients are expected to live longer. Prostate cancer treatment basically depends on the disease status. Cancer stage, age, and patient health are very important to choose the treatment. There are several therapies used to treat PC:

- Surgery: Extraction of complete prostate and surrounding tissues. Sometimes, lymph nodes of the pelvic area are extracted too. These intervention is known as radical prostatectomy,

- Radiotherapy: This therapy can be combined with the previous one, either to prepare the affected area or to “clean” the region after surgery.

- Hormonal therapy: Tumor progression is linked to the testosterone levels and activity. This treatment has the aim of reduce testosterone level in organism or to block the effect of this hormone in the prostate, in general by using analogues or antagonists of the Luteinizing Hormone-Releasing Hormones (LHRH). This treatment is usually employed when the disease has already spread, when cancer relapses, or before and along with radiotherapy as initial therapy in men who have high risk of cancer recurrence. Hormonal therapy is usually effective in reducing tumor size or slowing down the growth of prostate cancer. However, in most cases it loses effectiveness over time, developing castration resistant prostate cancer (CRPC), which will progress despite the fact that hormonal therapy maintains testosterone under very low level.

A significant number of medications have been approved recently for the treatment of CRPC (Pezaro et al., 2013), including chemotherapy (docetaxel), the second generation androgen receptor antagonist enzalutamide, and immunotherapy's as Sipuleucel-T (Provenge®). Sipuleucel-T is a cancer vaccine approved by the FDA in 2010, used to treat advanced prostate cancer that is no longer responding to initial hormone therapy. It is based on DCs pulsed with the prostatic acid phosphatase antigen (PAP) (Kantoff et al., 2010a) in order to enhance immune recognition of this TAA, getting a life expectancy of 25,8 months versus 21,7 months of the placebo group. Actually there are others immunotherapy´s being tested against prostate cancer.

PROSTVAC (PSA-TRICOM) is a pox-viral vaccine developed to stimulate the immune system via in-vivo immunologic stimulation that encodes for PSA and three co-stimulatory molecules (B7-1, ICAM-1 and LFA-3) and is designed to enhance T cell recognition of PSA (Gulley et al., 2014). In phase II studies it demonstrated an

improvement in overall survival (Kantoff et al., 2010b) (25,1 vs 16,6 months), and is currently in phase III testing in metastatic CRPC patients (NCT01322490).

GVAX is an allogeneic cellular immunotherapy or whole cell vaccine, comprised of two PC cell lines, PC3 and LNCAP, genetically modified to secrete the granulocyte macrophage colony-stimulating factor (GM-CSF), which are irradiated before injection. Phase III trials have been disappointing (Small et al., 2007).

Targeting the “immune-checkpoint” (inhibitory molecules that inhibit immune response, which are usually over-expressed in tumors) represent a novel application in anticancer immunotherapy (Pardoll, 2012). Anti-CTL4 antibodies such us Ipilimumab are currently FDA approved for metastatic melanoma, and are now in phase III testing in a variety of settings in metastatic CRPC (ClinicalTrials.gov Identifier: NCT01057810), or in combination with other treatments as radiotherapy (ClinicalTrials.gov Identifier: NCT00861614). There is an ongoing phase I study using a combination of Ipilimumab and Sipuleucel-T (ClinicalTrials.gov Identifier: NCT01832870).

Although new treatments for metastatic CRPC are providing an improvement in overall survival, they are still far from a curative efficacy. The study of prostate cancer immune status, including HLA-I expression, seems to be necessary for the understanding of the unresponsiveness to new immunotherapies in PC patients. Nevertheless, published data on human PC are limited with regard to tumor HLA-I expression and its predictive value in disease progression. Research into PC pathogenesis and immune escape has long been limited by the lack of studies using cryopreserved tumor tissues (where antigenic determinants of highly polymorphic HLA class I complex are better preserved) and new cell lines with well-characterized HLA

expression. Only a few human PC cell lines with characterized HLA expression are available from ATCC, including Du145, LnCap and PC-3 (Sanda et al., 1995), while the development of a cancer vaccine requires tumor cells expressing specific HLA-I alleles that selectively present a particular tumor-associated peptide to CTLs. The presence of reversible/ irreversible HLA-I lesions in prostate cancer may be affecting the resistance to immunotherapy. In the last case, restoration of affected gene would help to improve the therapy

## 3.- CANCER GENE THERAPY

Gene therapy can be defined as "the process of treating a particular disease through the introduction of genetic material in order to elicit a therapeutic benefit" (Stone, 2010). A gene can be delivered to a cell using a carrier known as a vector. In gene therapy, the most common types of vectors used are viruses that have been genetically altered to carry normal human genes and to make them safe, eliminating or altering one or more essential viral functions, as its self-replication.

Since the first clinical trial using gene transfer in 1989, 2142 gene therapy trials has been developed worldwide until year 2014 (The Journal of Gene Medicine 2015). Cancer is the most targeted disease using gene therapy approaches, with a 64,2% of total trials, followed by the treatment of monogenic (9,2%) and infectious (8%) diseases.

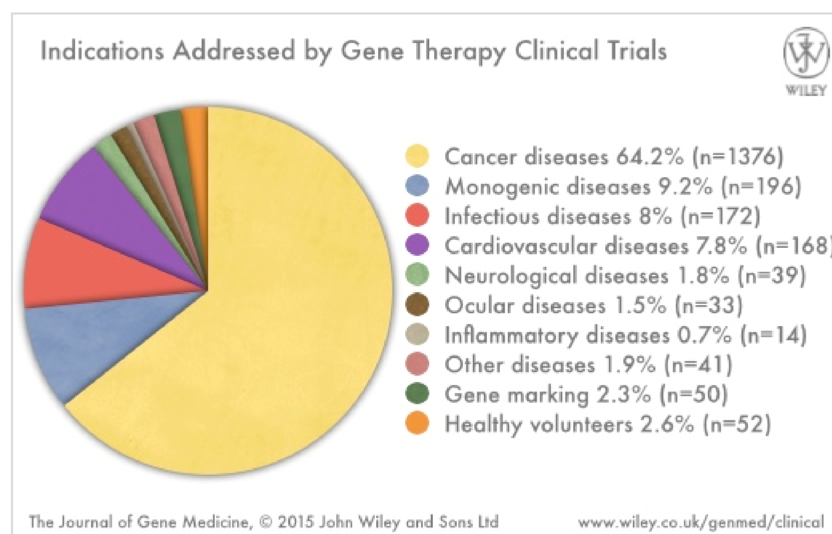



Figure 9: Indications addressed by gene therapy clinical trials. Reproduced from (Ginn et al., 2013).

## 4.1 Cancer gene therapy strategies

Many different types of cancers, including lung, gynecological, skin, urological, neurological and gastrointestinal tumors, as well as hematological malignancies and pediatric tumors are being treated in cancer gene therapy trials. A range of strategies has been applied to treat cancer, from inserting tumor suppressor genes, to cytokines, angiogenic factors, and gene-directed enzyme pro-drug therapy (Ginn et al., 2013), that can be classified depending on the target (cancer cell itself or tumor microenviroment) as it is depicted in table 2.

A separate group or strategy is formed by the cytolitic viruses, replicating or conditionally replicating viruses that mediate effects directly on transduced cells through viral replication and host cell lysis, and which may mediate effects on neighboring tumor cells by sequential propagation. These vectors have been modified by a variety of approaches to enable preferential replication in tumor cells or a specific tissue (Doronin et al., 2001; Wadler et al., 2003).

Table 2: Gene therapy approaches targeting cancer

| Transgenes or viral genes that mediate direct cytotoxic or cytostatic effects | |
|---|---|
| **Down-regulation of oncogenes:**<br>Retroviral vector that interferes with the cyclin G1 gene *(Rexin-G).*<br>Antisense oligonucleotides: *Genasense* blocks bcl-2 mRNA.<br>Ribozymes. The transgene encodes an enzymatically active RNA that can bind to and cleave the RNA from the oncogene<br>Single-chain antibodies that act intracellularly to bind to and disrupt the oncoprotein.<br>Dominant negative forms of the oncoprotein that serve to block its activity. | (Alvarez et al., 2000; Georges et al., 1993; Gordon et al., 2004; Gordon et al., 2000; Jansen et al., 2000; Russell et al., 1999; Thompson et al., 1995) |
| **Expression of tumor suppressor genes**<br>*Gendicine*: adenovirus vector carrying the p53 gene. | (Tazawa et al., 2013; Wilson, 2005) |
| **Expression of suicide genes:**<br>Herpes simplex type-1 thymidine kinase/ganciclovir (HSV-TK/GCV) and cytosine deaminase/5-fluorocytosin (CD/5FC).<br>Retrovirus-mediated HSV- 1TK suicide gene therapy for metastatic melanoma. | (Altaner, 2008; Klatzmann et al., 1998) |
| **Increase tumor immunity:**<br>Tumor antigens: Viral vectors encoding CEA, oncofetal antigen 5T4 (*Trovax*), MUC1 plus IL-2, PSA.<br>Use of co-stimulatory molecules, like B7, or the TRICOM system (B7-1, ICAM-1 and LFA-3). | (Conry et al., 1999; Kaufman et al., 2005; Kim et al., 2010; Lubaroff et al., 2009; Madan et al., 2012; Quoix et al., 2011) |
| Gene therapy producing modifications of tumor microenvironment | |
| **Anti-angiogenic effects**<br>Vectors carrying negative regulators of negative regulators of angiogenesis such as angiostatin, endostatin, PRP, IP10, VEGF, or chemokines such as CXCL10. | (Im et al., 1999; Li et al., 1999; Li et al., 1998; Li et al., 2008; Persano et al., 2007; Régulier et al., 2001; Takayama et al., 2000; Wang et al., 2009b) |
| **Immunomodulatory transgenes**<br>Cytokines: A retroviral vector has been used to deliver the human IFN-γ to patients with advanced melanoma in Phase I study. Adenoviral and canarypox vectors have both been used for intratumoral administration of the human IL-2 gene in Phase I studies in melanoma, lung and breast cancer.<br>Stimulate T cell response by CD40L administration in bladder cancer<br>T cell modification therapy: aimed to express tumor specific TCR genes in T cells (anti-MART-1, CEA or NY-ESO-1)<br>DC-based cancer gene therapy. Use of DC cells transduced with adenoviral vectors encoding IL23, IL-12 or CD40L or RNA pulsed encoding different TAAs | (Griscelli et al., 2003; Hu et al., 2006; Kass et al., 2001; Malmström et al., 2010; Mazzolini et al., 2005; Morgan et al., 2006; Nemunaitis et al., 1999a; Nemunaitis et al., 1999b; Park et al., 2011; Stewart et al., 1999; Tartaglia et al., 2001; Tomihara et al., 2008; Vacchelli et al., 2013) |

### 4.2 Choosing a viral vector

In gene therapy clinical trials the most commonly used gene delivery systems have been based on adenovirus (Ad), retrovirus, poxvirus, adeno-associated virus (AAV) and herpes simplex virus, which were cumulatively used in more than 66% of all clinical trials until to date (figure 10). Initially, gene delivery systems were developed from these viruses since they are easily manipulated in vitro and have been studied in great detail. These viral vectors have a wide range of attributes presenting different advantages and drawbacks that determine their suitability for different therapeutic applications (table 3).

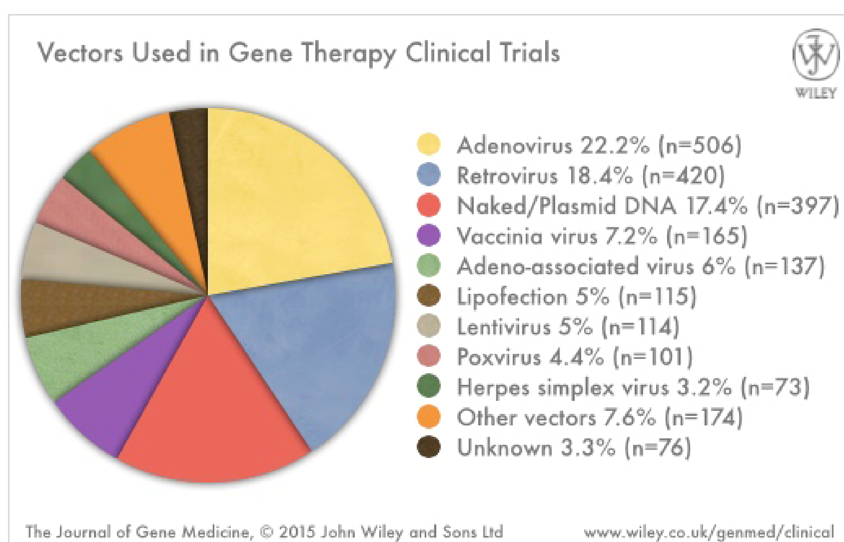



Figure 10: Vectors used in Gene Therapy Clinical Trials. Reproduced from (Ginn et al., 2013).

Although adenoviral vectors have been widely used in a vast number of different pre-clinical applications; in the clinic they have mainly been considered for use in applications such as cancer gene therapies or vaccination that require short-term gene transfer to specific cell types and/or organs [reviewed in (Stone and Lieber, 2006)]. The lack of integration and host immune responses limits their use for long-term therapies, while the relative toxicity and high levels of human exposure make it undesirable for

some therapies. Nevertheless, adenovirus can infect a wide variety of cells with high transfection efficiency.

On the other hand, adeno-associated virus (AAV) is considered to be the safest viral vector system since it is based on a non-pathogenic human virus that can only replicate in the presence of a helper virus co-infection. For this reason, AAV is quickly becoming the viral vector of choice in clinical trials and particularly those which require long-term correction or enhancement of a genetic defect. While AAV has a good safety profile and is able to facilitate long-term gene expression to several organs, it is limited by a number of factors. The small genome of AAV limits the size of potential therapeutic genes that can be packaged, while there is a clear inability to efficiently infect certain cell types (such as hematopoietic stem cells) that are considered essential targets for a number of genetic therapies (Daya and Berns, 2008; Mueller and Flotte, 2008).

Table 3: Characteristics of different viral vectors. Modified from (Vorburger and Hunt, 2002).

| | | Adenovirus | AAV | Alphavirus | Herpesvirus | Retrovirus | Lentivirus | Vaccinia virus |
|---|---|---|---|---|---|---|---|---|
| Particle characteristics | Genome | dSDNA | ssDNA | ssRNA | dsDNA | ssRNA | ssRNA | dsDNA |
| | Capsid | Icosahedral | Icosahedral | Icosahedral | Icosahedral | Icosahedral | Icosahedral | Complex |
| | Coat | Naked | Naked | Enveloped | Enveloped | Enveloped | Enveloped | Enveloped |
| | Virion polymerase | Negative | Negative | Negative | Negative | Positive | Positive | Positive |
| | Virion diameter | 70-90 nm | 18-26 nm | 60-70 nm | 150-200 nm | 80-130nm | 80-130nm | 300-450 nm |
| | Genome size | 38-39 kb | 5 kb | 12 kb | 120-200 kb | 3-9 kb | 3-9 kb | 130-280 kb |
| Gene therapy characteristics | Infect/tropism | Dividing and non dividing cells | Dividing and non dividing cells | Dividing and non dividing cells | Dividing and non dividing cells | Dividing cells | Dividing and non dividing cells | Dividing and non dividing cells |
| | Host genome interaction | Non integrating | Non integrating | Non integrating | Non integrating | Integrating | Integrating | Non integrating |
| | Transgene expression | Transient | Potential long lasting | Transient | Potential long lasting | Long lasting | Long lasting | Transient |
| | Packaging capacity | 7,5 kb | 4,5 kb | 7,5 kb | >30 kb | 8 kb | 8kb | 25kb |

We have selected an adenoviral vector as the method to restore HLA class I expression because this vector is considered to be the most effective vector in gene delivery (Cevher et al., 2012) and they have several biologic characteristics that can make them effective for cancer gene therapy: (1) a broad host/ cell range, (2) high levels of gene expression, (3) they infect dividing and non dividing cells (Seth, 2000), and (4) low risk of insertional mutagenesis (Jager and Ehrhardt, 2007). In addition, we have previous experience working with this vector. Moreover, we also decided to compare it with AAV, which has low pathogenicity, immunogenicity and toxicity. Among the drawbacks are small packaging capacity (up to 4,5kb) (Warrington and Herzog, 2006) and lesser transfection efficacy than adenovirus.

## 4.3 Adenovirus

Adenovirus is a DNA virus that contains about 36 kb of lineal double-stranded DNA encapsulated in an icosahedral protein capsid, constituted by about a dozen proteins and no lipid envelope (Shenk, 1996). The virion also has a unique fiber associated with each penton base of the capsid which is implicated in cell recognition and internalization. Figure 11 illustrates Adenovirus structure.

Viruses of the family *Adenoviridae* infect various species of animals, including humans. Adenoviruses were first isolated in human adenoids in 1953 (Rowe et al., 1953) and there are at least 57 serotypes of human adenovirus, Ad1-Ad57, which form seven “species”, each denominated from A to G (Horwitz, 1996). All serotypes are similar in general structure and the functions of most proteins, but certain unique protein functions contribute to the unique properties of the serotype and the species. Group C (Ad1, 2, 5, 6) are usually acquired in early childhood and whose infection can be asymptomatic or can lead to disease which is usually mild in immunocompetent

individuals, causing respiratory infections and to a lesser extent, infect the gastrointestinal and urinary tracts. Vectors based on this group are currently the most commonly used for gene transfer for cancer treatment.

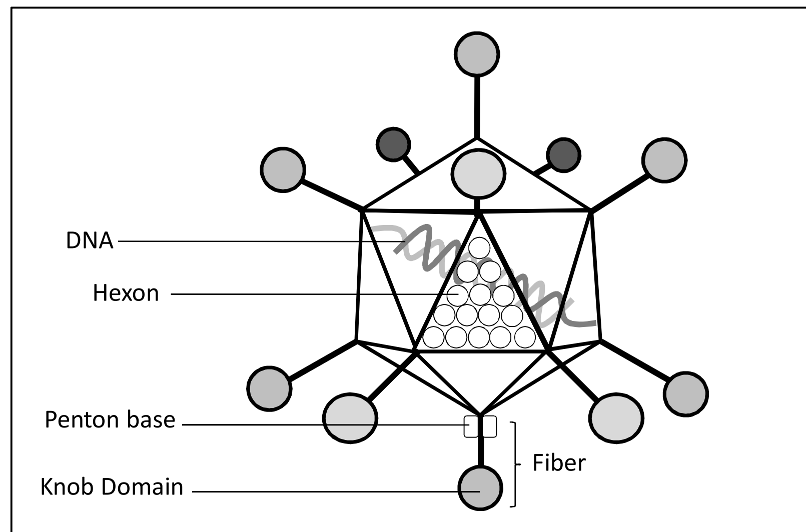



Figure 11. Schematic representation of adenovirus structure. Icosahedral structure is mainly formed by hexon proteins. The fibers are associated to the penton base and recognize cell receptor through the knob domain.

Viral cycle is initiated with the entry of adenoviruses into the host cell, which involves two sets of interactions between the virus and the host cell. Entry is initiated by the knob domain of the fiber protein binding to the cell receptor. The two currently established receptors are: CD46 for the group B human adenovirus serotypes and the coxsackievirus adenovirus receptor (CAR) for all other serotypes (Bergelson et al., 1997; McDonald et al., 1999). After anchoring at the CAR, the adenoviruses achieve internalization through a secondary interaction of a specialized motif in the penton base protein of the capsid with αv integrins (alpha v beta 3 and alpha v beta 5) present on the target cells, stimulating internalization of the adenovirus (Wickham et al., 1994). Binding to αv integrin results in endocytosis of the virus particle via clathrin-coated pits within an endosome. Once the virus has accessed into the cell, the endosome acidifies causing capsid components to disassociate. These changes results in the release of the virion into the cytoplasm. The virus is transported to the nuclear pore complex with the

help of cellular microtubules, whereby the adenovirus particle disassembles. Viral DNA is subsequently released and enters the nucleus via the nuclear pore (Ziello et al., 2010). After this the DNA associates with histone molecules, but it does not integrate into the host genome, remaining in the nucleus as an episomal element. Thus viral gene expression can occur and new virus particles can be generated. Figure 12 summarizes adenovirus infection and replication pathway.

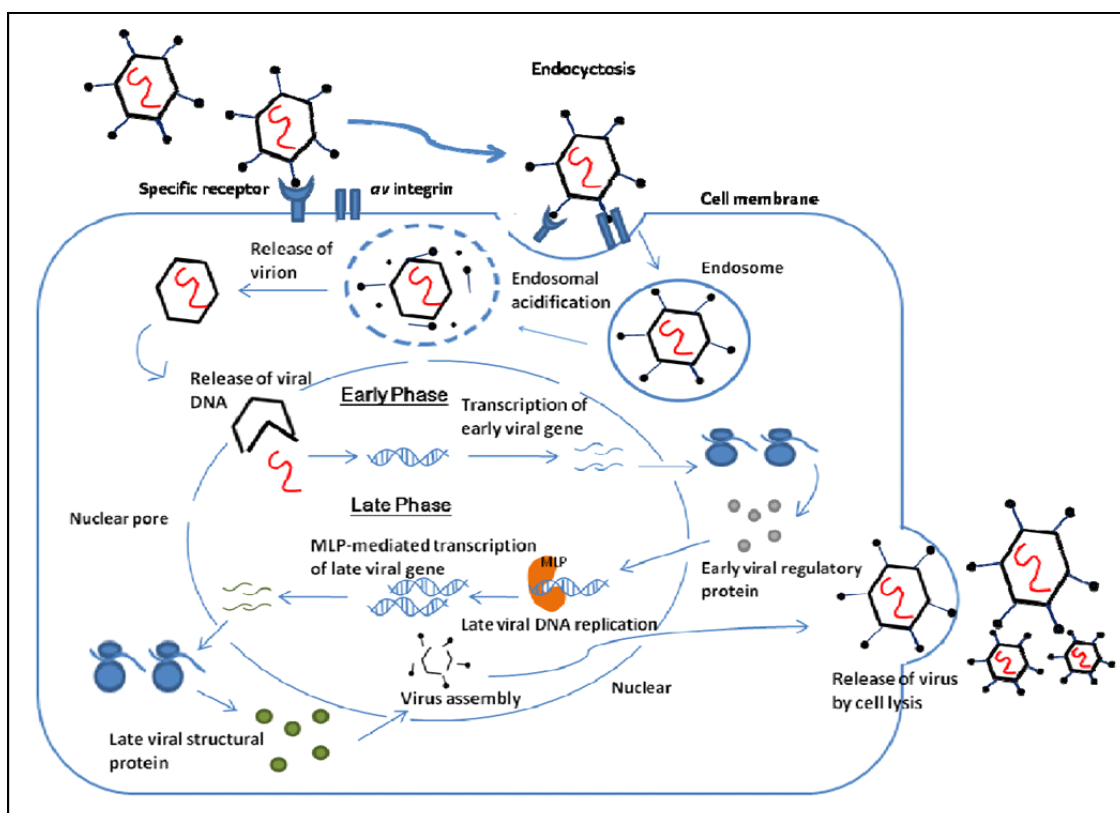



Figure 12. Adenovirus infection and replication pathway. Entry is initiated by fiber binding to the cell receptor (CAR) and is enhanced through a secondary interaction with αv integrins, which results in endocytosis of the virus particle. Once the virus has accessed into the cell, the endosome acidifies causing capsid components to disassociate. These changes results in the release of the virion into the cytoplasm. The virus is transported to the nuclear pore complex with the help of cellular microtubules, whereby. Viral DNA is subsequently released and enters the nucleus via the nuclear pore. After this the DNA associates with histone molecules, but it does not integrate into the host genome, remaining in the nucleus as an episomal element. Viral gene expression occur in two phases, early phase, where regulatory genes are encoded, and late phase, where the strong major late promoter (MLP) mediates the transcription of late virus genes which encode the viral structural proteins and proteins for the maturation of viral particles. Finally all the genetic material produced is packed and new virus particles can be generated. Reproduced from (Waye and Sing, 2010).

After entry into the nucleus, adenovirus genome initiates transcription. The genome of adenovirus, flanqued by ITRs encodes approximately 35 proteins that are

expressed in two general phases: early phase, which occurs prior to the initiation of viral DNA replication at about 7 hours post-infection, and late phase, which occurs following the initiation of DNA replication. In both phases a primary transcript is generated which is alternatively spliced to generate monocistronic mRNAs compatible with the host's ribosome, allowing for the products to be translated. During the early phase of viral replication, four non-contiguous regions of the viral genome are quickly expressed (E1 to E4), and transduce 20 early proteins which have regulatory functions that allow the virus to alter the expression of host proteins that are necessary for DNA synthesis, to activate other virus genes (such as the virus-encoded DNA polymerase), and to avoid premature death of the infected cell by the host-immune defenses (Bennett et al., 1999; Chatterjee-Kishore et al., 2000). In the late phase, late genes codify mainly structural proteins of the virus to pack all the genetic material produced. Once the viral components have successfully been replicated the virus is assembled into its protein shells and released from the cell as a result of virally induced cell lysis. Virions assemble in the nucleus starting at about 1 day postinfection, and after several days the cell lyses to release infectious virus. About 10,000 progeny virions are produced in permissive cells.

**Using Adenovirus as a vector**

At least three regions of the viral genome can accept insertions or substitutions of DNA to generate a helper independent virus: a region in E1, a region in E3, and a short region between E4 and the end of the genome (Hitt and Graham, 2000; Imperiale and Kochanek, 2004). From the first adenoviral vector generated for gene therapy to nowadays, several generations of adenovirus have been developed, improving some capacities and trying to solve drawbacks.

In the first-generation vectors, the E1 region was removed to avoid viral replication, and a heterologous transgene is substituted for the viral E1 genes. Vectors have also been constructed in which the viral E3 region has been deleted to provide additional free space, or is substituted by a second transgene. Adenoviral vectors that have deletions in the E1 and E3 regions can carry 7000-8000 base pairs of genetic material (Bett et al., 1994). One of the approaches to construct E1 deleted adenovirus is homologous recombination (Hardy et al., 1997) where a purified adenoviral genome with deleted E1 and/or E3 regions, is co-transfected with a shuttle vector that contains the left handed ITR, the E1 enhancer, the encapsidation signal, the cytomegalovirus promoter, a multicloning site for insertion of the therapeutic gene, and the SV40 poly(A) signal followed by sequences from the adenoviral genome located 3´ of the E1 domain. Replication-impaired adenoviral vectors must be grown on cell lines that complement the missing E1 functions, such as 293, an embryonic kidney cell line whose genome carries the adenoviral E1 region (Graham et al., 1977). However, even in the absence of E1 gene products, there was low-level transcription of the remaining viral genes, resulting in early innate host cytokine transcription followed by antigen-dependent immune responses (Hartman et al., 2008). This resulted in a reduction of the period of gene expression because of cell-mediated destruction of the transduced cells (Kay et al., 1995; Yang et al., 1995; Yang and Wilson, 1995).

Replicating or conditionally replicating vectors (CRAdV) employed in cancer treatments typically retain all or most of these regions but have modifications to allow for preferential replication in particular cell types. These modifications typically fall into two classes, those where expression of critical viral functions, such as those of the viral E4 region, is transcriptionally regulated by a tissue- or tumor-specific promoter

and those where modifications have been made in the E1 region to allow for preferential replication in tumor cells but not normal cells (Everts and van der Poel, 2005).

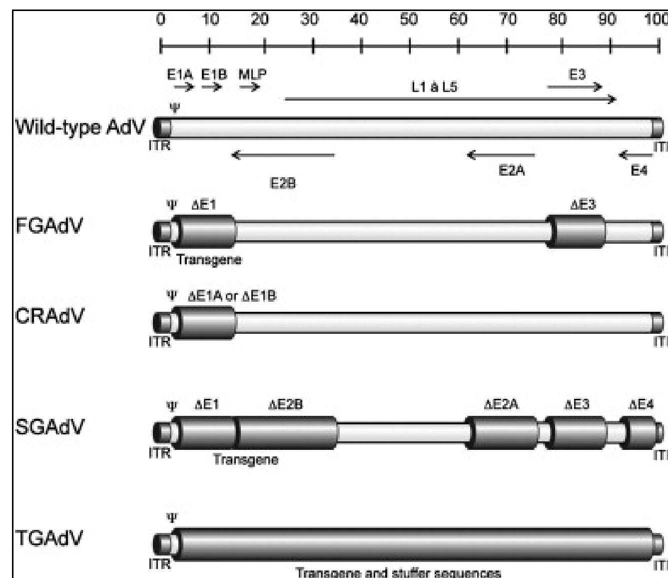



Figure 13. Schematic representation of wild-type adenovirus genome and different generations of adenovirus. Genomes are divided into 100 map units (28 to 38 kb). E1 to E4: early transcript units, L1 to L5: late transcript units, ITR: inverted terminal repeats, MLP: major late promoter, ψ: packaging signal, FGAdV: first generation adenoviral vectors, CRAdV: conditionally replicative adenovirus, SGAdV: second generation adenovirus, TGAdV: third generation adenovirus. Reproduced from (Dormond et al., 2009)

Second generation adenoviral vectors have deletions of various E1, E2, and E4 genes (Lusky et al., 1998), because viral proteins encoded by these DNA sequences were shown to induce most of the host immune response. These new adenovirus vectors have decreased toxicity and show prolonged gene expression in vivo (Engelhardt et al., 1994; Schiedner et al., 1998) , but they need the isolation of a complementary cell line (Dormond et al., 2009). However, an important limitation in the use of recombinant adenoviruses has been the difficulty to obtaining efficient gene transfer upon a second administration of virus due to the strong humoral and cellular immune responses it elicits (Bangari and Mittal, 2006).

To minimize the immune responses, the third generation of adenovirus or “gutless” vectors was developed. Gutless vector contain only the viral terminal repeats

and the packaging sequence (Kochanek et al., 1996) meanwhile all other viral components are deleted, thus it can accommodate up to 36 kb of exogenous DNA and does not trigger strong immune responses. However, gutless adenovirus vector, due to the deletion of most viral components, requires helper plasmid or virus for production, making the production process more complicated. Furthermore, the transgene expression appears to be weakened (Evans et al., 2006).

**Adenoviral cancer gene therapy**

Adenovirus potential as anti-tumor therapy was evident after the observation of tumor regression in patients with cervical carcinoma following adenovirus administration (Huebner et al., 1956). Despite the concern over safety of use adenovirus vectors in relation to immune responses and after the first death reported subsequent to adenovirus administration, adenoviral therapeutic applications have demonstrated to be safe in a large number of clinical trials (Wold and Toth, 2013).

Different adenoviral vectors have been tested in clinical trials with promising results as showed by the approval of two vectors in China for cancer treatment, both carrying p53 gene: *Gendicine* ®, in 2003, being the first gene therapy vector for commercial use in combination with chemotherapy (Wilson, 2005), and *ADVEXIN* (Senzer and Nemunaitis, 2009). Conditionally replicative vectors has also obtained promising results in the treatment of different types of cancer, as ONYX-015 (Lamont et al., 2000; Nemunaitis et al., 2001), Ad5-RGD-D24-GMCSF (Pesonen et al., 2012), ICOVIR-5 (Cascallo et al., 2007), the oncolytic adenovirus vector CV706 evaluated for recurrent prostate cancer (Chen et al., 2001; DeWeese et al., 2001), or the conditionally replication-competent adenovirus DELTA-24-RGD in recurrent glioblastoma multiforme (EudraCT Number: 2007-001104-21).

Adenoviral vectors have also been developed with the purpose to boost anticancer immunity against cancer, by delivering different cytokines, including IL-12 (Cao et al., 2013), a truncated form of survivin in combination with IL-2 (Zhang et al., 2012) or IFN-γ (ClinicalTrials.gov Identifier:NCT01082887) in melanoma, but also in prostate cancer, injecting adenovirus encoding IL-12 after radiation therapy in prostate resistant cancer (ClinicalTrials.gov Identifier: NCT00406939), or GM-CSF in Patients with non-muscle invasive bladder cancer who have failed BCG (ClinicalTrials.gov Identifier: NCT01438112). Co-stimulatory molecules have been also tested using adenoviral vectors as the co-expression of B7-1 with cytokines like GM-CSF using an oncolytic virus (Kanerva et al., 2013), or the transduction of CD40 ligand to orthotopic bladder cancer model that elicited strong anti-tumor immunity and suppressed tumor growth (Loskog et al., 2004; Loskog et al., 2005; Malmström et al., 2010).

### 4.5 Adeno associated virus

AAV are single-stranded DNA virus which belongs to the family Parvoviridae and are placed in the genus Dependovirus. Discovered in 1965 (Atchison et al., 1965), they are called associated virus because productive infection by AAV requires the presence of an adenovirus or members of the herpes virus family for efficient replication (Buller et al., 1981; Casto et al., 1967). Although most people have been exposed to wild-type AAV and are seropositive for AAV antibodies, AAV infection is not associated with any known disease (Berns and Linden, 1995). Until to date, more than 100 human and non-human primate AAVs have been identified, including 12 human serotypes.

The viral genome of 4,7 kb consists of two genes, each encoding multiple polypeptides: *rep*, required for viral genome replication; and *cap*, which produces

structural proteins. These two genes are flanked by viral ITRs that are composed by 145 nucleotides in length. The first 125 nucleotides of the ITR constitute a palindrome, which folds upon itself to maximize base pairing and forms a T-shaped hairpin structure. The ITRs are important cis-active sequences in the biology of AAV, constitute the origin of replication and serves as a primer for second-strand synthesis by DNA polymerase [reviewed in (Daya and Berns, 2008)]. The four Rep proteins are crucial for genome replication, transcription, integration and encapsidation. The three structural capsid proteins assembly into a 60-subunit icosahedral capsid with help of an essential assembly-activating protein (AAP) (Naumer et al., 2012). Following infection in the absence of helper virus, the double strand DNA of AAV (serotype 2) is retained in circular episomal form or can set up latency by integrating into AAVS1 site in human cells (Kotin et al., 1990) localized in chromosome 19q13.4, until subsequent rescue upon helper virus co-infection.

The intracellular transport of AAV has been previously reviewed (Nonnenmacher and Weber, 2012). The target cell specificity of AAV relies on capsid interactions with cell surface receptors. Importantly, most sequence variability between different AAV serotypes is located in the surface-exposed regions of the capsid (Xie et al., 2002), which may explains the different tropism between serotypes. AAV2, the most studied AAV serotype, gains entry into target cells by using the cellular receptor heparan sulfate proteoglycan (HSPG) (Summerford and Samulski, 1998). Internalization is enhanced by interactions with one or more of at least six known co-receptors including αVβ5 and αVβ1integrins (Asokan et al., 2006; Summerford et al., 1999), fibroblast growth factor receptor 1 (Qing et al., 1999), hepatocyte growth factor receptor (Kashiwakura et al., 2005) and laminin receptor (Akache et al., 2006), suggesting that AAV2 infection requires a primary glycan receptor together with a co-

receptor for optimal attachment and internalization. After AAV binding to the surface of the target cell via one or more receptors/co-receptors, the virus/receptor complex will be uptake by endocytosis with subsequent invagination of the cell membrane following different pathways (Doherty and McMahon, 2009; Mayor et al., 2014).

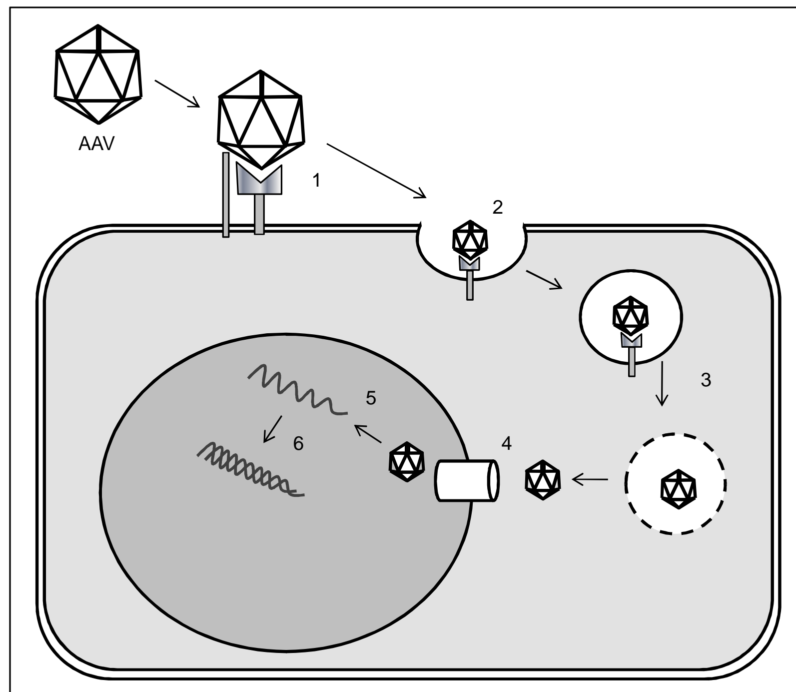



Figure 14. Course of AAV infection. 1) Viral particles bind to cell membrane specific receptor and co-receptor. 2) Endocytosis of the virus/receptor complex. 3) Endosomal trafficking of virus. 4) Import of virus through the nuclear pore complex. 5) Uncoating of virus and DNA release. 6) Replication of the single strand DNA to double strand. The presence of helper virus will determine if AAV DNA remains as an episomal element, or it will be integrated in host genome.

The next steps which can be collectively characterized as “endosomal trafficking”, include the maturation of virus-containing vesicles through the acidification of endocytic compartments, either late endosomes, recycling tubular endosomes or lysosomes (Bartlett et al., 2000; Douar et al., 2001; Sonntag et al., 2006), followed by retrograde transport to Golgi and/or the endoplasmic reticulum.

Endosomal trafficking is most likely followed by the nuclear import of the viral particle through the nuclear pore complex. Once the virus has gained access to the nucleus, it is readily transported to the nucleolus, in which it is maintained as an intact

particle until its egress into the nucleoplasm (Johnson and Samulski, 2009). After nuclear translocation, single-stranded DNA is released by capsid uncoating and converted into double-stranded DNA to allow transgene expression.

**Using AAVs as vectors**

AAVs are non pathogenic to humans and show relatively low host immune response and weak toxicity. AAV can mediate long term transgene expression in a wide variety of cells, including dividing and non-dividing cells. These advantages have inspired the wide application of recombinant AAV vectors for gene delivery (Mingozzi and High, 2011). Currently, AAV appears to be one of the most promising vectors for gene therapy, and may offer the best compromise between safety and efficacy for in vivo gene transfer (Evans, 2012; Evans et al., 2006). In 2012, Glybera®, an AAV vector designed to treat lipoprotein lipase deficiency, becomes the first gene therapy product approved in the Western world (Ylä-Herttuala, 2012).

Initially recombinant AAV (rAAV) vectors have been based mostly on AAV2, but today more serotypes (1 to 9) are being tested. The efficiency of rAAV transduction is dependent on the efficiency at each step of AAV infection. Vectorization of wild-type AAV has been usually done by removing all viral coding sequences (*cap* and *rep* genes) and keeping the ITRs, since they contain the necessary cis-acting sequences for replication and packaging, and have been adapted for gene delivery by placing heterologous DNA sequences of choice between flanking ITRs (Daya and Berns, 2008). Replication and assembly of recombinant rAAV virions is produced by adding to productive cells several plasmids containing the ITRs flanking the therapeutic gene cassette, the rep/cap genes, and the addition of adenoviral helper genes, which initially where provided by an adenovirus, but latter approaches improved the AAV production

by adding the essential adenoviral helper genes inserted in a third plasmid (Matsushita et al., 1998; Xiao et al., 1998). The packaging capacity of AAV is about 5.0 kb, which is a major limitation of this vector system. Nevertheless, the discovery of vector genome linkage has allowed to double the limited coding capacity by splitting a gene or expression cassette into two vectors and simultaneously administering them to muscle or liver (Nakai et al., 2000). The wild-type virus in the presence of rep has a propensity to integrate into a specific region of human chromosome 19, but this property is lost in AAV vectors due to the absence of the *rep* gene. In cells transduced by rAAV, vector genomes are maintained predominantly as episomal circular monomers and concatemers.

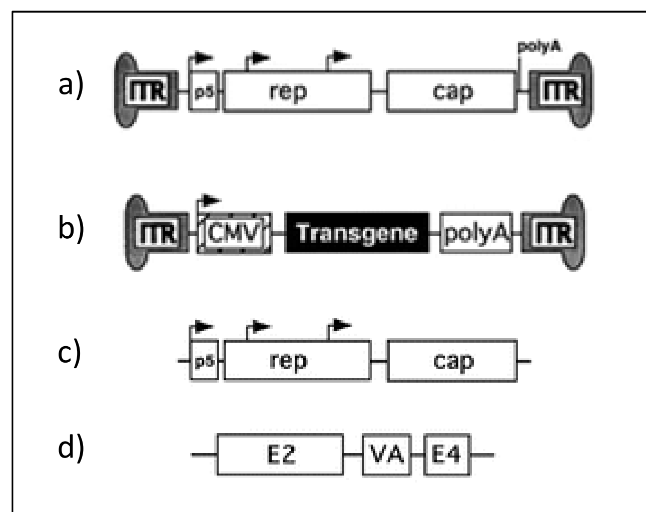



Figure 15. AAV genetic structure and derived vectors. a) Wild type AAV; b) AAV vector containing only the ITRs of the wild virus, the transgene and its promoter and the polyadenilation signal sequences; c) AAV packaging construct including cap and rep genes. d) Minimal helper construct derived from adenovirus. Modified from (Pfeifer and Verma, 2001).

Several approaches have been realized in order to improve rAAV as gene delivering vectors, focusing in modify AAV tropism and enhance transduction, and decrease the host immune response. A critical step in AAV vector development was the discovery that the prototypic AAV2 vector genome could be cross-packaged into the capsids of multiple different AAV isolates from a diversity of species (Rabinowitz et

al., 2002). This process, known as pseudo-serotyping, has proven to confer novel tropism and biology on individual genome/capsid configurations, and enhance the utility and flexibility of the AAV vector system.

The design and use of self-complementary AAV (scAAV) vectors to bypass the limiting aspects of second-strand synthesis have been described previously (McCarty et al., 2001). The rationale underlying the scAAV vector is to shorten the lag time before transgene expression and potentially to increase the biological efficiency of the vector. scAAV vectors can fold upon themselves, immediately forming transcriptionally competent double-stranded DNA. One consequence of the use of scAAV is that the maximal size of the transgene is reduced by 50% (2.4-kb capacity), but up to 3.3 kb of DNA can be encapsidated (Wu et al., 2007). Rapid transduction has been observed using scAAV in both tissue culture and in vivo experiments.

Other development in order to enhance AAV transduction came from a series of studies on the role of tyrosine phosphorylation in viral trafficking and gene expression. Highly efficient transduction could be achieved in human cells *in vitro* and in murine hepatocytes *in vivo* using AAV vectors containing single-point mutations of surface-exposed tyrosine residues in the AAV2 VP3 capsid (Zhong et al., 2008).

One challenge to the clinical application of AAV is that a large portion of human population has been previously exposed to AAV and possesses neutralizing antibodies against AAV diminishing the in vivo efficacy of AAV (Frank et al., 2009; Nayak and Herzog, 2010). Several studies have shown that recombinant AAV vectors can be modified in terms of either the expression cassette or the capsid structure with the aim of mitigating cellular and humoral immunity. Restricting the expression of transgene to the target tissue would allow enhanced vector performance and prevent antigen

presentation by APC and subsequent immune activation (Nathwani et al., 2006; Ziegler et al., 2004). Capsid engineering of AAV vectors is also an effective approach to evade immune response and augment their transduction potential. Covalent attachment of synthetic polymers onto the virion surface can shield the antigen binding sites from neutralizing effects of anti-AAV antibodies and circumvent the challenge of pre-existing immunity (Le et al., 2005; Lee et al., 2005). Genetic modification of the capsid by mutating the neutralizing antibodies epitopes is an alternate way to generate low immunogenic AAV. Rational design utilizes either insertion of peptides at specific positions that disrupt the antibody binding site of viral capsid or site-directed mutagenesis of specific residues of immunogenic peptides on AAV2 capsid (Lochrie et al., 2006; Wobus et al., 2000).

**Cancer gene therapy using AAV**

In recent years, gene therapies with different subtypes of AAV vectors have been reported for treatments of a variety of diseases, from cystic fibrosis (Flotte et al., 1996)), deficiency in lipoprotein lipase (*Glybera)*, hemophilia B (Nathwani et al., 2006), muscular dystrophies (Anisimov et al., 2009), Alzheimer disease´s (Kou et al., 2015), to liver cancer (Glushakova et al., 2009).

AAVs have been successfully used to deliver and transfer a variety of therapeutic genes to cancer cells in mice models with different approaches to inhibit tumor initiation, growth, and metastasis. One of the most promising is the use of suicide genes, as the rAAV carrying the herpes simplex virus thymidine kinase gene/ganciclovir pro-drug (HSV-tk/GCV) system, which have shown to generates strong antitumor efficacy (Kim et al., 2011; Pan et al., 2012). The AAV-mediated anti-angiogenesis gene therapy strategy is headed by the transfection of VEGF, showing that

single intravenous administration of AAV/VEGF led to long-term efficacy permitted suppression of primary tumor growth and prevention of pulmonary metastasis (Lu et al., 2012). AAV-mediated transduction of other anti-angiogenic genes, such as pigment epithelium derived factor (PEDF) also showed significant inhibition of tumor angiogenesis, tumor growth, and metastasis (He et al., 2012; Wu et al., 2012).

AAV-mediated immune gene therapy is becoming an important approach in the treatment of different cancers, for example the use of AAV2 transfecting PSA in prostate cancer (Mahadevan et al., 2007), IL-12 in glioblastoma (Chiu et al., 2011) or IL-15 in cervical cancer (Yiang et al., 2009) and breast cancer (Yiang et al., 2012). Other serotypes also have been used as AAV8 transferring (CCR4) in lymphoma (Han et al., 2012), AAV5 and AAV9 carrying HPV16 L1/E7 in HPV tumors (Nieto et al., 2009) or AAV6 and AAV2 transducing LMP1 and LMP2 in nasopharyngeal carcinoma (Pan et al., 2009). Other AAV-mediated gene therapies include the gene delivery of the extracellular domain of murine PD-1 (Elhag et al., 2012), FasL in human laryngeal carcinoma Hep2-bearing nude mice (Sun et al., 2012) or the cell binding domain and C-terminal heparin-binding domain (CBDHep II) recombinant polypeptide of human fibronectin in breast carcinoma (He et al., 2014).

Some novel phase I cancer gene therapy clinical trials using AAV are active or are recruiting new participant. The first one uses DCs infected with AAV carrying carcinoembryonic antigen (CEA) gene to induct cytotoxic T lymphocytes against tumor cells in the treatment of stage IV gastric cancer patients (ClinicalTrials.gov Identifier: NCT01637805). The second one uses AAV carrying the human aquaporin-1 gene to infect parotid salivary gland of squamous cell head and neck cancer patients with irradiation-induced parotid salivary hypofunction (ClinicalTrials.gov Identifier: NCT02446249)

## 4.6 Targeting HLA in cancer gene therapy

It has been described above that the inability of the immune system to recognize malignant cells may be due to the loss of tumor HLA-I expression and lack of presentation of TAA to T cells. Moreover, immunotherapy treatments can lead to immune selection of cancer cells with irreversible alterations in HLA-I genes, like LOH in chromosome 6 and 15, which finally develop malignant lesions resistant to therapy (Aptsiauri et al., 2008; Carretero et al., 2011; Carretero et al., 2008; Carretero et al., 2012).

HLA downregulation can be restored by cytokine treatment and patients may therefore benefit from T-cell-based immunotherapy. However, patients with tumors harboring irreversible HLA class I loss due to structural defects, have a low probability of benefiting from this therapeutic approach. Only restoration of damaged genes by means of gene therapy can recover normal HLA-I expression.

Restoration of HLA-I expression has been employed previously to show the relevance of class I molecules in specific tumor lysis by CTL and NK cells (Tanaka et al., 1985). In various experimental systems, introduction of the MHC class I molecules into class I-negative tumor cells led to higher immunogenicity and decreased tumorigenicity of these cells (Hui et al., 1984; Wallich et al., 1985). In our laboratory, we constructed previously a replication-deficient adenoviral vector with human β2m gene in order to recover HLA class I expression in various HLA class I-negative cancer cell lines with a double knockout of the two β2m gene copies (del Campo et al., 2009). The functional ability of the newly expressed HLA class I complex to present peptides to T cells in HLA-restricted manner was confirmed in ELISPOT and CTL assays. In addition, in vivo experiments using human tumor xenograft model, the intra-tumoral

injection of β2m carrying vector led to the restoration of normal HLA class I expression. We showed that this replication-deficient adenoviral vector-carrying human β2m gene is effective in restoration of HLA class I expression in various types of β2m-negative human tumor cells.

Nevertheless, loss of an HLA-I haplotype or allele caused by mutations or LOH-6 are also frequent, and can be detected alone or in combination with chromosome 15 aberrations (Chang et al., 2005; Koopman et al., 2000). Loss of expression of one particular allele, HLA-A2, has been reported in different types of cancer (Brady et al., 2000; Geertsen et al., 2002; Masuda et al., 2007; Norell et al., 2006; Serrano et al., 2000) with important clinical implications since HLA-A2 allele has one of the widest peptide repertoires among human class I molecules (Maecker et al., 2005; Paul et al., 2013). Moreover, many of cancer vaccines and immunotherapies use HLA-A2-specific tumor peptides to stimulate A2-restricted CTL responses in patients who are positive for HLA-A2 allele (Mackensen et al., 2006). Hence, loss of this particular allele in tumor could compromise the efficacy of immunotherapy, and the recovery of HLA-A2 expression using gene transfer methods might improve the clinical outcome of the treatment.

# OBJECTIVES

HLA class I antigens are key molecules in tumor cells recognition by cytotoxic CD8+ T-lymphocytes. Loss of HLA class I expression is a well documented phenomenon in human cancer associated with cancer immune escape. It has been described in a wide variety of primary tumors, metastatic lesion and tumor cell lines derived from them. However, in some types of malignant diseases, including prostate cancer, this knowledge is limited due to various factors, such as lack of cryopreserved primary tumor samples and few prostate tumor cell lines available for HLA analysis and modulation of its expression. The use of cancer cell lines facilitates the study of HLA-I alterations, as well as allows manipulation of different factors implicated in its expression.

Molecular mechanisms causing aberrant HLA class I expression can range from total loss to partial alterations (locus and allelic losses) and can be classified, according to the type of underlying molecular defect, as regulatory alterations (“soft”) or structural alterations (‘hard’). Structural defects are caused by mutations and structural aberrations in HLA genes leading to permanent loss of expression, which can be recovered only by gene transfer methods. Due to the HLA allele-specific restriction of tumor associated antigenic peptide presentation, it is believed that the loss of only one HLA allele can lead the tumor to escape.

Prostate cancer is one of the most common types of cancer in men. HLA-I alterations have been previously reported in prostate cancer; however, information regarding the frequency and the associated molecular mechanisms is incomplete. With the development of immunotherapy in the era of personalized medicine molecular analysis of tumor HLA expression becomes essential for optimization of cancer treatment. Restoration of HLA-I genes trough gene therapy is the only way to recover

tumor HLA class I expression, and might therefore correct the inability of CTLs to recognize and eliminate tumor cells. Different viral vectors, including adeno-associated vectors (AAV) have emerged recently as good candidates for cancer gene therapy, providing some advantages over other well established vectors as adenovirus. Testing their suitability for HLA restoration in cancer cells could be useful for future applications. HLA-A2 is the most common allele in European population with one of the widest peptide repertoires among human class I molecules, which makes this allele an attractive target for HLA class I recuperation using gene therapy.

**Objectives:**

a. Analysis of the frequency of HLA alterations in prostate primary tumors and association of HLA alterations with clinico-pathological data.

b. Analysis of the molecular mechanisms implied in HLA loss in prostate primary tumors and cancer cell lines.

c. Comparative analysis of viral vectors (different AAV serotypes and adenovirus) for the optimization of the effectiveness of HLA gene transfer in tumor cells with different histological origin.

d. Recovery of total and allelic losses of HLA class I expression in human cancer cell lines using viral vectors carrying HLA-A2 and/or Beta-2-microglobulin genes.

# MATERIAL AND METHODS

## 1.- Patients and tumour samples

We analyzed 42 cryopreserved human prostate tumours and 12 prostatic benign hyperplasia (BH) samples collected from patients after radical prostatectomy between 1994 and 2006 in the Virgen de las Nieves University Hospital of Granada (Spain). We also analyzed a separate group of 6 samples collected between 2008 and 2010 (primary prostate tumours with adjacent normal epithelium). The research protocol was approved by the hospital institutional review board and ethical committee. Tumour Gleason grade and TNM were characterized according to the guidelines of the European Association of Urology. Tumours were also classified by disease progression risk according to the D'Amico criteria (D'Amico et al., 1998) based on known risk factors: clinical stage, Prostate Specific Antigen (PSA) level and Gleason tumour grade. Perineural invasion (PNI) was determined by histopathological examination. Clinical-pathological characteristics of the patients are summarized in Table 1.

Table 1. Clinico-pathological characteristics of the patients and tumours

| | | n |
|---|---|---|
| Stage | | |
| | pT1-2c | 26 |
| | pT3a-4 | 14 |
| | n.a | 2 |
| PSA | | |
| | <10 | 18 |
| | ≥10, <20 | 15 |
| | ≥20 | 2 |
| | n.a | 7 |
| Gleason | | |
| | <7 | 23 |
| | =7 | 12 |
| | >7 | 5 |
| | n.a | 2 |
| Cancer Risk | | |
| | Low | 5 |
| | Medium | 28 |
| | High | 7 |
| | n.a | 2 |
| Perineural Invasion | | |
| | yes | 15 |
| | no | 20 |
| | n.a | 7 |
| Recurrence | | |
| | yes | 25 |
| | no | 15 |
| | n.a | 2 |

*n*: number of cases analyzed
*n.a:* not analyzed

## 2.- Cell lines

We used four groups of human tumour cell lines for virus-mediated recovery/upregulation of HLA-A2 and/or β2m based on the baseline expression status of these molecules (Table 2). In addition, we studied the HLA expression of twelve

previously unreported prostate cell lines. Ando-2 was kindly provided by Dr P. Coulie (Université Catholique de Louvain (UCL), Brussels, Belgium) and is characterized by the loss of HLA-A2 expression due to HLA-I haplotype loss (Paco et al., 2007). Prostate cancer cell lines DU145 and PC3, bladder cancer cell lines T24, WIL and RT112, and Burkitt lymphoma DAUDI were obtained from American Type Culture Collection (ATCC). All melanoma cell lines, except for Ando-2, were obtained from the European Searchable Tumour Cell Line Data Base (ESTDAB) (Pawelec and Marsh, 2006) and have been previously genotyped for HLA-I and characterized for HLA phenotype (Méndez et al., 2008). Twelve prostate cell lines derived from cancerous and/or normal prostate epithelia of eight different PC patients (and immortalized by HPV16/18-E6/E7 gene transfer) were provided by our collaborators from Onyvax Limited (St George's Hospital, UK) as a part of the European ENACT project (European Network for the identification and validation of Antigens and biomarkers in Cancer and their application in clinical Tumour immunology, LSHC-CT-2004-503306). Malignant prostate cells in culture retained neoplastic phenotypes and prostate-specific markers. Cell line identity was confirmed by HLA typing using genomic sequencing technique.

Bladder cancer cells were cultured in Dulbecco media supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine and 1% penicillin/streptomycin. Prostate cancer cells, except for DU145, PC3 and LnCaP, were cultured in keratinocyte medium with a growth supplement (Gibco, Paisley, UK; Invitrogen, Carlsbad, CA, USA). All melanoma cells, and prostate cells DU145 and PC3, were grown in RPMI-1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% FCS, 2% glutamine (Biochrom KG) and 1% penicillin /streptomycin (Biochrom KG) at 37 ºC in a humidified atmosphere with 5% CO2.

**Table 2. Genomic HLA-I typing in the studied human cancer cell lines**

| Cell line | HLA-I genomic typing | | | Origin |
|---|---|---|---|---|
| | Locus A | Locus B | Locus C | |
| **Cell line negative for HLA-A2 due to HLA-I haplotype loss** | | | | |
| Ando-2* | 3201 | 4001 | 0304 | Melanoma |
| Cell lines negative for genomic *HLA-A2* | | | | |
| PC3 | 2402/0101 | 1302/5501 | 0102/0602 | Prostate |
| DU145 | 0301/3303 | 5001/5701 | 0602/0602 | Prostate |
| OPCN1 | 0101/2301 | 3701/4001 | 0304/0602 | Prostate |
| OPCT1 | 0101/2301 | 3701/4001 | 0304/0602 | Prostate |
| WIL** | 6802 | 1503 | 1203 | Bladder |
| T24** | 0101 | 1801 | 0501 | Bladder |
| RT112** | 2601 | 270305 | 0102 | Bladder |
| E130** | 1101 | 4001 | 30401 | Melanoma |
| **Cell lines negative for genomic *HLA-A2* allele with total HLA-I loss caused by *β2m* alterations** | | | | |
| E038 | 0101/2902 | 3501/4403 | 40101 | Melanoma |
| E109 | 0101/2501 | 0801/1801 | 070101/120402 | Melanoma |
| OPCN3 | 0101/2501 | 5701/5701 | 0602/0602 | Prostate |
| **Cell lines positive for genomic *HLA-A2*** | | | | |
| E050 | 0201/2601 | 1402/3801 | 0802/1203 | Melanoma |
| E102 | 0201/0301 | 1801/4001 | 0304/070101 | Melanoma |
| E120 | 0101/0201 | 0702/0801 | 0701/0702 | Melanoma |
| E013 | 0101/0201 | 0801/15010101 | 070101/0304 | Melanoma |
| E019 | 0101/0201 | 0801/0702 | 0701/0702 | Melanoma |
| E025 | 0101/0201 | 0801/15010101 | 0304/070101 | Melanoma |
| E002 | 0201 | 0702/4402 | 0501 | Melanoma |
| E006 | 0101/0201 | 0801/15010101 | 0701/0702 | Melanoma |
| E007 | 0201 | 0702/4402 | 0501 | Melanoma |
| E009 | 0201/0301 | 0702/4402 | 0702/0501 | Melanoma |
| E012 | 0201/0101 | 0801/15010101 | 0304/070101 | Melanoma |
| E014 | 0101/0201 | 0801/15010101 | 0304/070101 | Melanoma |
| E021 | 0201 | 0702/1801 | 0501/0702 | Melanoma |
| E026 | 0201/6802 | 1501/4402 | 0303/0704 | Melanoma |
| E027 | 0101/0201 | 0801/15010101 | 0704/0304 | Melanoma |
| E029 | 0201/0205 | 2704/4901 | 0102/070101 | Melanoma |
| E043 | 0201/6801 | 4001/4002 | 0304/0202 | Melanoma |
| E049 | 0201/0301 | 0702/1401 | 0702/0802 | Melanoma |
| E064** | 0201 | 0702 | 0702 | Melanoma |
| E073 | 0201/0301 | 0702/1302 | 0602/0702 | Melanoma |
| E074 | 0201 | 0702/4402 | 0501/0702 | Melanoma |
| E076 | 0201/2902 | 1302/440301 | 1302/440301 | Melanoma |
| E077 | 0201/2601 | 1302/3801 | 0602/1203 | Melanoma |
| E084 | 0301/0201 | 1501/0702 | 0304/0702 | Melanoma |
| E100 | 0201/0301 | 1402/44020101 | 0501/0802 | Melanoma |
| E107 | 0201/1101 | 15010101/55 | ND/ND | Melanoma |

*Ando-2 cells have loss of HLA haplotype caused by LOH-6, as confirmed by the analysis of autologous PBMC; ** homozygote or a probable loss of heterozygocity, not confirmed due to the absence of autologous material

## 3.- HLA genomic typing

Genomic HLA typing of all cell lines and autologous PBMCs corresponding to prostate tumours was performed using Dynal RELI®SSO Typing Trays and Dynal RELI®SSO Strip Detection Reagent kit, following the manufacturer´s instructions.

## 4.- Immunohistochemistry and antibodies

The Novolink Polymer Detection System (Leica Mycrosystems) was used for tissue immunohistochemistry and for immunocytochemical staining of tumour cells grown on glass slides using a wide panel of anti-HLA monoclonal antibodies (mAb) (Table 3). Results were interpreted as described previously (Carretero et al., 2011), using immunolabeling grading system in accordance to the HLA & Cancer Component of the 12th International Histocompatibility Workshop (IHW)) (Garrido F et al., 1996) as negative (when <25% cells were stained), heterogeneous (when 25–75% of cells were stained), or positive (when >75% of cells were stained). In some cases tumours with negative and heterogeneous patterns of HLA-I labeling were grouped together as oppose to HLA-I-positive ones. We also analyzed HLA-I/ β2m positive tumours to identify undetected partial locus and allelic alterations. Based on the obtained results we classified tumours into different phenotypes: positive, PhI (total loss), and partial losses - PhIII (locus loss) and PhIV (allelic loss).

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (BioSigma, St Louis, MO, USA) mAb was used as secondary Ab for FACS analysis.

**Table 3: List of monoclonal antibodies**

| Use | Ab | Recognized Antigen | Reference |
|---|---|---|---|
| IHC/FACS | W6/32 | HLA-I/ β2m Complex | (Barnstable et al., 1978) |
| IHC/FACS | GRH1 | β2m | (Lopez-Nevot et al., 1986) |
| IHC/FACS | 1082C5 | HLA-A | (Lozano et al., 1989) |
| IHC/FACS | 421B5 | HLA-B | (Lozano et al., 1990) |
| IHC/FACS | HC-10 | HLA-I free Heavy Chain | (Stam et al., 1990) |
| IHC/FACS | MARB3 | HLA-Bw4 specificities | (Rehm et al., 2000) |
| IHC/FACS | HB152 | HLA- Bw6 specificities | ATCC |
| IHC/FACS | HA41 | HLA- A23, A24 | One Lambda |
| IHC/FACS | CR11 | HLA- A2 | (Russo et al., 1983) |
| IHC/FACS | HB122 | HLA-A3 | ATCC |
| IHC/FACS | HB56 | HLA- B7 | ATCC |
| IHC/FACS | MRE4 | HLA- B8 | kindly provided by Dr Renèe Fauchet, France |
| IHC/FACS | GRB1 | HLA-II DR | (Sáenz-López et al., 2010) |
| IHC/FACS | TU155 | HLA- A | (Hutter et al., 1996) |
| FACS | DT9 | HLA-C | Millipore |
| FACS | 6B11 | HLA- A1 | (Carrel et al., 1994) |
| FACS | B721 | HLA-II DP | (Rodríguez et al., 2007) |
| FACS | TU22 | HLA-II DQ | (Maeda et al., 1986) |
| FACS | L368 | β2M | (Lampson et al., 1983) |

## 5.- <u>Flow cytometry</u>

HLA expression on prostate cell lines was done by flow cytometry (BD FACS Canto (Becton Dickinson)) using a panel of specific antibodies directed against HLA-I and II antigens in baseline conditions and after 48-hr incubation with IFN-γ (800 U/ml) as previously described (Méndez et al., 2008). FITC-conjugated goat anti-mouse Ab (Sigma) was used as a secondary Ab. Results are shown either as representative FACS plots or based on fluorescence intensity as follows: - negative (MFI 0-1), + weakly positive (MFI 1-10), ++ positive (MFI 10-100), +++ strongly positive (MFI >100).

Transgene expression after infection of the cells with recombinant vectors was also evaluated by FACS. The expression of GFP was evaluated directly, while the cell surface expression of HLA class I complex, and of HLA-A2 and β2m molecules was evaluated by indirect immunofluorescence using specific antibodies (table 2). Results

were presented as representative fluorescence plots, as mean fluorescence intensity (MFI) bars or as percentage of positive cells. Data were analysed using *Flowing Software* (University of Turku, Finland).

## 6.- Tissue microdissection, DNA/RNA isolation, and reverse transcription

Cryopreserved 6-µm thick tissue sections were stained with a 0,05% wt/vol solution of toluidine blue and microdissected using the Laser micromanipulator PALM Microlaser System (Zeiss). Microdissected fragments were collected in PALM Adhesive Caps and used for RNA and DNA isolation. DNA and RNA extraction of microdissected tissue was performed using the QIAmp DNA mini kit (QIAGEN) and the miRNeasy mini kit (QIAGEN), respectively. In cell lines, total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed with the iScript$^{TM}$ cDNA Syntheis Kit (BIORAD) following the manufacturer´s instructions.

Cells and tumour samples were analyzed for the expression of *β2m, HLA-A, HLA-B, Tap1, Tap2, Tapasin, LMP2, LMP7, calnexin, calreticulin, IRF1,* and *NLRC5* mRNA by quantitative real-time PCR (Q-PCR) on Applied Biosystems Fast 7500 apparatus using TaqMan PCR master mix and target-specific primers/probes from Applied Biosystems (UK). Results are expressed relative to β-*glucuronidase* (GUSB) gene expression to control for variations in amounts of mRNA. In some cases we included results obtained at high amplification cycles with CT cutoff of 39 due to the small RNA quantities obtained from after tissue microdissection.

## 7.- PCR of genomic DNA and *β2m* sequencing

DNA from microdissected HLA-I negative tumours was used to amplify specific "hot-spot" sequences of *β2m*, using the following primers: fw (5´-gtccctctctctaacctggc -3´) and bw (5´-cagagcgggagggtaggaga-3´) to amplify exon 1, and fw (5´-taccctggcaatattaatgtgtc-3´) and bw (5´-catacacaactttcagcagcttac -3´) to amplify exon 2, with annealing temperatures of 64ºC and 53ºC, respectively. DNA from OPCN3 cells was isolated with the QIAamp DNA Mini Kit (Qiagen) and was used to amplify *β2m* gene sequences with the following primers: fw (5´- aattgctatgtcccaggcac-3´) and bw (5´-acacaactttcagcagcttac-3´), at an annealing temperature of 53 ºC.  PCR products were amplified using Bigdye Terminator v1.1 cycle sequencing kit *(*Applied Biosystems), followed by sequence analysis in an Abi Prism 377 DNA sequencer.

## 8.- Microsatellite analysis

To determine loss of heterozygosity (LOH) in chromosome 6 and 15 (LOH-6 and LOH-15), DNA obtained from microdissected tumour specimens, peripheral blood, and cell lines was studied using eight short tandem repeats (STRs) mapping the *HLA-I* region of chromosome 6 (D6S291, D6S273, C.1.2.C, C.1.2.5, D6S265, D6S105, D6S276 and D6S311) and five markers flanking the *β2m* on chromosome 15 (DS15209, DS15126, DS15146, DS151028 and DS15152). The STRs, PCR, electrophoresis methods and data interpretation were previously described (del Campo et al., 2014; Maleno et al., 2006). LOH was assigned when a signal reduction of >25% in three alleles was seen in tumour sample versus analogous control DNA sample.

## 9.- Viral vectors

Adenovirus serotype 5 (Ad5) carrying *GFP* and *β2m* gene under the control of cytomegalovirus (CMV) (AdCMV-GFP and AdCMV-β2m) were produced previously in our laboratory as described earlier (del Campo et al., 2009) according to a published protocol. Same method with minor modifications was used to construct a novel replication-deficient Ad5 coding for human *HLA-A0201* (AdCMV-HLA-A2) gene by homologous recombination between ψ5 (as a donor virus to supply the viral backbone) and pAdlox-A2 (a shuttle plasmid with a single loxP site carrying the HLA-A0201 gene) using the Cre-lox recombination system (**Hardy et al, 1997**). HLA-A0201 gene was initially amplified from pclnx-A2 plasmid (obtained from ATCC) using specific primers for human *HLA-A0201* (forward primer: 5´-AAGCTTGCCACCATGGCCGTCATGGCGCCCCGAA -3´; reverse primer: 5´-AATTGGATCCCCTACAGGTGGGGTCTTTC - 3´) with restriction sites HindIII and BamHI in 5´and 3´end, respectively. KOZAK sequence was added to facilitate the recognition of the initiation sequence. The 1500 base pairs fragment corresponding to HLA-A0201 cDNA was cloned into pCR4-TOPO plasmid generating TOPO-A2 (TOPO TA cloning Kit; Invitrogen). The HindIII ⁄ BamHI HLA-A0201 fragment excised from TOPO-A2 was ligated into HindIII ⁄ BamHI-digested pAdlox. New pAdlox-A2, linearized with SfiI restriction enzyme, was co-transfected along with ψ5 DNA (ψ5 is an E1–E3 deleted version of Ad5-containing loxP sites flanking the packaging site) into 116 cells using jetPrime™ (Polyplus Transfection). After development of complete cytopathic effect (5 days), the cell lysate was passaged three times in 116 cells to reduce the ψ5 virus presence. A single AdCMVA2 clone was expanded to produce a large-scale concentrated stock in HEK293 cells, which later was purified on CsCl gradient, desalted using PD-10 columns (Amersham Biosciences) and

stored in Tris–HCl 0,1 M and 10% glycerol at -80 ºC. Virus titres were determined by plaque assay in HEK293 cells based on the visual detection of infected cells stained with the mouse anti-adenovirus monoclonal antibody blend (Chemicon Int.) and the titre was expressed as plaque-forming units (pfu).

Adeno-associated vectors of various serotypes, AAV1, AAV2, AAV5, AAV6, AAV8 and AAV 9, carrying GFP gene under the control of CMV were kindly provided by Drs. S. Zolotukhin, I. Zolotukhin, and G. Aslanidi and A. Dinculescu from the University of Florida, USA. AAV-Triple, AAV- Quadruple and AAV-Sextuple, AAVs which have mutations in tyrosin/phenilalanin groups of the viral proteins responsible for improved attachment to the target cell surface (**Ryals et al, 2011**), were kindly provided by Dr. Astra Dinculescu, from the University of Florida. Mutations in these vectors are: Triple mutant (Y444F+Y730F+Y500F), Quadruple mutant (Y444F+Y730F+Y500F+Y272F) Sextuple mutant (Y444F+Y730F+Y500F+Y272F+Y704F+Y252F). Recombinant AAV2 carrying *HLA-A0201* gene (AAV2-HLA-A2) under the control of CMV promoter was constructed using method described previously (Zolotukhin et al., 1999) by cotransfection of three plasmids: phelper (containing adenoviral genes required for AAV replication), PACG2 (containing Rep and Cap genes, the AAV genes required for replication and capsid production) and UF11-A2, which includes ITR regions and the HLA-A0201 gene, into 293T cells. UF11-A2 was previously constructed by excising HLA-A0201 gene from TOPO-A2 using restriction enzymes, and cloned into UF11 plasmid, kindly provided by Dr. Zolotukhin. 72 hours later, when cytopatic effect was observed, cells were collected and lysed by freezing/thawing. Cell lysate was purified on Iodixanol discontinued gradient (Optiprep Density Gradient Medium, Sigma) and purified viral fraction was recovered and rinsed with *Lactated Ringer´s solution*. Vector quantification was

determined by Q-PCR of specific AAV sequences and was expressed by viral-genome per mL.

A schematic representation of different steps involved in the construction of AdCMV-HLA-A2 and AAV2-HLA-A2 vectors is depicted in figure 1.

### 10.-<u>Cell infection in vitro</u>

The purified virus was added either directly to the cell culture flasks in a 2% FCS supplemented media or was first used to infect cells in a small volume (200 µl) for 2 hrs at 37ºC before transferring to a bigger flask. Viral vectors were used at following doses or multiplicity of infection (MOI): 200, 500 or 1000 MOI for AAVs, and 20, 50, 100, 200 MOI for Ad5. Comparable transduction effect was observed at 100 MOI for AdCMV-GFP and 1000 MOI in case of AAVs. 100% of cells infected with AdCMV-GFP at 200 MOI were GFP-positive. However, cytopathic effect prevailed limiting the possibility of increasing the virus dose. Therefore, two optimal viral doses with maximum efficacy were selected for cell infection: 100 MOI for Ad5 and 1000 MOI for AAV2. In some experiments tumour cells were simultaneously infected with two adenoviral vectors, AdCMV-HLA-A2 and AdCMVHLA-β2m. The expression of the transgenes was determined by flow cytometry using specific antibodies. To examine the time course of transgene expression, cells were harvested on days 3, 6, 10, 15, 20, 25, 30 and 35 after infection, and HLA class I surface expression using W6/32 mAb was evaluated by flow cytometry.

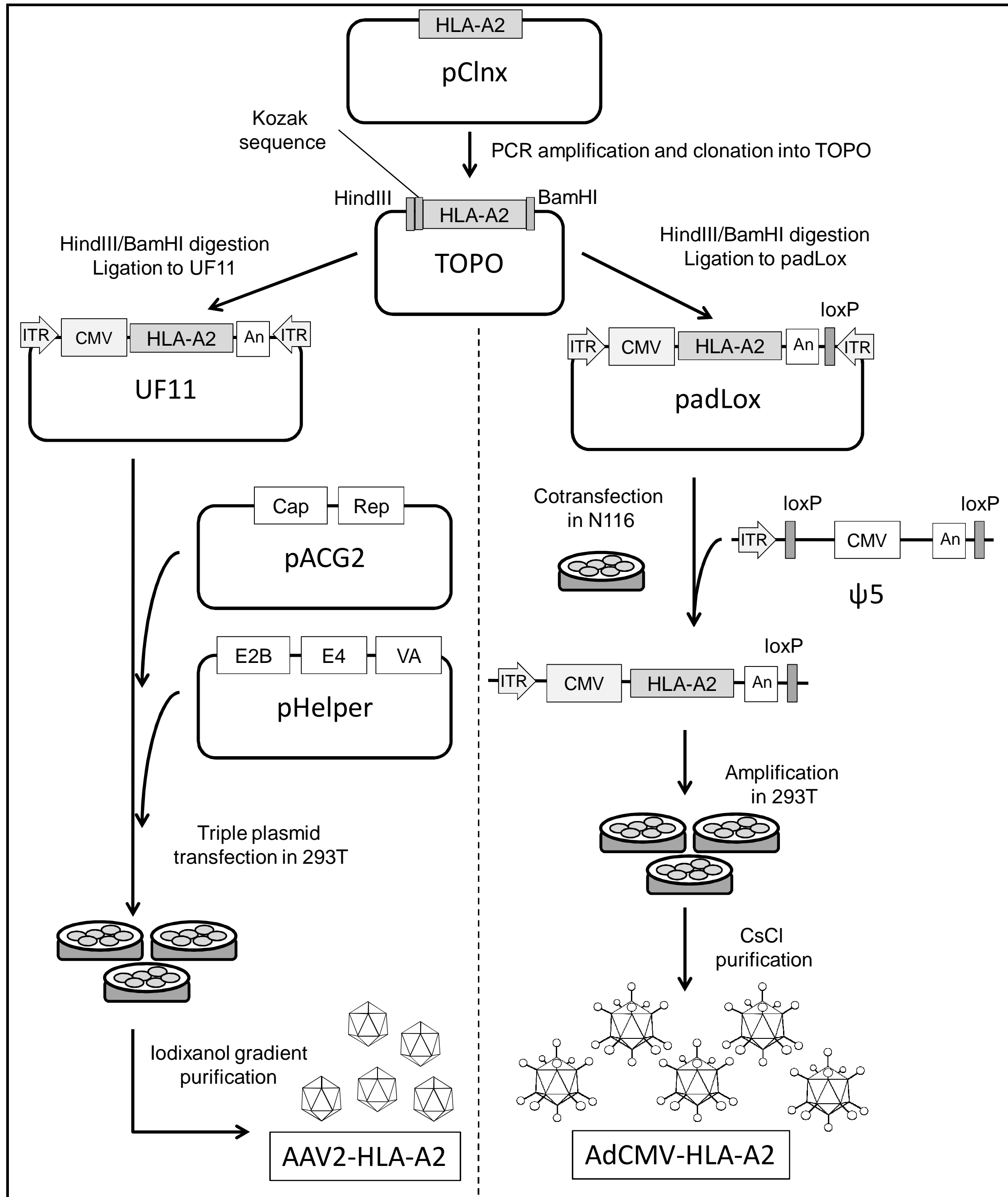



Figure 1. Schematic representation of the construction of recombinant vectors AdVCM-HLA-A2 and AAV2-HLA-A2. Initially, pClnx plasmid with HLA-A2 gene was obtained from ATCC. PCR amplification was performed with introduction of additional restriction sites HindIII and BamHI, and of KOZAK sequence at 5´end. PCR product was cloned into TOPO plasmid. For the production of adenovirus, HLA-A2 gene was isolated from TOPO, and ligated to padLox plasmid. Next it was co-transfected with ψ5 DNA (donor virus to supply the viral backbone) into N116 cells (source of E1 adenoviral genes) using the Cre-Lox recombination system. Recombinant adenovirus was re-amplified several times in 293T cells, and purified on CsCl gradient. For AAV2 construction, HLA-A2 gene was isolated using restriction enzymes and ligated to UF11 plasmid, which contains necessary ITRs. Later it was co-transfected together with pACG2 (which carry Cap and Rep genes) and phelper (source of adenoviral genes) into 293T cells. The resultant rAAV was purified on Iodixanol gradient. CMV: Cytomegalovirus promoter; An: poly-adenilation signal; ITR: Inverted Terminal Repeats.

## 11.- In vivo vector gene transfer using human tumour xenograft model

Six to nine week old male nude mice were obtained from Charles River Labs (Barcelona, Spain). Cells in concentration of 5 x $10^6$ in a volume about 50–100 µl in phosphate-buffered saline (PBS) were injected subcutaneously into a thigh area. After 2–3 weeks, when the tumours reached about 7–10 mm in diameter, recombinant adenovirus and various AAV2 mutants were injected intratumurally at a dose of 3 x $10^{10}$ vg diluted in PBS in a volume of 10 µl. Seven days after virus administration each tumour was excised and was embedded into optimal cutting compound and frozen in liquid nitrogen for further immunohistochemical examination. Expression of GFP in tumours after gene transfer was evaluated by immunohistochemistry using anti-GFP antibody.

## 12.- Statistical analysis

We used the Chi-square and Fisher´s exact test with a significance threshold of $p < 0.05$ to evaluate possible association of the tumour HLA expression with clinico-pathological data. We used the Student’s t-test with a significance threshold of $p < 0.05$ to evaluate possible differences in mRNA expression levels between BH controls and: group 1- total HLA-I loss (including heterogeneous samples); and group 2- other types of HLA alterations (locus or allelic losses). SPSS v17.0 software was used for all statistical analyses.

# RESULTS

# 1.- Frequent HLA class I alterations in human prostate cancer: molecular mechanisms and clinical relevance.

In order to analyze the frequency of HLA alterations in prostate primary tumours and its association with clinical data, we studied HLA expression in 42 cryopreserved prostate cancer samples and evaluated its correlation with cancer recurrence, perineural invasion, stage and Gleason grade. Molecular mechanisms underlying HLA loses were also examined. In addition, we studied HLA expression in 12 previously unreported prostate cell lines and analysed the molecular mechanisms implied in HLA loss.

## 1.1.- Analysis of HLA loss in cryopreserved prostate tumors

### Immunohistochemical analysis of HLA-I and -II expression in PC

Around 88% of studied tumors had at least one type of HLA-I alteration. Using anti-HLA-I/ β2m antibody w6/32 we found that 50% of the studied tumors showed positive immunostaining, while another 50% had total loss of HLA-I/ β2m complex expression with either complete (16.7%) or a heterogeneous (33.3%) pattern. β2m loss was found in 50% of all the studied samples, and loss of the free HLA-I heavy chain (HC-10 Ab) in 75% of the cases (Table 1a). HC-10 antibody detects free HLA heavy chains not associated with β2m protein, therefore, the percentage of HC-10 positive labeling can differ from that of the cell surface HLA-I/ β2m complex. Immunohistochemical analysis of all studied tumors using anti-locus-specific antibodies, revealed negative labeling of locus -A and -B in 34,1% and 28,6% of all the specimens, respectively (Table 1a). In contrast, all BH samples were positive for class I expression.

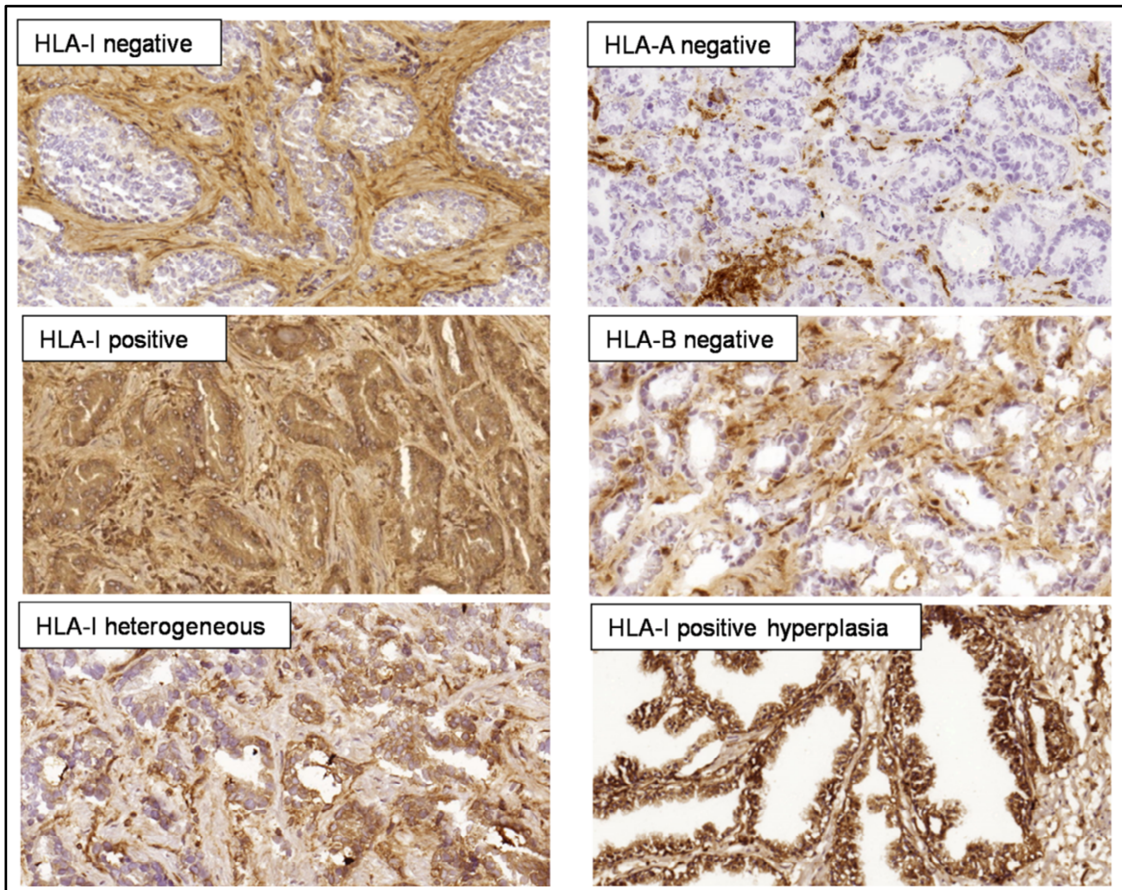



**Figure 1.**Representative immunohistochemistry images of HLA-I expression in cryopreserved PC and BH: negative, positive and heterogeneous expression patterns of HLA-I/ β2m complex in PC (w6/32 mAb); loss of HLA-A (Tu155 mAb) and loss of HLA-B (421b5 mAb) in PC; and positive HLA-I expression in benign prostate hyperplasia (w6/32).

Based on HLA-I genotyping results of each tumor, we selected locus- and allele-specific mAbs to detect unnoticed partial alterations in HLA-I/ β2m positive tumors and found locus loss in 26% and allelic losses in 12% of the cases, respectively. Consequently, we classified the studied PC tumors into different phenotypes with the following incidence: PhI (total loss) in 50%, PhIII (locus loss) in 26%, and PhIV (allelic loss) in 12% (Table 1b). There may have been additional undetected allelic losses, due to the lack of Abs against all possible allelic specificities. Representative images of different patterns of HLA-I immunolabeling in PC and BH are depicted in Fig.1 HLA-DR expression was negative in the majority of BH samples and in around 75% of tumors as compared to the stroma. Approximately 22% of tumors showed a

heterogeneous HLA-DR labeling pattern, and only one tumor was graded as DR-positive.

**Table 1a**: Immunohistological analysis of HLA expression patterns in prostate tumors

| PC % | HLA-I/β2m | β2m | free HLA-I heavy chain | locus A | locus B | HLA-DR |
|---|---|---|---|---|---|---|
| negative | 16,7% | 21,4% | 35% | 34,1% | 28,6% | 75,7% |
| heterogeneous | 33,3% | 28,6% | 40% | 36,6% | 30,9% | 21,6% |
| positive | 50% | 50% | 25% | 29,3% | 40,5% | 2,7% |
| n of samples | 42 | 42 | 40 | 42 | 42 | 37 |

**Table 1b**: Frequency of different HLA-I altered phenotypes (immunohistochemistry) and LOH-6/LOH-15 (microsatellite analysis) in prostate tumors

| phenotype | total loss (PhI) | locus loss (PhIII) | allelic loss (PhIV) | positive | total alterations |
|---|---|---|---|---|---|
| tumors (n=42) | 21 (50%) | 11 (26%) | 5 (12%) | 5 (12%) | 37 (88%) |
| LOH-6 (n=25) | 5/13 | 0/5 | 1/3 | 2/4 | 8/25 |
| LOH-15 (n=25) | 1/13 | 0/5 | 0/3 | 0/4 | 1/25 |

**Table 1c**: Immunohistological analysis of HLA expression in tumors (T) versus matching normal epithelium (N)

| **sample** | **HLA-I** | **β2m** | **heavy chain** | **locus A** | **locus B** | **HLA-DR** |
|---|---|---|---|---|---|---|
| 12241 N | ++ | +++ | +++ | +++ | +++ | ++ |
| 12241 T | - | - | - | - | - | - |
| 8723 N | ++ | ++ | ++ | ++ | +/- | - |
| 8723 T | - | - | + | - | - | - |
| 9238 N | ++ | ++ | ++ | ++ | +/- | +/- |
| 9238 T | +/- | +/- | +/- | +/- | +/- | - |
| 8722 N | ++ | +++ | ++ | ++ | ++ | +/- |
| 8722 T | +/- | +/- | +/- | - | - | - |
| 2489 N | ++ | ++ | ++ | ++ | ++ | ++ |
| 2489 T | +++ | +++ | ++ | +/- | - | +/- |
| 10216 N | ++ | ++ | ++ | ++ | ++ | +/- |
| 10216 T | ++ | ++ | ++ | ++ | ++ | +/- |

+ weakly positive ; ++ positive ; +++ strongly positive; +/- heterogeneous; - negative

From a separate set of six PC patients we were able to compare cryopreserved tumor and matching normal tissues and observed a higher incidence of HLA alterations in cancerous samples (Table 1c). Four tumors showed a loss of HLA-I expression (two totally negative and two heterogeneous, w6/32 mAb) and one tumor showed loss of locus B. Only one tumor had the same positive labeling pattern as the adjacent normal epithelium (Table 1c).

**Analysis of the association of HLA-I and -II expression with tumor recurrence, perineural invasion, and high D´Amico cancer risk**

Tumors negative for HLA-I/ β2m complex, β2m, locus A or locus B expression showed a strong tendency to relapse and develop PNI, and were also linked to high D´Amico risk (as compared to HLA-I positive lesions) (Fig. 2), although the number of cases was too low for statistical significance to be reached. In addition, tumor recurrence was more frequent among DR-negative tumors than in tumors with heterogeneous DR expression pattern (73% versus 37%, respectively). All the results are summarized in Table 2.

Table 2: correlation between HLA expression and clinico-pathological parameters

| % | | Recurrence | PNI | D´Amico | | |
|---|---|---|---|---|---|---|
| | | | | Low | Medium | High |
| Total HLA | - | 100,0 | 60,0 | 0,0 | 50,0 | 50,0 |
| | Het | 71,4 | 41,7 | 21,4 | 64,3 | 14,3 |
| | + | 47,6 | 38,9 | 10,0 | 80,0 | 10,0 |
| B2M | - | 100,0 | 87,3 | 0,0 | 62,5 | 37,5 |
| | Het | 66,7 | 61,9 | 25,0 | 58,3 | 16,7 |
| | + | 54,5 | 49,0 | 10,0 | 80,0 | 10,0 |
| Locus A | - | 91,7 | 63,6 | 0,0 | 53,8 | 46,2 |
| | Het | 52,6 | 31,3 | 21,1 | 73,7 | 5,3 |
| | + | 50,0 | 28,6 | 14,3 | 85,7 | 0,0 |
| Locus B | - | 80,0 | 55,6 | 0,0 | 63,6 | 36,4 |
| | Het | 69,2 | 33,3 | 30,8 | 53,8 | 15,4 |
| | + | 47,1 | 42,9 | 6,3 | 87,5 | 6,3 |
| HLA class II DR | - | 73,1 | 45,5 | 11,5 | 69,2 | 19,2 |
| | Het | 37,5 | 28,6 | 12,5 | 75,0 | 12,5 |
| | + (n=1) | 100,0 | 100,0 | 0,0 | 0,0 | 100,0 |

Interestingly, when we grouped together heterogeneous and positive samples versus negative samples, we observed a statistical significance for the correlation of locus A loss with high D´Amico risk (p=0,005).

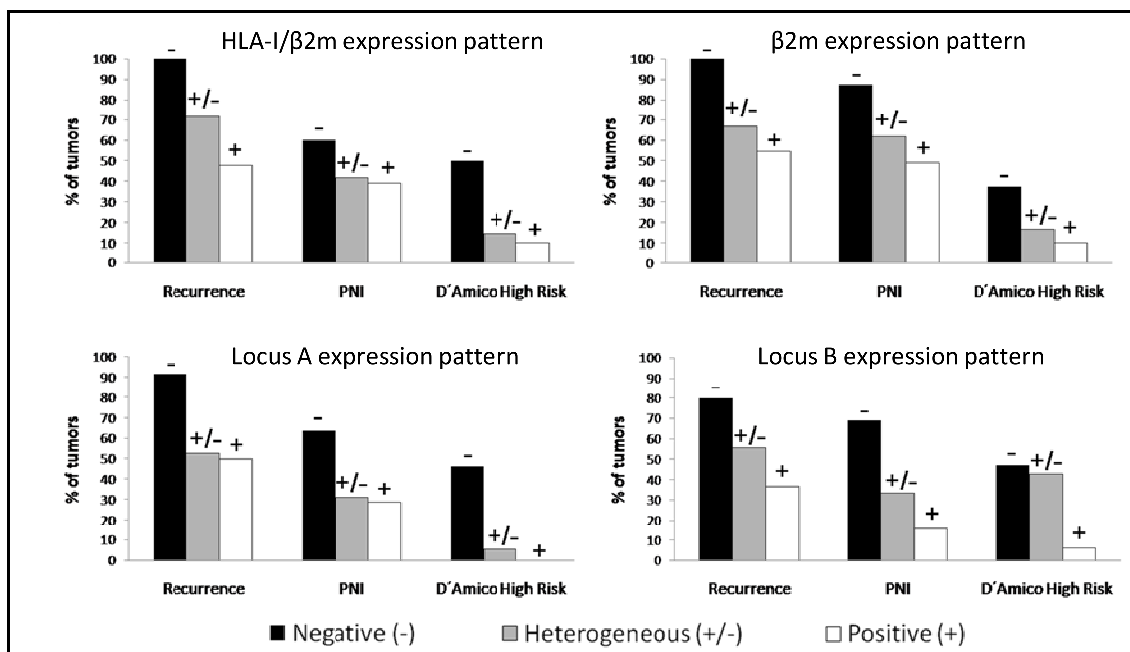



**Figure 2.** Correlation between the expression of HLA-I, β2m, HLA-A and HLA-B and clinico-pathological characteristics: PC recurrence, PNI and D`Amico high cancer risk. Tumors with negative and heterogeneous expression of HLA-I/ β2m, β2m, locus A, and locus B have a strong tendency to relapse and develop PNI.

## mRNA expression of *β2m*, APM*, IRF-1, and NLRC5* in microdissected PCs and BHs

cDNA from microdissected tissues was analyzed for mRNA expression of β2m, *Tap1, Tap2, tapasin, LMP2, LMP7, NLRC5* and *IRF-1* genes. The results were compared among three groups: Control – benign hyperplasia; group 1 - total HLA-I loss (combined negative and heterogeneous samples); and group 2 - other types of HLA alterations (locus or allelic losses). A positive correlation was found between tumor HLA-I alterations and reduced transcriptional level of all studied genes in each group versus BH control. Notably, in tumors with total HLA-I loss (group1), we found a statistically significant decrease in mRNA expression of *β2m* (p=0.046), *tapasin*

(p=0.003), *NLRC5* (p=0.001) and *TAP2* (p=0.05) for but also reduced levels of LMP2, LMP7 and IRF-1 (Fig.3), while transcriptional levels of *NLRC5* (p=0.003) and *tapasin* (p=0,003) were significantly lower in group 2.

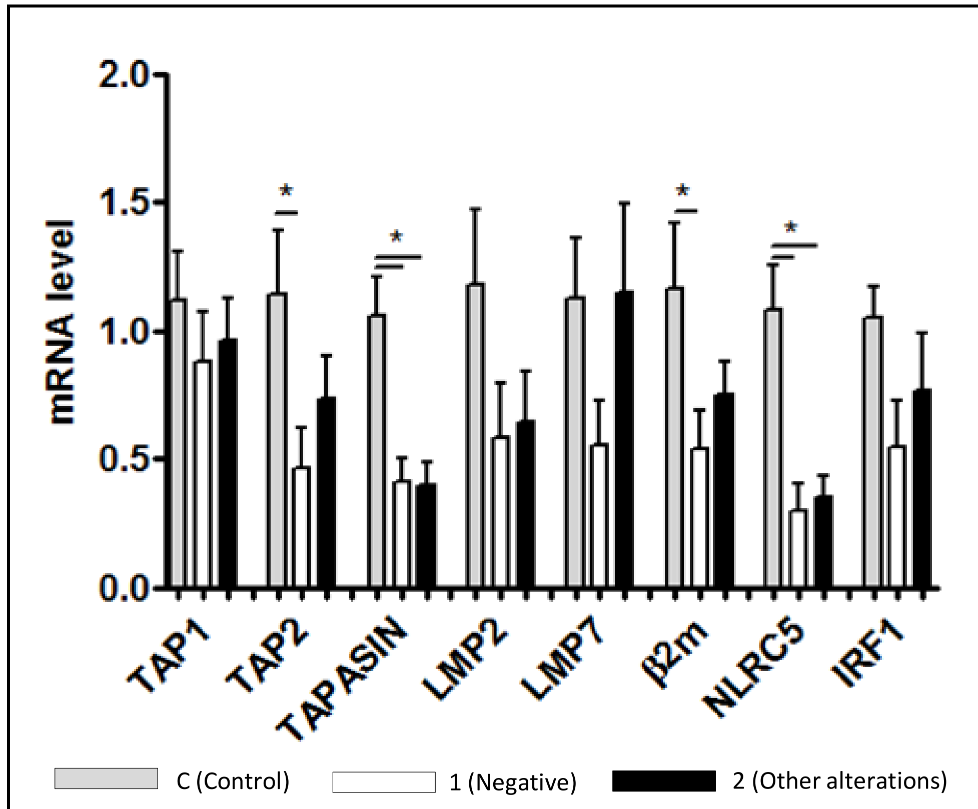



**Figure 3.** Correlation of the tumor HLA-I loss with the reduced mRNA expression of the APM components, β2m, IRF-1 and NRLC5 in two groups of PC compared to control BH (C). Tumor group 1- total HLA loss (including heterogeneous samples); and group 2- other types of HLA alterations (locus or allelic losses). Statistically significant decrease in the expression of β2m (p=0.046), tapasin (p=0.003), NLRC5 (p=0.001) and TAP2 (p=0.05) was found in tumors with total HLA-I loss (group1). In addition, mRNA expression levels of NLRC5 (p=0,003) and tapasin (p=0,003) were also significantly lower in group 2 than in control. * Statistically significant difference compared to control.

## Microsatellite analysis and *β2m* sequencing in prostate tumors

We performed microsatellite analysis of DNA isolated from 25 microdissected tumor samples to detect LOH-6 and LOH-15. LOH-6 was observed in 32% (8/25) of the tumors, and only one showed LOH-15. Among 8 tumors with LOH-6, five had total HLA-I loss, one demonstrated allelic loss, while two tumors were positive for HLA-I (Table 1b). We sequenced *β2m* in 11 selected microdissected tumors with total HLA-I

loss (including tumors with heterogeneous expression pattern) but did not find aberrations in *β2m* (data not shown).

### 1.2.- Analysis of HLA expression in prostate cell lines

**Analysis of HLA-I and -II expression in cell lines from PC patients**

Using flow cytometry we characterized altered HLA-I/II expression in 12 previously unreported cell lines derived from primary tumors and normal epithelia of PC patients. Various types of HLA-I alterations were found, ranging from locus or allelic downregulation to a total loss of expression (Table 3), with both regulatory and structural underlying molecular defects. HLA-I locus loss (PhIII) was found in six cell lines. MPH0203HT cells showed reversible downregulation of locus-B and -C (Fig.4a). IFN-inducible locus-C loss was also detected in SVP0303-T, Q0057T, and PC3 cells (ATCC), while locus-C downregulation in MPH0503PN-T, SVPO703OE, and OPCN1 cells was resistant to IFN-γ, suggesting a structural defect. Three cell lines demonstrated allelic losses (PhIV) (Fig.4b), most of them reversible with IFN-γ except for HLA-B40 loss in OPCN1. In OPCN3 cells, we found a total loss of HLA-I expression (PhI), not inducible by IFN-γ (Table 3 and Fig. 5a).

Constitutive HLA-DP, -DQ and -DR expression was negative in practically all studied cell lines, although inducible with IFN-γ in some cases (Table 3).

HLA expression in PC-3, DU-145, and LnCAP (ATCC), was previously characterized by our group and others. These cell lines are widely used by many research groups and have been studied from various standpoints. In comparison to DU145 and PC3 cells, LnCAP has very low expression weakly inducible by IFN-γ. DU145 cells lack HLA-A3 allele, due to a mutation (Jiménez et al., 2001).

**Table 3:** Constitutive and IFN-γ inducible cell surface HLA expression and genomic typing of prostate cell lines

| | HLA-I | | | | | | | | | | HLA-II | | | | | | HLA genomic Typing | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | total HLA | | β2m | | locus A | | locus B | | locus C | | DP | | DQ | | DR | | locus A | locus B | locus C |
| **IFN-γ** | - | + | - | + | - | + | - | + | - | + | - | + | - | + | - | + | | | |
| DU145 (ATCC) | ++ | ↑ | ++ | ↑ | ++ | ↑ | + | ↑ | ++ | ↑ | - | = | - | = | - | ↑ | 0301/3303 | 5001/5701 | 0602/0602 |
| PC3 (ATCC) | + | ↑ | + | ↑ | + | ↑ | + | ↑ | - | ↑ | - | = | - | = | - | = | 2402/0101 | 1302/5501 | 0102/0602 |
| LnCAP (ATCC) | + | = | + | = | + | = | + | = | + | = | - | = | + | = | - | = | 0101/0201 | 0801/3701 | 0602/0701 |
| MPH0203HT (N) | ++ | ↑ | ++ | ↑ | ++ | ↑ | - | ↑ | - | ↑ | - | ↑ | - | = | - | ↑ | 0201/0206 | 2703/4402 | 0303/0501 |
| SVP0703OE (N) | ++ | ↑ | ++ | ↑ | ++ | ↑ | + | ↑ | - | = | - | = | - | ↑ | - | ↑ | 0101/6801 | 0702/0802 | 0701/0702 |
| Q0057T (T) | ++ | ↑ | +++ | ↑ | ++ | ↑ | + | ↑ | - | ↑ | - | = | - | = | - | ↑ | 0201/0201 | 4001/5701 | 0304/0602 |
| MPH0503PN-N (N) | ++ | ↑ | +++ | ↑ | + | ↑ | + | ↑ | + | ↑ | - | = | - | = | - | ↑ | 0101/0101 | 0801/4002 | 0202/0701 |
| MPH0503PN-T (T) | + | ↑ | ++ | ↑ | + | ↑ | + | ↑ | - | = | - | = | - | = | - | ↑ | 0101/0101 | 0801/4002 | 0202/0701 |
| SVP0303-N (N) | ++ | = | +++ | ↑ | ++ | ↑ | ++ | ↑ | + | ↑ | - | = | - | = | - | ↑ | 0101/2901 | 0801/4403 | 0701/1601 |
| SVP0303-T (T) | ++ | ↑ | ++ | ↑ | ++ | ↑ | ++ | ↑ | - | ↑ | - | ↑ | - | = | - | ↑ | 0101/2901 | 0801/4403 | 0701/1601 |
| OPCN1 (N) | + | ↑ | ++ | ↑ | + | ↑ | + | ↑ | - | = | - | = | - | = | - | = | 0101/2301 | 3701/4001 | 0304/0602 |
| OPCT1 (T) | ++ | ↑ | +++ | ↑ | + | ↑ | ++ | ↑ | + | ↑ | - | ↑ | - | = | - | ↑ | 0101/2301 | 3701/4001 | 0304/0602 |
| OPCN2 (N) | ++ | ↑ | ++ | ↑ | ++ | ↑ | + | ↑ | ND | ND | - | = | - | = | - | = | 0207/1101 | 4002/4601 | 0102/0702 |
| OPCT2 (T) | + | ↑ | ++ | ↑ | + | ↑ | + | ↑ | + | ↑ | - | = | - | = | - | ↑ | 0207/1101 | 4002/4601 | 0102/0702 |
| OPCN3 (N) | - | = | - | = | - | = | - | = | - | = | - | = | - | = | - | ↑ | 0101/2501 | 5701/5701 | 0602/0602 |

HLA expression was measured by flow cytometry in baseline conditions and after incubation with IFN-γ using several antibodies directed against different HLA specificities

- no expression; + weak expression; ++ strong expression; +++ very strong expression; ↑ - IFN-γ-induced HLA upregulation; = resistance to IFN-γ, no HLA upregulation; ND - not done

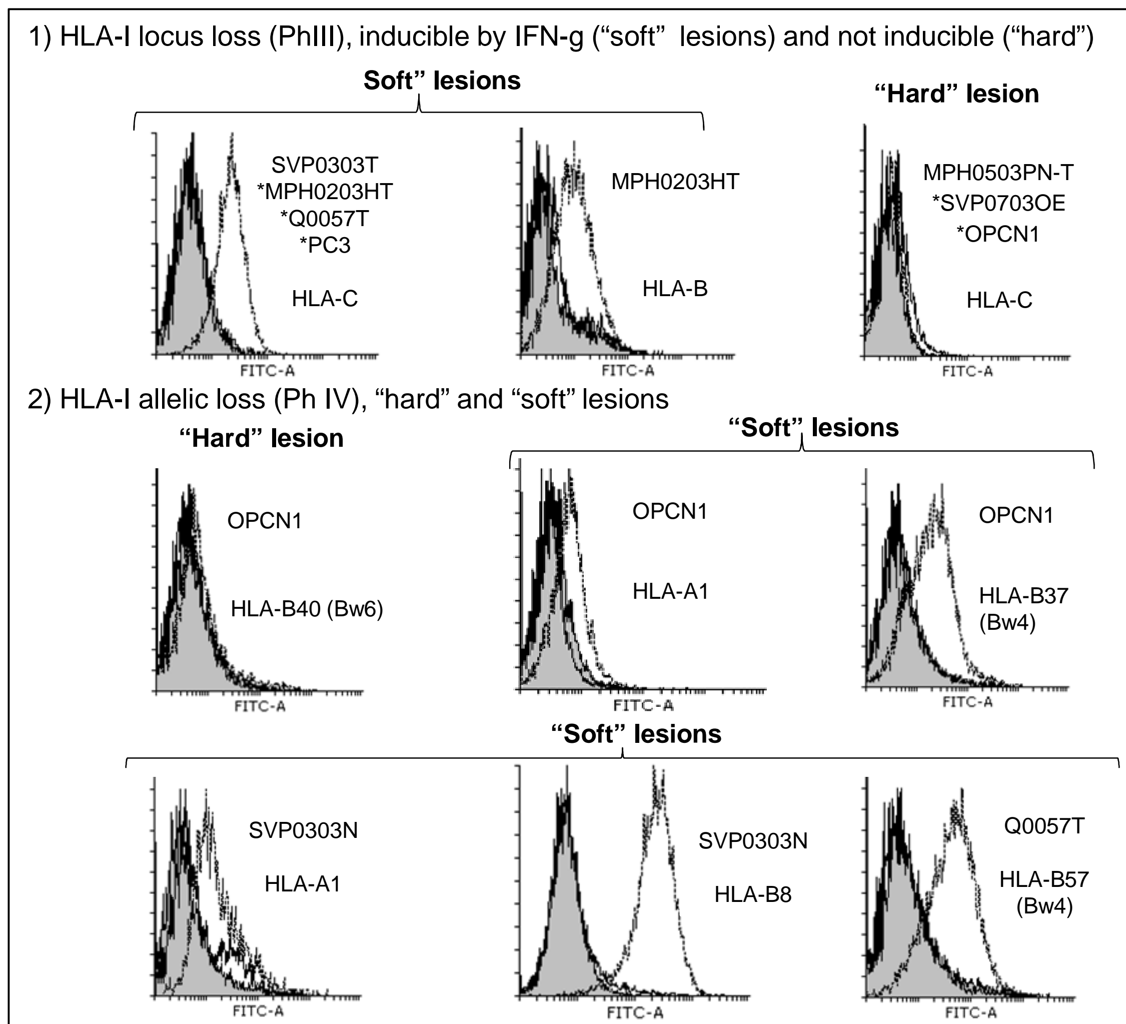



**Figure 4.** Different patterns of HLA-I loss resistant to IFN-γ ("hard" lesions) or inducible by IFN-γ ("soft" lesions) in PC cell lines determined by FACS. **(a)** Irreversible locus C loss and IFN-γ–inducible downregulation of locus C and B (PhIII); **(b)** irreversible loss of HLA-B40 allele and IFN-γ-inducible downregulation of A1, B37, B57 and B8 (Ph IV). Grey fill – only secondary Ab control, solid line - baseline conditions, dashed line - IFN-γ treatment. mAb used: w6/32 (HLA-I/ β2m complex), 421B5 (Locus B), Hb122 (HLA-A3), Marb3 (bw4 specificities) and HB152 (bw6 specificities).
* Representative FACS histogram for several cell lines.

## Total loss of HLA-I expression in OPCN3 cells caused by *β2m* gene alterations

Total loss of HLA-I found in OPCN3 cells was not inducible by IFN-γ, but was recovered after adenovirus-mediated *β2m* gene transfer (Fig.5a). Amplification of the DNA isolated from these cells produced a short fragment, approximately 500pb smaller than in the control (Fig.5b). Sequencing of the fragment showed a 480pb microdeletion between nucleotides +3591 and +4071, including first half of exon 2 (Fig.5b and Fig. 6a), promoting a splicing error in mRNA maturation in which exon 2 is lost, as shown

by β2m cDNA sequence (Fig 5b). This deletion generates a premature stop codon producing a truncated 26 aminoacids-long β2m protein. In addition, microsatellite analysis showed LOH-15 for three specific markers on the long arm (q) of chromosome 15, pointing to a macrodeletion in this region (Fig. 5c). Therefore, the total loss of HLA-I expression in this PC cell line is caused by two structural defects in β2m gene, a microdeletion in one gene copy and a macrodeletion (LOH-15) in another one.

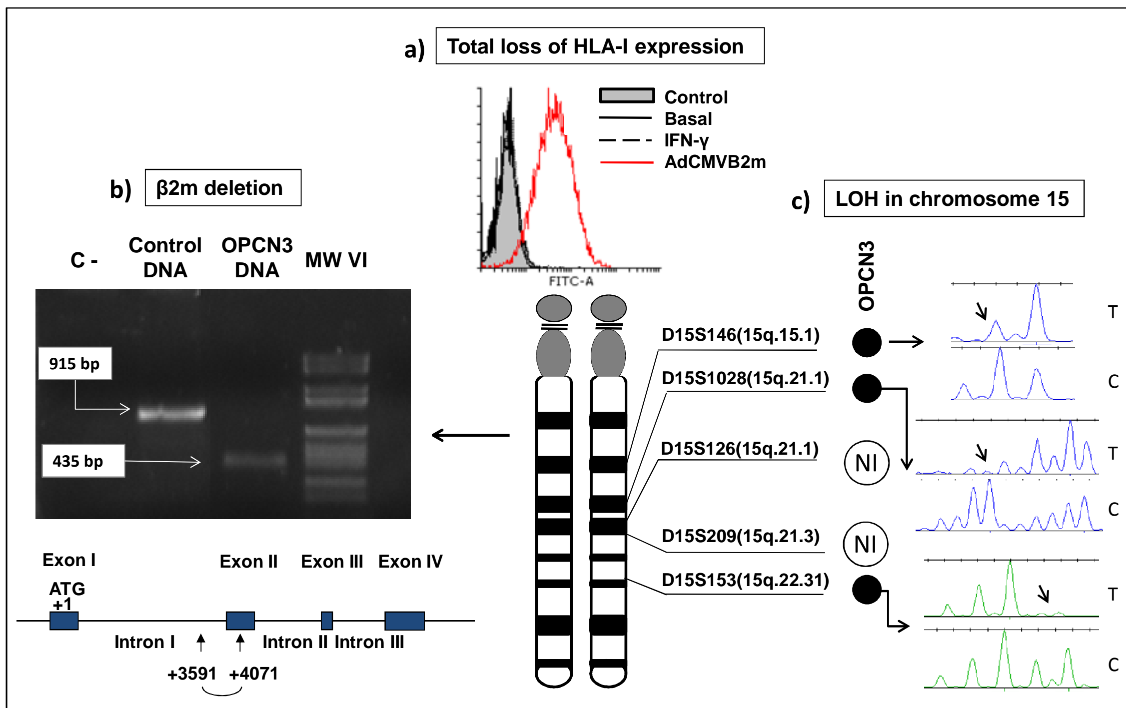



**Figure 5.** Total loss of HLA-I expression in OPCN3 cells caused by a deletion in *β2m* gene and LOH-15. **a)** FACS histogram demonstrating loss of HLA-I cell surface expression (w6/32 mAb) not inducible by IFN-γ. Adenovirus vector coding for human *β2m* gene recovers HLA-I expression. Grey fill – control; solid line – baseline expression; dotted line - IFN-γ treatment; dashed line - after AdCMVβ2m transfection. **b)** PCR amplification of *β2m* gene fragment (including intron 1 and exon 2) using DNA from OPCN3 cells. *β2m* amplicon from OPCN3 cells is smaller than in control; sequencing of the amplicon showed a 480pb microdeletion that includes the first half of exon 2 (schematic representation). **c)** Loss of heterozygocity at chromosome 15 was determined by microsatellite analysis using five specific markers flanking *β2m* region. (NI- not informative reading).

The detected deletion in the β2m gene in OPCN3 cells generates a 26 amino-acid long truncated protein, while normal β2m protein has 119 aminoacids. The stop codon is located three amino acids after the junction of the exon 1 to exon 3, while the exon 2 is lost.

Wild type β2m amino acid sequence:

MSRSVALAVLALLSLSGLEAIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDI

EVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYACRVNHVTLSQ

PKIVKWDRDM

β2m amino acid sequence in OPCN3 cells:

MSRSVALAVLALLSLSGLEAIQHRDM

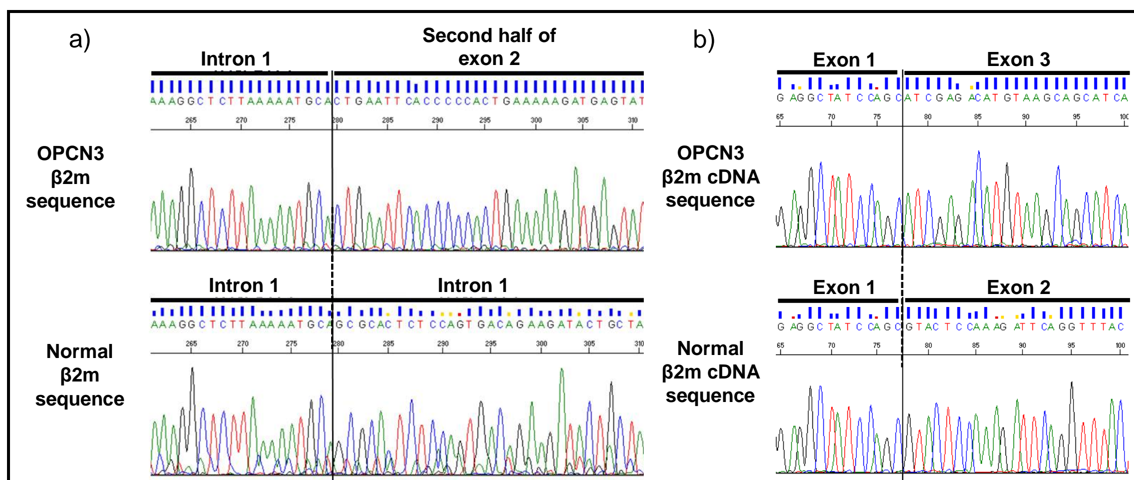



**Figure 6**: Sequence histograms illustrating a microdeletion in *β2m* gene in OPCN3 cells. **a)** loss of DNA fragment between the end of intron 1 and the second half of exon 2 in OPCN3 cells as compared to control. **b)** cDNA sequence of *β2m* in OPCN3 demonstrates that exon 3 is positioned right after exon 1, while exon 2 is missing. Sequence of the wild type *β2m* gene was used as a control.

## HLA-I cell surface expression on cell lines correlates with transcriptional levels of HLA-I and APM genes

We analyzed the mRNA expression of the following genes: *HLA-I heavy chain*, *β2m, TAP1, TAP2, tapasin, LMP2, LMP7, calreticulin, calnexin, IRF-1* and *NLRC5*. Protein and mRNA expression patterns of HLA-I loci coincided in the majority of the studied cell lines with frequently observed increased expression of *calreticulin*. Interestingly, LnCAP cells showed a coordinated downregulation of all studied APM and HLA-I genes, as well as of the transcriptional factors *NLRC5* and *IRF-1* (Table 4).

**Table 4:** mRNA transcription of APM, β2m, HLA, NLRC5 and IRF-1 genes in prostate cell lines in baseline conditions and after IFN-gamma treatment

| CL | IFN | Tapasin | Tap2 | Tap1 | Calreticulin | Calnexin | LMP7 | LMP2 | HLA-A | HLA-B | HLA-C | IRF1 | NLRC5 | B2M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DU145 | - | 18,59 | 2,32 | 2,27 | 47,76 | 6,07 | 1,43 | 2,01 | 0,66 | 0,66 | 0,00 | 2,07 | 0,18 | 16,39 |
| | + | 28,03 | 1,89 | 12,55 | 21,84 | 4,74 | 5,90 | 12,21 | 3,11 | 5,79 | 0,00 | 7,79 | 0,78 | 58,27 |
| PC3 | - | 6,88 | 1,55 | 0,87 | 128,56 | 5,14 | 0,55 | 0,32 | 0,02 | 0,08 | 0,00 | 0,77 | 0,01 | 10,00 |
| | + | 13,46 | 4,73 | 13,20 | 76,92 | 4,37 | 3,56 | 10,92 | 0,02 | 0,18 | 0,00 | 14,32 | 0,65 | 63,87 |
| LNCAP | - | 1,27 | 0,23 | 0,41 | 45,64 | 4,52 | 0,01 | 0,01 | 0,06 | 0,22 | 2,60 | 0,10 | 0,01 | 3,15 |
| | + | 1,86 | 0,28 | 0,51 | 65,04 | 4,83 | 0,02 | 0,10 | 0,12 | 0,46 | 3,84 | 0,28 | 0,04 | 2,89 |
| 3PNN | - | 7,30 | 2,46 | 1,65 | 44,16 | 5,43 | 1,31 | 2,43 | 0,08 | 3,90 | 7,23 | 2,00 | 0,05 | 15,07 |
| | + | 13,16 | 3,77 | 13,50 | 40,48 | 4,73 | 4,03 | 11,51 | 0,28 | 16,43 | 17,88 | 15,68 | 0,72 | 38,75 |
| 3PNT | - | 7,71 | 3,96 | 2,95 | 64,88 | 6,74 | 1,70 | 3,06 | 0,14 | 4,14 | 9,18 | 4,49 | 0,08 | 13,32 |
| | + | 14,25 | 6,69 | 20,38 | 64,92 | 10,77 | 6,42 | 22,45 | 0,71 | 29,93 | 36,78 | 11,71 | 0,94 | 71,46 |
| SVPN | - | 8,78 | 2,41 | 4,04 | 28,65 | 11,58 | 1,38 | 5,05 | 0,98 | 11,31 | 12,91 | 2,68 | 0,25 | 30,55 |
| | + | 13,89 | 3,34 | 16,86 | 24,57 | 8,81 | 3,27 | 14,77 | 2,19 | 21,87 | 26,12 | 14,17 | 0,69 | 61,91 |
| SVPT | - | 7,52 | 1,27 | 0,90 | 29,83 | 10,95 | 1,13 | 2,87 | 0,20 | 1,56 | 5,17 | 2,84 | 0,03 | 8,25 |
| | + | 12,41 | 2,55 | 9,05 | 26,35 | 10,77 | 3,42 | 13,63 | 1,04 | 15,01 | 19,14 | 11,42 | 0,61 | 35,85 |
| OPCN1 | - | 8,77 | 1,01 | 0,74 | 28,63 | 17,90 | 0,36 | 1,33 | 0,10 | 0,00 | 0,00 | 5,76 | 0,05 | 17,06 |
| | + | 24,02 | 7,36 | 28,82 | 23,91 | 13,08 | 4,21 | 22,81 | 0,45 | 0,41 | 0,00 | 63,78 | 1,57 | 76,44 |
| OPCT1 | - | 16,56 | 2,32 | 1,08 | 70,62 | 6,31 | 1,13 | 1,94 | 0,02 | 0,56 | 0,00 | 3,08 | 0,04 | 10,25 |
| | + | 23,79 | 5,34 | 18,00 | 14,39 | 3,41 | 5,75 | 23,48 | 0,18 | 10,09 | 0,00 | 27,67 | 1,30 | 79,10 |
| OPCT2 | - | 7,27 | 1,43 | 1,47 | 28,68 | 6,73 | 0,78 | 2,08 | 0,15 | 0,95 | 5,63 | 3,00 | 0,02 | 7,07 |
| | + | 14,88 | 4,86 | 13,14 | 11,82 | 6,98 | 4,05 | 14,41 | 0,81 | 7,21 | 29,05 | 21,27 | 1,37 | 41,84 |
| OPCN3 | - | 9,09 | 1,29 | 1,60 | 35,05 | 9,89 | 1,89 | 4,19 | 0,12 | 0,28 | 0,00 | 1,49 | 0,08 | 9,78 |
| | + | 4,83 | 1,78 | 3,90 | 7,92 | 3,31 | 2,70 | 5,53 | 0,92 | 3,47 | 0,00 | 1,11 | 0,18 | 0,00 |
| 3HT | - | 8,71 | 1,04 | 1,15 | 53,84 | 4,01 | 0,92 | 2,22 | 0,50 | 1,33 | 0,00 | 3,56 | 0,05 | 17,84 |
| | + | 18,08 | 4,36 | 12,79 | 30,87 | 2,99 | 4,40 | 15,12 | 2,44 | 13,26 | 0,00 | 25,36 | 1,77 | 64,62 |
| 57T | - | 6,90 | 2,16 | 1,45 | 38,14 | 8,58 | 0,60 | 2,03 | 1,25 | 0,63 | 0,00 | 3,70 | 0,04 | 6,86 |
| | + | 12,09 | 3,48 | 9,77 | 30,17 | 6,52 | 3,05 | 8,99 | 1,93 | 10,69 | 0,00 | 19,32 | 0,63 | 40,53 |
| 3OE | - | 15,90 | 1,79 | 3,22 | 72,50 | 5,07 | 2,84 | 10,59 | 1,11 | 8,01 | 27,43 | 5,68 | 0,40 | 28,15 |
| | + | 17,29 | 2,68 | 11,88 | 17,30 | 3,29 | 3,15 | 16,45 | 1,45 | 23,11 | 55,90 | 11,83 | 0,78 | 51,40 |

## 2.- Reconstitution of HLA-A2 and beta2-microglobulin expression in human tumor cells using adeno- and adeno-associated recombinant viruses

With the aim of develope a useful tool for HLA restoration, we compared the transfection efficacy of adenovirus and several adeno-associated virus serotypes after infection *in vitro* of tumor cells with different origin. Then we constructed adenoviral and adeno-associated viral vectors carrying HLA-A2 gene with the objective to recover or upregulate tumor HLA expression. We evaluated HLA-A2 expression in different cancer cell lines after infection with A2-coding vectors alone oo together with adenovirus carrying β2m gene.

### 2.1.- Comparison of AAV serotypes and adenovirus vectors

**Comparative analysis of the transduction efficacy of adenoviral and adeno-associated viral vectors driving GFP expression in human tumor cells**

In order to optimize viral vector application and choose a vector with the best transduction efficacy we compared adenovirus and six AAV serotype variants (AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9) driving GFP expression by infecting one melanoma, two prostate, and two bladder cancer cell using different virus doses. GFP expression after infection of PC3 cells was dose-dependent as shown on Fig.7a with highest transgene expression at 1000 MOI. We discovered that AAV serotype 2 (AAV2) has the highest transduction efficiency of GFP gene among the studies AAV serotypes 1, 2, 5, 6, 8 and 9. However, Ad5 demonstrated similar results at a ten times lower MOI (100) indicating superior transduction efficacy. Infection with AAV1 and AAV9 serotypes induced GFP expression in around 10% of PC3 cells. Similar dose-dependent pattern was obtained in all studied cell lines, however, the percentage of positive cells varied. We found that the highest transduction rate in most of the cell lines

was achieved using adeno-GFP. Among adeno-associated-viral vectors AAV2-GFP showed the best results with varying efficacy depending on the cell line. AAV serotypes 1, 5, 6, 8, and 9 showed none or very little ability to induce GFP expression in the studied cells (Fig. 7).

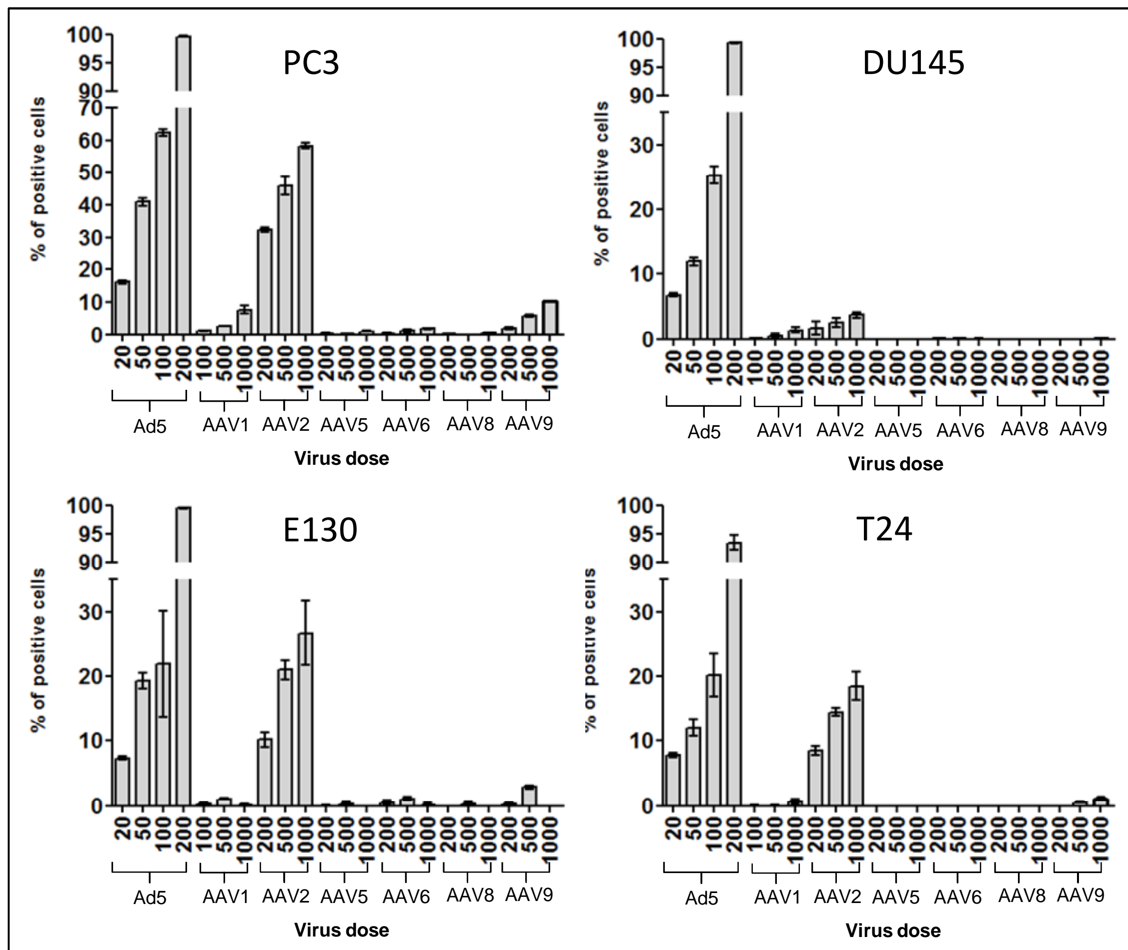



**Figure 7:** Comparative analysis of transduction efficiency of recombinant adenovirus Ad5 and AAV viruses of serotypes 1, 2, 5, 6, 8 and 9. **a)** Dose-dependent GFP transgene expression (MOI 400, 1000 or 2000) in different tumor cells driven by different viral vectors showing superior efficacy of AdV5 and AAV2. Similar pattern was obtained in all cell lines. **b)** Percentage of GFP-positive tumor cells after infection with different viral vectors at highest dose. Transduction efficacy of the studied viral vectors varies among infected tumor cell lines: prostate (Du145 and PC3), bladder (T24 and RT112) and melanoma (E130), with the highest efficacy of adenovirus and AAV2.

We also checked the level of GFP expression in two melanoma and one prostate cell lines during 30 days after infection with an AAV2-GFP. As shown in figure 8, in all three cell lines the maximum percentage of GFP-positive cells was observed on day 9, followed by a graduate decrease until day 30.

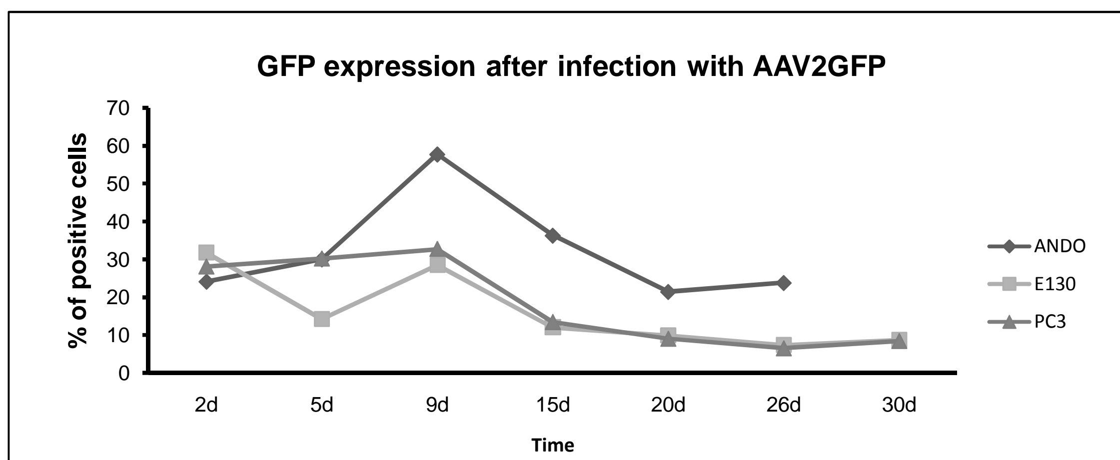



**Figure 8**: Kinetics of GFP expression in two melanoma (E130 and Ando-2) and one prostate cell line (PC3) after infection with AAV2-GFP measured at days 2, 5, 9, 15, 20, 26 and 30.

## Efficacy of mutant AAV2 in cancer cell lines

We also compared the ability of three variants of AAV2-GFP with tyrosine/phenylalanine mutations, introduced to improve virus attachment to the target cell surface, to induce GFP transgene expression in E130 melanoma cell line at the highest dose previously tested for all AAV vector serotypes (1000 MOI) (Fig. 9). Figure 9 shows that Quadruple mutant has the highest efficacy of transfection, followed by Sextuple mutant with the highest transfection rate on day two. Using different doses of Quadruple and Sextuple mutants (1.000, 5.000 and 10.000 MOI) we observed a dose dependent variations in the transfection efficacy more apparent for the Sextuple mutant, increasing from 35% to 65% at day 2, and from 17% to 54% at day 5 (Fig.9b). At the same time, Triple mutant did not induce transgene expression in melanoma cells E-130.

The AAV2 Quadruple mutant was used to inject human melanoma xenografts in vivo (E-007 melanoma cells grown in nude mice) and compared with Adenovirus. Immunohistochemistry of the injected tumors tissue showed that adenoviral GFP transduction has higher efficiancy than AAV2 Quadruple mutant. Nevertheless, liver immunohistochemistry in adenovirus-treated mice showed stronger immunolabeling as

compared to AAV-injected amnimals. Representative images of GFP expression in tumor and liver after intratumoral injection of viral vectors are shown in figure 9c.

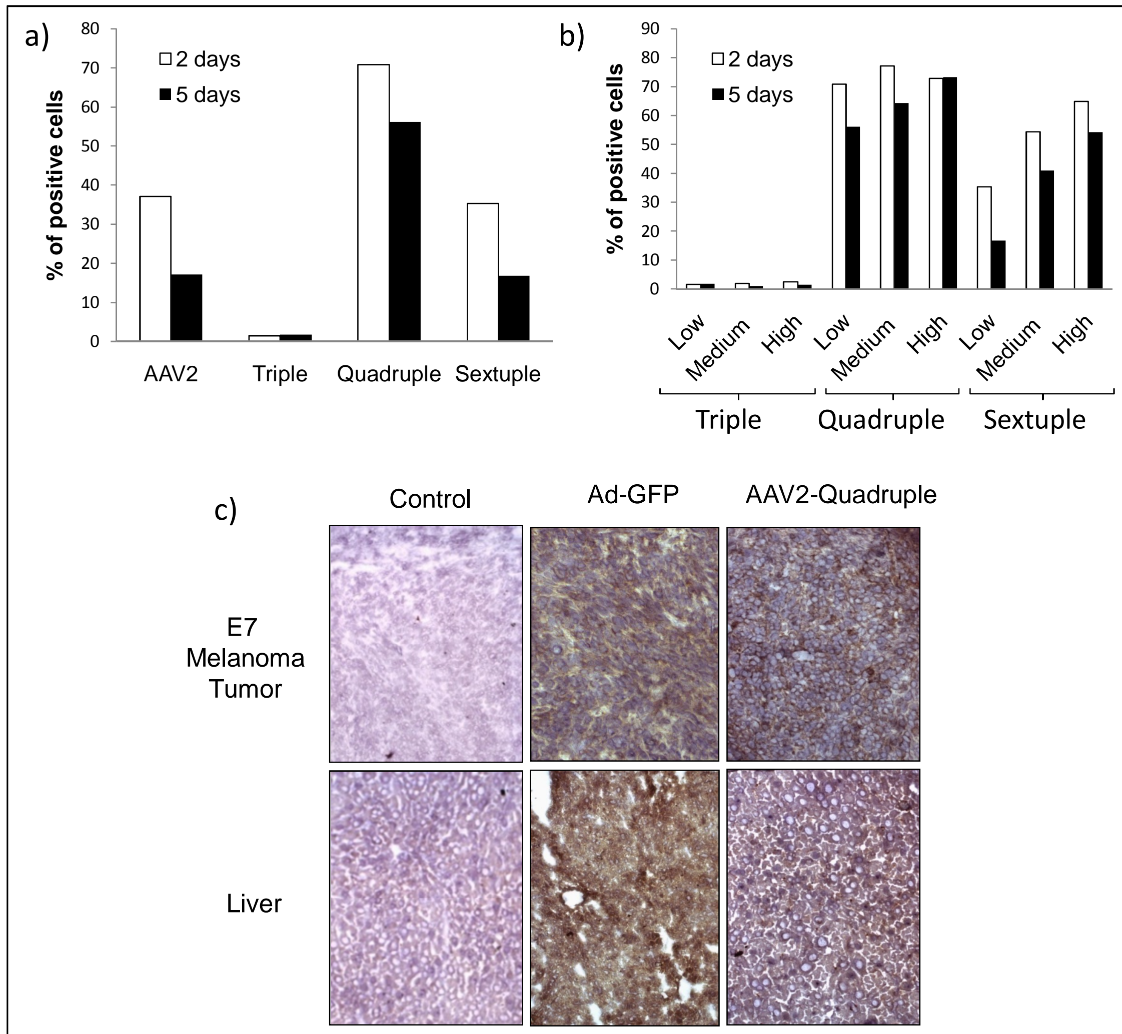



**Figure 9:** Comparative analysis of the transduction efficacy of a native AAV2-GFP vector and three different AAV2-GFP variants with tyrosine/phenylalanine point mutations: Triple, Quadruple and Sextuple. **a)** Percentage of GFP-positive E130 melanoma cells 2 and 5 days after in vitro infection with the studied AAV vectors at MOI 1.000. Highest transgene expression was observed in case of Quadruple AAV2, while Triple mutant failed to induce GFP expression. **b)** Percentage of transduced E130 cells after infection with Quadruple and Sextuple mutants increases with higher dose of the virus (MOI 1.000, 5.000 and 10.000). **c)** Representative immunohistochemical images of GFP immunolabeling (using rabbit anti-GFP mAb) in E-007 human melanoma xenograft tumors and corresponding mouse liver tissues after intratumoral injection of GFP-coding adenovirus, Triple and Quadruple AAV2 mutant viruses.

## 2.2.- Expression of HLA-A2 in cancer cell lines after infection with AdCMV-HLA-A2 and AAV2-HLA-A2

### Both vectors, AAV2-HLA-A2 and AdCMV-HLA-A2, recover HLA-A2 expression in A2-deficient cells

Based on the results described above, we have selected the most effective viral vectors for *HLA-A2* transfer into tumor cells: replication-deficient AdV5 and AAV2. We constructed both vectors carrying *HLA-A2* gene and compared their efficacy to induce A2 transgene expression in different tumor cell lines. Using these viral vectors we successfully recovered HLA-A2 expression in melanoma cell line Ando-2 which has been characterized by a lack of endogenous *HLA-A2* due to a haplotype loss caused by LOH at chromosome 6 (Table 1) (Paco et al., 2007). Figure 10 depicts recovery of A2 expression after infection with AdCMV-HLA-A2 and AAV2-HLA-A2 as demonstrated by FACS (Fig.5a) and by immunocytochemistry (Fig.5b).

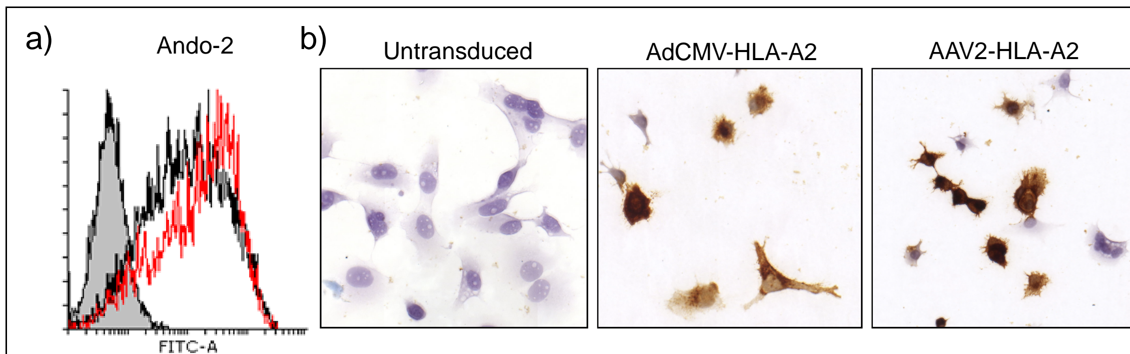



**Figure 10:** Recuperation of HLA-A2 expression in melanoma cells Ando-2 (cells lack A2 allele due to the loss of HLA haplotype caused by LOH-6) as determined **a)** by FACS or **b)** by immunocytochemistry, using anti-HLA-A2 mAb CR11.

**_De novo_ expression of HLA-A2 expression in tumor cells negative for endogenous genomic _HLA-A2_**

Infection with AdCMV-HLA-A2 or AAV2-HLA-A2 induced expression of A2 allele in various types of human tumor cells (melanoma, bladder, and prostate cancer cells) naturally lacking *HLA-A2* allele based on HLA class I genotyping results (Table 1). A2 expression was measured by flow cytometry 48 hours after infection, confirming a successful introduction of a novel HLA allele in addition to the existing ones (Fig. 11a). Comparable results were also obtained when we analyzed the percentage of the transduced cells in all cell lines, except for DU145 cells where AAV2-HLA-A2 demonstrated much lower efficacy than adenovirus (Fig. 11b). The level of transgene expression was comparable only when MOI of AAV2 was ten time higher that of Ad5.

**Kinetics of _de novo_ HLA-A2 expression induced by AdCMV-HLA-A2 or AAV2-HLA-A2**

Seven cell lines negative for endogenous HLA-A2 were infected with AdCMV-HLA-A2 and AAV2-HLA-A2 and transgene A2 expression was analysed by FACS on days 3, 6, 10, 15, 20, 25, 30 and 35 after infection (Fig. 12) demonstrating overall similar expression level and kinetics for both types of vectors. Most cell lines had maximum HLA-A2 expression on day 6, decreasing gradually until day 15-25. Only WIL retained more than 50% of HLA-A2-positive cells until day 30 (Fig. 12). In DU145, RT 112 and T24 cells with similar time-curves for both vectors types, the adenovirus induced transgene expression in a higher percentage of infected cells during the first days. On the contrary, Ando-2 and PC3 cells showed higher percentage of HLA-A2 expressing cells when infected with the AAV2.

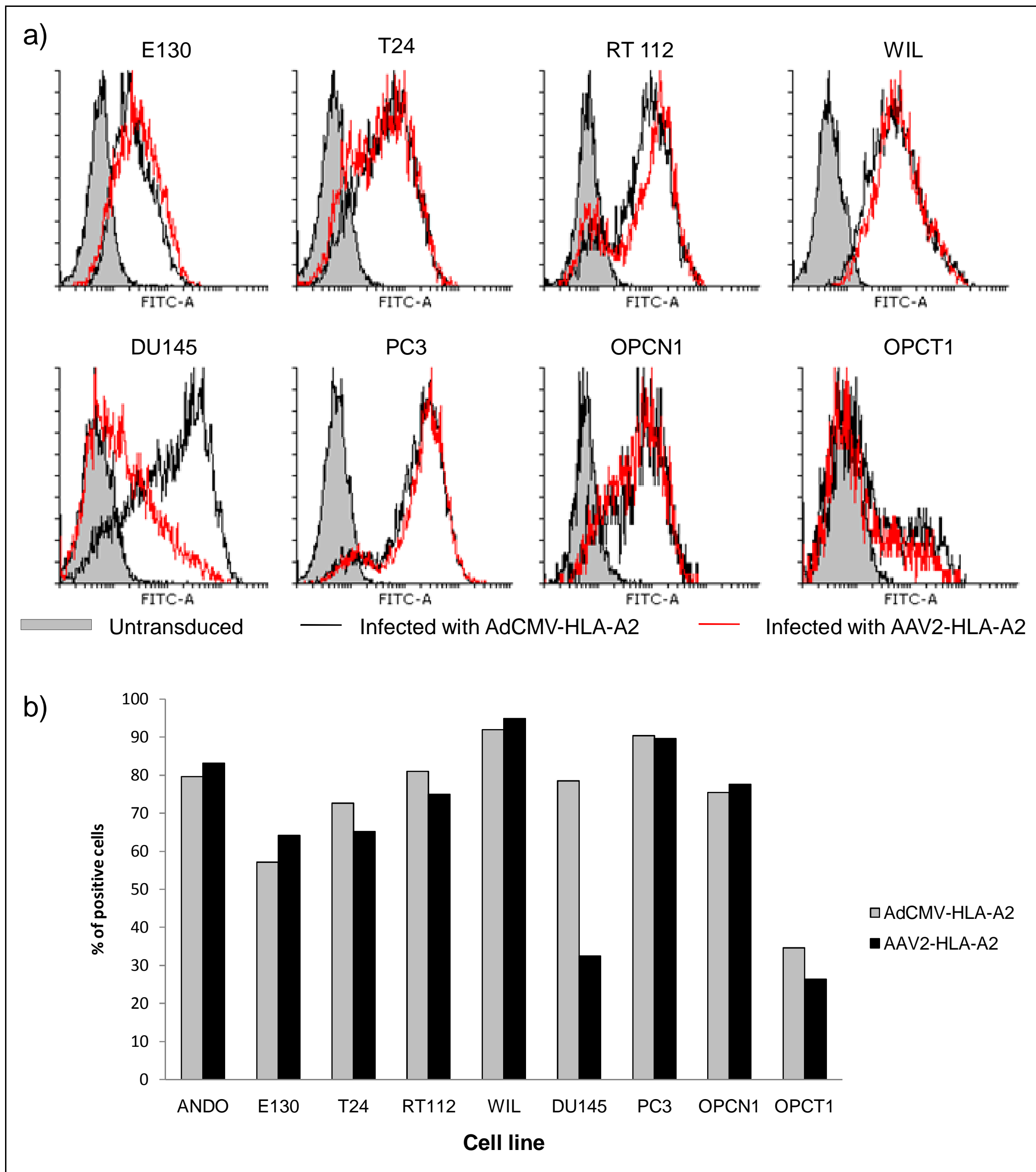



**Figure 11:** HLA-A2 expression in A2-negative cancer cells negative for genomic HLA-A2 allele after infection with AdCMV-HLA-A2 or AAV2-HLA-A2 at the highest optimal dose for each vector (100 and 1000 MOI, respectively). **a)** *de novo* HLA-A2 expression in cells was measured by flow cytometry and depicted as FACS plots or **(b)** as a percentage of A2- transgene-positive cells. Grey fill: Control – not infected cells; black lines - cells infected with AdCMV-HLA-A2; red lines - cells infected with AAV2-HLA-A2.

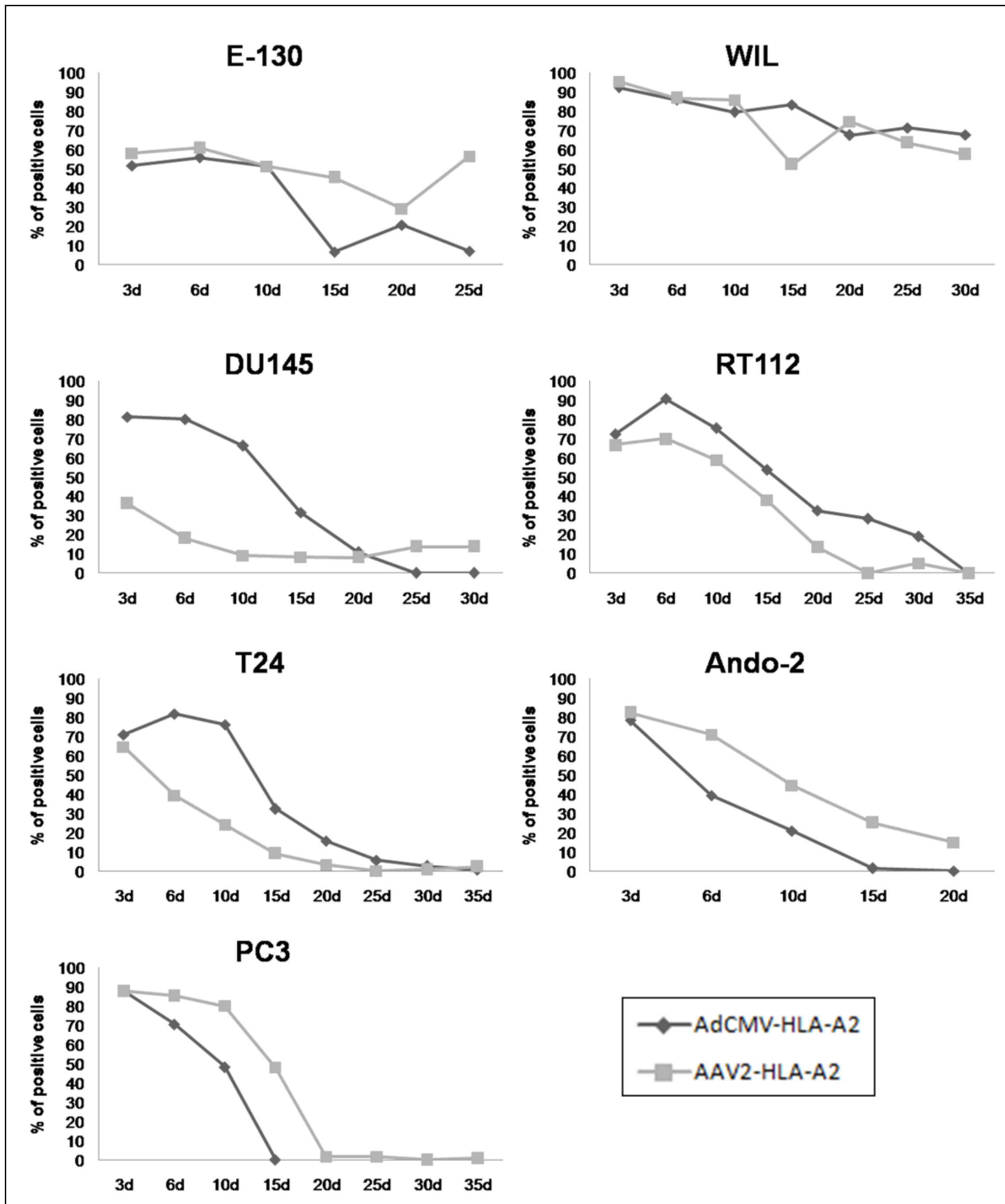



**Figure 12:** Kinetics of HLA-A2 expression in bladder (RT112, T24 and WIL), prostate (PC3 and DU145) and melanoma (E130 and Ando-2) cell lines after infection with AdCMV-HLA-A2 or AAV2-HLA-A2, measured by FACS at days 3, 6, 10, 15, 10, 25, 30 and 35 post-infection. Comparable kinetics was observed for most of the studies cell lines, except for Du145 cells, with the maximum expression on day 6 followed by a gradual decrease until day 15-20. WIL cell line maintained high level of the transgene up to day 30.

**Recuperation of cell surface HLA class I expression on β2m-deficient tumor cells negative for genomic HLA-A2 after co-infection with AdCMV-HLA-A2 and AdCMV-β2m**

To confirm the capacity of *de novo* expressed HLA-A2 heavy chain to form a complex with β2m on the cell surface, we co-infected with both AdCMV-HLA-A2 and AdCMV- β2m four tumor cell lines (E038, E109, OPCN3 and DAUDI) all β2m-deficient and with HLA genotype naturally lacking HLA-A2 allele. As shown in figure 13, all cell lines are negative for total HLA-I, β2m, and HLA-A2 in baseline conditions (Fig. 13a). Cells infected with AdCMV-β2m, as expected, recover HLA-I expression (Fig. 13b). Infection with AdCMV-HLA-A2 renders these cells HLA-I negative (Fig. 13c) since the pre-existing lack of β2m prevents formation of cell surface HLA-I complex with newly expressed HLA-A2 protein. Interestingly, DAUDI cells, despite the lack of β2m, became positive for HLA-I, A2 and even for β2m expression after transfer of A2 gene. This line has been previously reported to have an unusual molecular mechanism of HLA class I complex formation, including free heavy chain folding, transport and assembly on the cell surface in the absence of β2m (Martayan et al., 2009). Only co-transfection with both vectors, cell surface HLA-HLA-I/β2m complex was recovered and A2 expression was detected by flow cytometry using specific antibodies CR11 (Fig. 13d) suggesting that simultaneous use of both adenoviral vectors lead to an association of newly expressed HLA-A2 and β2m molecules to form a cell surface HLA-I complex.

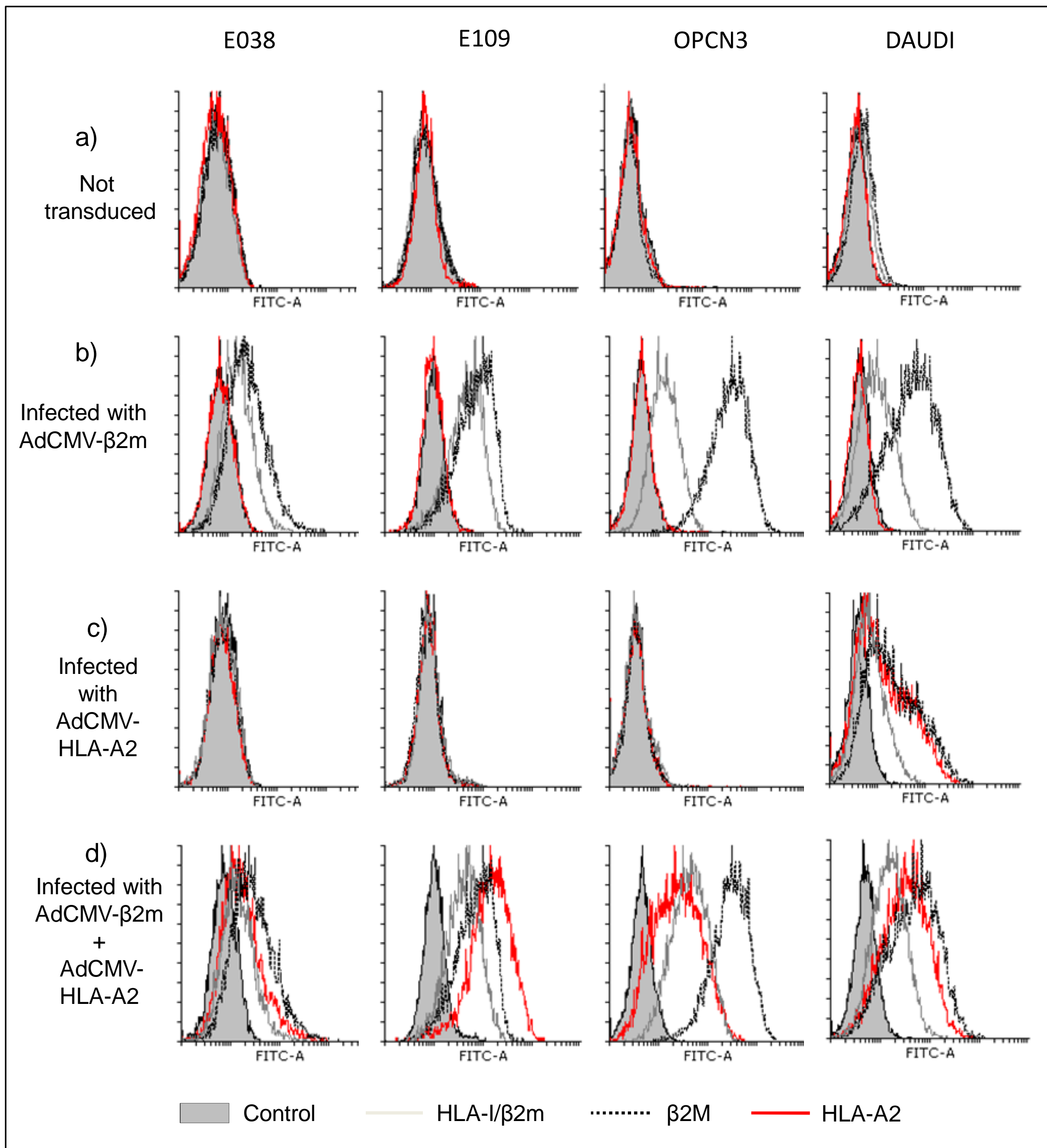
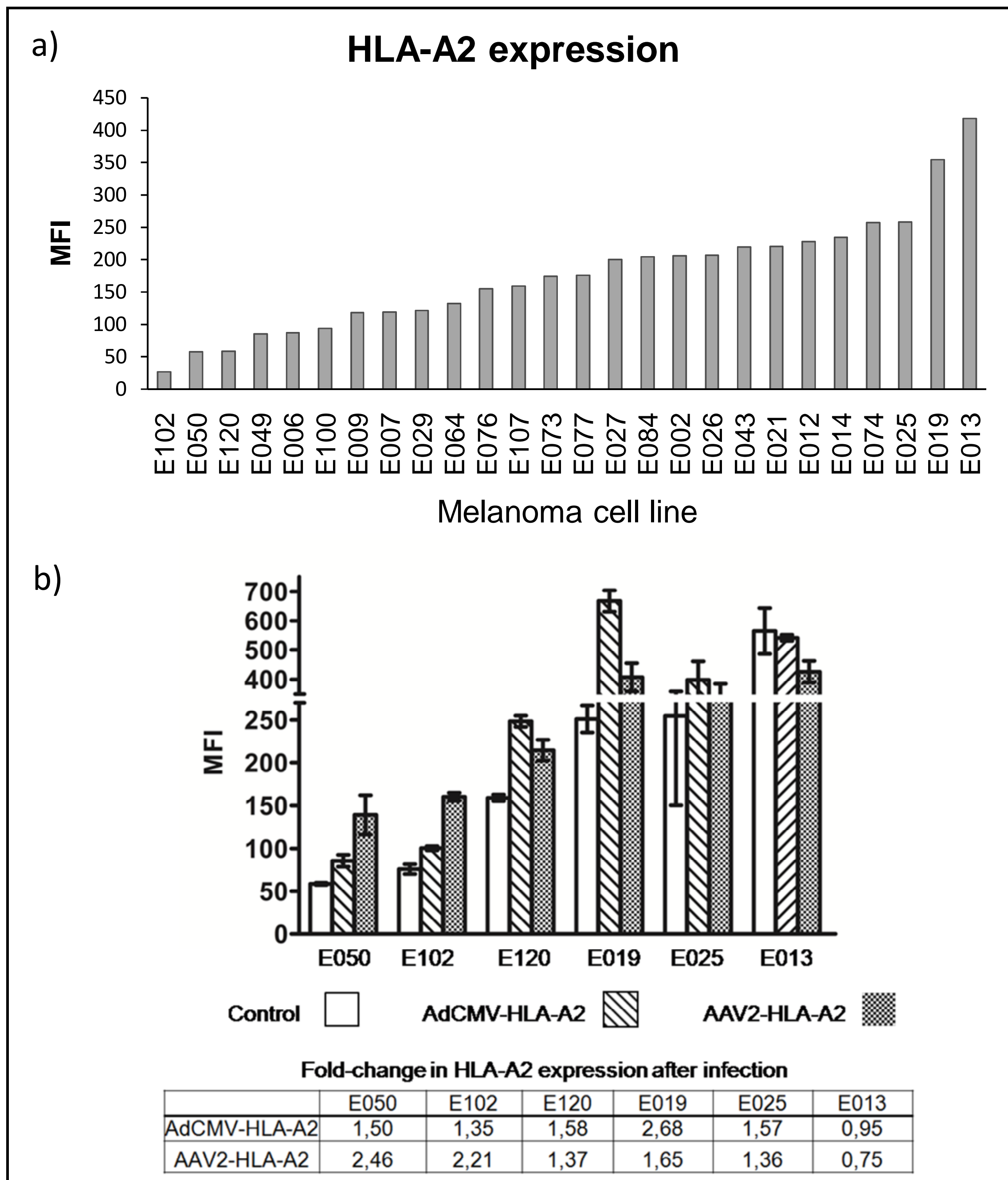



**Figure 13:** Recovery of tumor cell surface HLA-I expression by co-infection of β2m-deficient tumor cells negative for genomic A2 allele with two adenoviral vectors AdCMV-HLA-A2 and AdCMV-B2M. **a)** FACS plots demonstrating absence of A2 allele (CR-11 mAb), of β2m (L-368 mAb), and of cell surface HLA-I/B2M complex (w6/32 mAb) in baseline conditions. **b)** AdCMV-B2M-mediated recovery of B2M and HLA-I/β2m complex expression. **c)** Infection with AdCMV-HLA-A2 did not render cells positive for HLA-I/ β2m, except for DAUDI. **d)** Co-infection with both vectors induced HLA-A2 and recovered HLA-I/ β2m complex expression. Grey fill - control with only secondary antibody; red line - HLA-A2; grey line - HLA-I/β2m complex; and dotted line - β2m.

**Virus-mediated upregulation of HLA-A2 expression on tumor cells positive for genomic A2 allele**

We also analysed how HLA-A2 upregulation after viral transduction depends on the pre-existing baseline level of endogenous A2 expression in HLA-A2-positive cells. We used melanoma cell lines positive for A2 according to HLA class I genotyping (Méndez et al., 2008). Based on the level of HLA-A2 expression measured by FACS (fig. 14a), we selected three cell lines with high and three cell lines with low baseline A2 expression to infect with AdCMV-HLA-A2 or AAV2-HLA-A2. As shown in figure 14b, in low-HLA-A2 expressing cells (E050, E102 and E120) virus-mediated upregulation of A2 is much more evident than in cells with higher A2 level, with AAV2-HLA-A2 demonstrating superior transduction efficacy than adenovirus in two out of three cell lines. On the contrary, viral vectors showed different efficacy to upregulate A2 in cells with the high endogenous baseline HLA-A2: almost none or very little efficacy in E-025 and E-013 cells (Fig. 14b), and significant effectiveness of transgene expression in E-019 cells, especially when adenoviral vector was used (Fig 14b). MFI fold-change increment of HLA-A2 expression after infection of the cells is shown in Fig 14b.




| | E050 | E102 | E120 | E019 | E025 | E013 |
|---|---|---|---|---|---|---|
| AdCMV-HLA-A2 | 1,50 | 1,35 | 1,58 | 2,68 | 1,57 | 0,95 |
| AAV2-HLA-A2 | 2,46 | 2,21 | 1,37 | 1,65 | 1,36 | 0,75 |

**Figure 14: a)** HLA-A2 baseline expression of 26 melanoma cell lines sorted by fluorescence intensity, measured by FACS using CR11 anti-HLA-A2 mAb. **b)** Virus-mediated upregulation of HLA-A2 expression on tumor cells positive for genomic A2 allele. In cell lines (E050, E102 and E120) with low HLA-A2 expression in baseline conditions (control) virus-mediated upregulation of A2 is greater than in high HLA-A2 expressing cells, as measured by FACS. In cells with high endogenous baseline HLA-A2, the transduction efficacy varied, i.e. almost no transduction of E-013 cells, very little efficacy in E-025 cells, and high level of transgene expression in E-019 cells, especially when adenoviral vector was used. The table summarizes the vector-induced fold-change in HLA-A2 expression on cells with low and high baseline HLA-A2 expression.

# DISCUSSION

It has become increasingly accepted that the immune system plays a key role in controlling malignant growth and dissemination, and, in many cases, it is able to eradicate cancer. One of the most important players of anti-tumor immunity are cytotoxic T-lymphocytes that are able to recognize and eliminate malignant cells through the interaction between T-cell receptor (TCR) and tumor-specific antigen peptide associated with HLA class I molecular complex. Thus, HLA class I antigens represent an important molecules in the adaptive immune response against cancer and tumor rejection. There is accumulating evidence that normal HLA class I expression on tumor cells also plays an important role in the response to cancer immunotherapy. Despite the progress in cancer immunotherapy and vaccination that have lead to a certain clinical improvement in some patients, no significant increase in cancer patient survival has been achieved yet. Understanding 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-I molecules 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.

HLA class I abnormalities in tumor cells, with a frequency up to 90% in some types of tumor, have been well documented in different types of cancer and are caused by distinct molecular mechanisms (Aptsiauri et al., 2007; Aptsiauri et al., 2014; Campoli et al., 2002; Garrido et al., 1997; Paschen et al., 2006; Seliger et al., 2002). Hence, tumor cells with aberrant cell surface HLA-I phenotype are able to escape T-cell mediated immune attack. Accumulating experimental and clinical evidence suggests that the nature of HLA-I alterations predetermines cancer progression and clinical response to different types of immunotherapy, as well as selection of a method for

HLA-I upregulation (Garrido et al., 2010). Some HLA-I alterations can be reversed by cytokines leading to regression of tumor lesion. In contrast, tumor cells with permanent HLA losses due to genetic defects (‘hard” lesions) may fail to increase HLA-I expression in response to therapy and eventually progress. Therefore, immunotherapy, in addition to curative anti-cancer effects, may promote immune-selection of tumor variants with irreversible structural aberrations in HLA class I molecules, creating mixed responses and resistance to therapy. To circumvent this problem, development of a strategy to recuperate HLA-I expression on malignant cells is essential, which requires better understanding of tissue- and organ-specific molecular mechanisms of tumor HLA class I alterations. Data coming from different studies indicate that most of the known human cancers, including breast (Cabrera et al., 1996), colorectal (Cabrera et al., 1998), cervical (Koopman et al., 2000), laryngeal (Cabrera et al., 2000), melanoma (Kageshita et al., 2005) and bladder cancer (Cabrera et al., 2003), are characterized by very high incidence of HLA class I alterations, which, in the majority of cases, is associated with poor prognosis and aggressive cancer development (Kaneko et al., 2011; Meissner et al., 2005; Speetjens et al., 2008). On the contrary, renal cancer has an inverse correlation between HLA class I expression and cancer progression, with high expression in tumor tissue and low in normal adjacent stroma (Sáenz-López et al., 2010). However, current knowledge about the frequency and prognostic value of HLA class I alterations in prostate cancer is controversial and the molecular mechanisms responsible are undefined

In the present study using tumor immunohistochemistry we observed a high percentage (88%) of HLA-I alterations in cryopreserved PC tumors as compared to HLA-I-positive BH or normal matching prostate epithelium. Half of the studied tumors showed total loss of HLA-I expression (phenotype I) associated with a tendency to

increased tumor recurrence, high D'Amico cancer risk, and PNI. HLA-I downregulation linked to a higher tumor grade was previously reported in different types of cancer (Chang and Ferrone, 2007; Garrido and Algarra, 2001).

The overall frequency of HLA-I aberrations in this study is similar to that found in other types of malignancy (Aptsiauri et al., 2014) and is higher than in earlier reports in PC. The published frequency of HLA-I loss in prostate tumors varies depending on the type of tissue processing and antibodies used. Thus, HLA-I heavy chain expression in paraffin-embedded tumors was positive in some studies (Seliger et al., 2010), whereas others reported loss of expression in 50% (Zhang et al., 2003), 74% (Kitamura et al., 2007), and 90% (Lu et al., 2000) of cases. Analysis of HLA-I expression in cryopreserved prostate tumors allows more detailed analysis; thus, when individual allelic expression was assessed, loss of one or more alleles occurred in 78% and cumulative HLA-I loses in 85% of the cases (Blades et al., 1995). In contrast, Nanda and colleagues reported that all 27 cryopreserved human prostate tumors studied were HLA-I-positive, although they used a different primary antibody (Nanda et al., 2006).

Reduced HLA-I heavy chain expression in PC has been previously reported to correlate with tumor relapse, while loss of calnexin and tapasin has been linked to early recurrence and aggressive tumor phenotype (Seliger et al., 2010). In our study, we also observed a significant association between total HLA-I loss and reduced mRNA expression of APM molecules, including *tapasin, TAP2*, *LMP2*, *LMP7*, *β2M, IRF1* and *NLRC5*, while tumors with partial alterations showed a less evident tendency towards this correlation. Reduced expression of *NLRC5*, a transcription factor known to regulate HLA expression (Neerincx et al., 2012), suggests a possible defect in the coordinated regulation of HLA expression.

Loss of a single functional HLA haplotype, locus, or allele required to present a particular antigen to CTLs may be responsible for the failure of cancer immune surveillance and may be underestimated in tumors with positive immunolabeling for HLA-I/β2m, since the remaining alleles associated with surface β2m remain positively labelled with w6/32 mAb.

Even though 50% of the tumors positively expressed cell surface HLA-I/β2m complex, many of them demonstrated locus or allelic HLA-I losses. In addition, we detected LOH-6 in about 32% of the studied tumors, suggesting an accumulation of chromosomal loss in *HLA-I* genes linked to haplotype loss. A similar frequency of LOH-6 was previously reported in other types of malignancy (Maleno et al., 2011). The overall frequency of HLA-I aberrations in this study is similar to that found in other types of malignancy (Aptsiauri et al., 2014) and is higher than in earlier reports in PC. The published frequency of HLA-I loss in prostate tumors varies depending on the type of tissue processing and antibodies used. Thus, HLA-I heavy chain expression in paraffin-embedded tumors was positive in some studies (Seliger et al., 2010), whereas others reported loss of expression in 50% (Zhang et al., 2003), 74% (Kitamura et al., 2007), and 90% (Lu et al., 2000) of cases. Analysis of HLA-I expression in cryopreserved prostate tumors allows more detailed analysis; thus, when individual allelic expression was assessed, loss of one or more alleles occurred in 78% and cumulative HLA-I loses in 85% of the cases (Blades et al., 1995). In contrast, Nanda and colleagues reported that all 27 cryopreserved human prostate tumors studied were HLA-I-positive, although they used a different primary antibody (Nanda et al., 2006).

In the present study, loss of β2m expression was observed in 50% of the HLA-I negative tumors versus 65% reported by Seliger *et al* and 24.8% by Kitamura and co-

authors and was correlated with poor prognosis. Moreover, we found that β2m mRNA expression was significantly lower in tumors totally negative for HLA-I.

Total loss of HLA-I expression associated with structural genetic aberrations, such as *β2m* mutations, has been reported in melanoma and in colorectal carcinoma with microsatellite instability with a mutational "hot-spot" detected in the CT repeat region of exon 1 reflecting an increased genetic instability in this region (Bernal et al., 2012; del Campo et al., 2014; Kloor et al., 2007; Paschen et al., 2003). In our study we did not see any *β2m* mutations/deletions in microdissected primary prostate tumors and only one case was with LOH-15. However, we found a *β2m* mutation and LOH-15 in one HLA-I-negative prostate cell line, which is to our knowledge, the first report of β2m gene deletion in human cell line derived from a cancerous human prostate. Interestingly, this deletion lies close to the "hot spot" described in other cancers. The presence of such irreversible "hard" HLA lesion could represent a serious obstacle to the efficacy of PC immunotherapy (Aptsiauri et al., 2008).

Notably, the role of β2m expression in PC is controversial. Some groups associate β2m downregulation with bone metastasis (Huang et al., 2006), whereas others correlated elevated β2m expression with clinically aggressive PC (Mink et al., 2010). It has also been proposed that the downregulation of prostate tumor HLA-I increases the secretion of free soluble β2m, leading to increased serum and urine levels (Abdul and Hoosein, 2000), which have been correlated with clinical stage, Gleason grade, PSA, distant metastasis, and therapeutic efficacy (Zhang et al., 2013). Some reports suggest that soluble β2m is a growth-promoting factor in hematological malignancy and PC, and anti-β2m blocking antibody has been proposed as a treatment for myeloma and PC and has been evaluated in *in vivo* models (Yang and Yi, 2010). However, this approach could impair anti-tumor T-cell activity, because HLA-positive

tumor cells can be eliminated by CTLs, while HLA-negative cells escape immune surveillance and disseminate.

We also analyzed HLA class II expression in the studied PC tissue samples. HLA-DR expression was negative in most BH and majority of prostate tumors. Approximately 22% of tumors showed a heterogeneous HLA-DR labelling pattern, which appeared to be associated with decreased relapse and lesser PNI. *De novo* class II expression has been associated with a more aggressive phenotype in melanoma (Moretti et al., 1997) but with a better prognosis in colorectal (Sconocchia et al., 2014) and laryngeal carcinoma.

Characterization of the nature of molecular defects causing HLA altered expression is important in patient selection for immunotherapy treatment. More detailed molecular analysis, including responsiveness to cytokine treatment and modulation of transcriptional and cell surface tumor HLA class I expression can be done only *in vitro* using cell lines derived from patients with prostate cancer. However, current knowledge on HLA-I expression in human PC cell lines is incomplete, partially due to a limited availability. In addition, novel human prostate cancer cell lines with characterized HLA class I expression can be useful in research related to HLA loss in cancer immune escape, providing an in vitro model for cancer immunotherapy or gene therapy aimed at recovery normal HLA expression.

In this work we characterized HLA class I and II expression in twelve previously unreported cell lines derived from cancerous prostate tissues (both malignant and normal epithelial cells) obtained from our collaborators in Onyvax company in London UK as a part of European collaborative project ENACT.

These cell lines displayed various allele- and locus-specific HLA-I losses, in some cases resistant to IFN-γ (mostly locus-C). Constitutive HLA-DP, -DQ and -DR expression was negative in all studied cell lines but was inducible by IFN-γ in some cases. Expression of HLA-II has been shown to be lacking on human PC cells, while upregulation of class II molecules on prostate tumor cells have been reported by others (Bander et al., 1997). Interestingly, we detected HLA alterations both in malignant cells and in cells derived from normal epithelia of PC patients, which could be attributable to the immortalization of the cells with HPV, since it has been described that transfection with E6/E7 is able to cause chromosomal instability. Similarly, HLA-I expression in melanoma cells was found to be higher than in normal epidermal keratinocytes (Boegel et al., 2014), although a very high percentage of melanoma primary tumors and metastatic lesions are known to have altered HLA-I expression.

Many studies have used LNCaP and DU145 as target cells for T-cell immune response analysis, despite the fact that LNCaP has HLA-I downregulation caused by intracellular defect in the assembly of the HLA-I complex (Carlsson et al., 2004), while DU145 has HLA-A3 allelic loss due to a mutation (Jiménez et al., 2001). Altered expression of HLA class I molecules can also be caused by reduced expression of APM proteins, but we did not see it in the studied cell lines (even in immortalized ones), and this expression was always inducible by IFN-γ.

Our findings demonstrate that HLA-I altered expression is a very frequent event in human prostate cancer, ranging from total loss to reduced expression of single loci and alleles, and it is associated with more aggressive clinical course. Notably, a downregulation of the mRNA levels of HLA-I and some APM molecules was the most frequent molecular mechanism responsible for these alterations, although a significant

number of the studied tumors (32%) displayed “hard” lesions in a form of LOH-6 and one tumor had LOH-15. Our group previously reported a coordinated downregulation of HLA-I and APM molecules to be responsible for HLA-I loss in bladder carcinoma (Romero et al., 2005). The present findings suggest a similar regulatory molecular mechanism of HLA loss in PC to that in bladder cancer. However, we believe that genetic defects, including LOH affecting heavy and light chains of HLA complex, play a role in generating tumor escape variants during natural cancer development and after immunotherapy. In addition, the marked differences in clinical responses among cancer patients receiving adoptive TILs, peptide-based immunotherapy or antibodies blocking immune checkpoint inhibitors, highlight the need for further research into the role of HLA molecules in the resistance to therapy.

Given the impact of structural HLA-I alterations in the tumor resistance to immunotherapy, correction of antigen presentation in these tumor types using gene transfer methods may increase the efficacy of treatment. Recovery of HLA-I expression on tumor cells would allow for the increased presentation of tumor antigens to CTL.

We developed an experimental approach to recover tumor HLA-I loss by using recombinant viral vectors. Total loss of HLA class I expression is frequently caused by mutations/deletions in one copy of the β2m gene and loss of another copy in chromosome 15 due to LOH (Bernal et al., 2012). We have reported previously that adenovirus-mediated recovery of β2m expression in tumor cells with genetic alterations in β2m gene recovers normal cell surface HLA class I expression and recognition by CTL (del Campo et al., 2009). LOH at chromosome 6p21 commonly generates HLA haplotype loss leading to the loss of specific HLA alleles in various human tumors (Maleno et al., 2002; Smith et al., 2012). Importantly, the loss or decreased expression of only one HLA allele could be sufficient to promote tumor immune escape with a

negative effect on the clinical efficacy of immunotherapy, because the presentation of many tumor-associated antigenic peptides are restricted to specific alleles. HLA-I heavy chain is highly polymorphic and some HLA alleles appear to be able to bind more epitopes than other. One particular allele, HLA-A2, plays an important role both in tumor rejection, since the affinity to a large number of known cancer-associated epitopes recognizes by CTLs is by far the highest for HLA-A*0201 allele (Paul et al., 2013). Selective loss of HLA-A2 has been described in transformed cells (Chang et al., 2005), in some reports it had a negative impact on the presentation of NY-ESO-1 peptide used for cancer immunotherapy (Klippel et al., 2014; Smith et al., 2012).

Based in this data we constructed two types of vectors (adenovirus serotyope 5(Ad5) and AAV serotype 2 (AAV2)) carrying HLA-A2 gene in order to recover or upregulate the expression of A2 allele on the surface of tumor cells.

Infection of cells with Ad5 or AAV2 coding for *HLA-A2* gene results in recuperation of A2 expression in tumor cells deficient for this allele due to a chromosomal loss. These gene transfer method can also up-regulate endogenous genomic HLA-A2 in cells with low baseline A2 expression, or introduce a new HLA-A2 allele in addition to the existing six HLA alleles in tumor cells with HLA genotype negative for A2. In addition, tumor cell co-transfection with Ad5 coding for *HLA-A2* and *β2m* genes demonstrated that the *de novo* expressed proteins can form a detectable HLA-I/β2m complex on the tumor cell surface.

In earlier experimental systems introduction of MHC class I molecules into class I negative tumor cells led to higher immunogenicity and decreased tumorigenicity (Wallich et al., 1985). In some reports *β2m* transfection led to recovery of MHC class I expression and escape from natural killer cells (Glas et al., 1992), other publications

describe the reversal of metastatic phenotype in murine carcinoma cells after transfection with syngeneic H-2 gene (Wallich et al., 1985). Allogeneic transfer of HLA-B7 together with *β2m* has been previously reported to stimulate CTL and innate immune responses in melanoma (Doukas and Rolland, 2012). However, this approach induces immune reactivity directed against allogeneic target, and does not recuperate altered natural HLA-I expression and specific peptide presentation in HLA-restricted manner.

Advances in cancer gene therapy involving viral vectors coding for a wide range of therapeutic genes has offered novel tools for boosting anti-cancer immunity. Both adenoviral and adeno-associated vectors are widely used for transfer of a wide range of therapeutic genes (CD40L, p53, APM genes, different cytokines, etc.) aimed at boosting anti-cancer immunity both in experimental models and in clinical trials (Loskog et al., 2005; Vorburger and Hunt, 2002). Most commonly used adenoviral vectors of type 5 serotype (Ad5) offer a number of advantages including a broad cellular tropism, efficient gene transfer, persistence of gene expression, and little toxicity. We have reported previously that adenovirus-mediated recovery of β2m expression in tumor cells with genetic alterations in *β2m* gene recovers normal cell surface HLA class I expression and recognition by CTL (del Campo et al., 2009).

To date, the most commonly AAV vectors have been based on AAV serotype 2 and it has been already used in clinical trials in humans with variable outcomes. Transduction in vitro with different AAV serotypes of a variety of human tumor cells, including small cell lung carcinoma, ovarian carcinoma, melanoma, colon carcinoma, pancreatic carcinoma, cervical carcinoma, and osteosarcoma, has been reported, but there is still much controversy with reference to the transduction efficiency of AAV vectors in human malignant cells (Chen et al., 2013; Maass et al., 1998; Teschendorf et

al., 2010). Here we report that AAV serotype 2 (AAV2) among other studied AAV serotypes has the highest GFP transduction efficiency in different human tumor cell lines, although, in general, inferior to adenovirus, since Ad5 demonstrated the same efficacy as AAV2 at a ten times lower dose. We also observed that the efficacy of Ad5 to drive a *de novo* expression of HLA-A2 allele is higher than that of AAV2. Using both these vectors we achieved a recovery of HLA-A2 expression on A2-deficient Ando-2 melanoma cells, and an increase in HLA-A2 expression even in tumor cells already positive for endogenous genomic A2 allele. Importantly, a simultaneous transfer of both *β2m* and *HLA-A2* genes into β2m-deficient/HLA-A2 negative tumor cells using Ad5 vector recovered cell surface HLA-I expression.

Increased HLA-I expression and tumor cell immunogenicity can be also achieved by the introduction of viral vectors encoding the peptide transporter TAP into APM-deficient tumor cells (Lou et al., 2007). Other strategies to counteract tumor HLA-I deficiencies have been suggested, including search for CTLs that are capable of recognizing low expressing MHC-I tumors (Lampen and van Hall, 2011), or development of a genetic vaccine based on introduction of a membrane-attached β2m (Cafri et al., 2013). Another interesting approach to stimulate recognition of HLA-deficient tumors is design of T-cells that express chimeric antigen receptors (CARs). Unlike for TCR recognition, the antigen does not need to be processed and presented by HLA (Park et al., 2011).

Here we have described a restoration/upregulation of HLA-A2 surface expression in different tumor cell lines by using a replication-deficient AdV5 or AAV2 vector that carry *HLA-A2* gene. Introduction of both *HLA-A2* and *β2m* genes using both types of viral vectors leads to an efficient association of both molecules into cell surface

HLA/β2m complex, which gives an option of correcting simultaneously multiple defect causing HLA-I loss.

We hypothesise that recuperation of normal endogenous HLA-I alleles expression will induce stronger immune response against cancer than other approaches, since it may recruits old circulating peptide-specific HLA-restricted CTLs previously primed against tumor cells, while other strategies, which promote allogeneic response or various T-cell based immunotherapies, may fail to induce new T-cell response against tumor cells deficient for HLA-I expression.

This scenario is schematically represented in Figure 1.

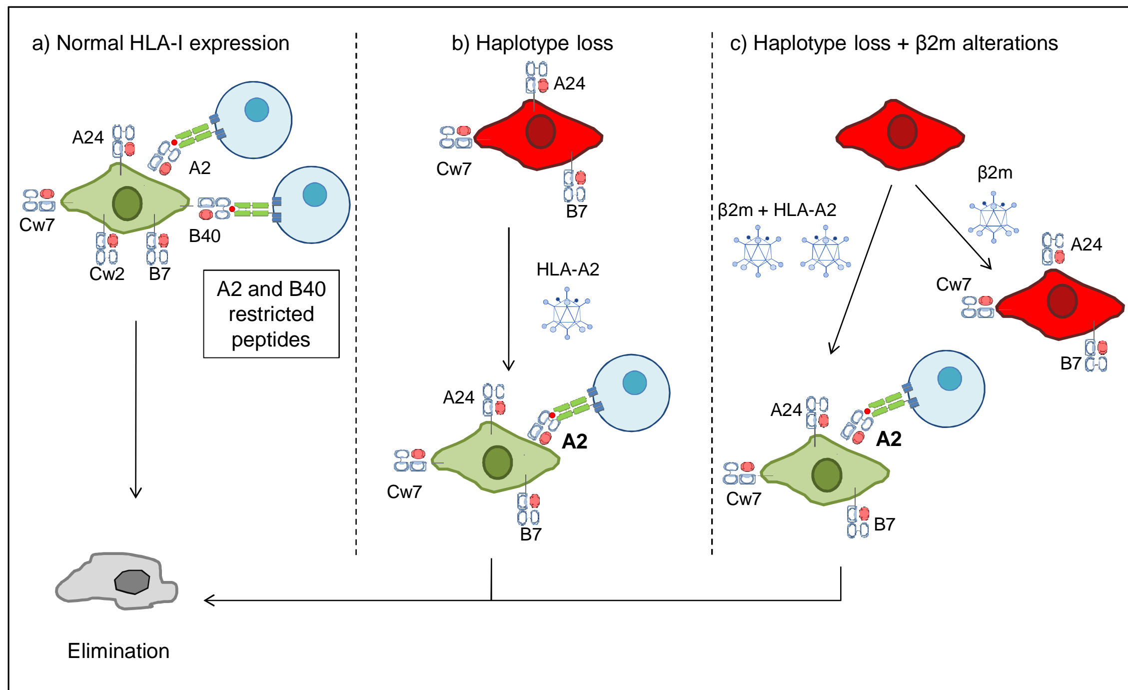



Figure 1. CTL response against cancer cells presenting tumor-associated peptide restricted by HLA-A2, either with normal or altered HLA-I expression, and after recuperation of HLA-A2 expression. **a)** Normal HLA-I expressing tumor cells present peptides in HLA restricted manner, which will lead to tumor cell recognition by CTLs and consequent elimination. **b)** Tumor cells with structural HLA-I alterations, such as haplotype loss, may lose the surface expression of the peptide specific for the lost haplotype and may escape T-cell recognition and proliferate. Recuperation of HLA-I endogenous allele (HLA-A2) via viral transfection recovers tumor peptide presentation by HLA-A2 and, as a consequence, reactivation of the pre-existing T cell clones specific for this peptide. **c)** This gene therapy approach is also feasible in case of accumulation of new “hard” lesions, including total loss of HLA-I expression due to β2m mutations. In some cases, the recuperation of HLA-I expression by β2m transfer alone may be not enough, since there could be other alterations preventing presentation of particular tumor associated peptides. Simultaneous correction of both β2m and HLA-A2 deficiencies could improve recognition and elimination of cancer cells by specific CTLs.

Primary tumors consist of heterogeneous populations of malignant 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 progression leads to the proliferation of tumor variants that have better survival options. The selective pressure leads to the expansion of new populations of cells capable of evading different immune responses. In this way, tumor cells with normal HLA-I expression are subjected to T-cell cytotoxic response restricted to an HLA-I allele (e.g. HLA-A2 restricted CTL reactivity) and eliminated, but HLA-altered clones may arise

due to structural defects as HLA haplotype loss and generation of HLA-A2 negative cells, that ultimately will progress, although new tumor escape variants can appear (Aptsiauri et al., 2013).

We believe that the correlation between clinical outcome of immunotherapy and HLA expression cannot be precisely defined without identification of the exact type of HLA defect in every given tumor and patient, which would predict the ability of CTLs to recognize tumor associated peptides. It has been shown that immune evasion strategies can be developed before the clinical application of immunotherapy (del Campo et al., 2014), making important the analysis of tumor HLA expression for selection of effective therapy. Unfortunately, the majority of cancer immunotherapy clinical trials do not include tumor HLA-I expression analysis before or during the treatment, reducing the number of patient with potentially positive clinical response. The importance of monitoring tumor HLA-I expression was suggested by a clinical trial report of the immune response associated with multiple tumor peptides used for vaccination in renal cell cancer (Walter et al., 2012), in which they treated 96 HLA-A*02 renal cancer patients with peptides presented by HLA-A2 without previous assessment of tumor 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. In fact, the study was continued with a previous selection of HLA-A2 positive patients, suggesting that the systematic use of T-cell response monitoring may constitute a valuable strategy to optimize immunotherapy (Walter et al., 2013).

In summary, our results indicate that viral vectors coding for HLA molecules represent a useful tool in upregulation of tumor HLA class I expression which can be used to increase rejection of tumors harboring structural genetic defects responsible for HLA class I loss. Tumor HLA-I upregulation also may enable cancer biologists to better

understand how to specifically target CTL-mediated immune responses and further improve therapeutic efficacy of cancer immunotherapy.

# CONCLUSIONS

1. High percentage (88%) of cryopreserved PC tumors show some type of HLA-I alteration. Half of the studied tumors showed loss of β-2-microglobulin (β2m) and total loss of HLA-I expression (phenotype I) associated with a tendency to increased tumor recurrence, high D'Amico cancer risk, and perineural invasion.

2. There was a significant association between total HLA-I loss and reduced mRNA expression of APM molecules, including *tapasin, TAP2, β2m,* and of a transcriptional factor *NLRC5,* all pointing to a coordinated downregulation of HLA expression.

3. Molecular analysis of the prostate tumors revealed that 32% of the studied samples have structural genetic alteration: a loss of material in chromosome 6 (LOH-6). One tumor had LOH-15, but sequencing of the *β2m* gene did not detect any structural aberrations.

4. The studied prostate cell lines displayed various allele- and locus-specific HLA-I losses, in some cases resistant to IFN-γ. One cell line showed total loss of HLA-I expression caused by two structural defects in *β2m* gene, a microdeletion in one gene copy and a chromosomal loss (LOH-15) affecting another copy.

5. AAV serotype 2 among other studied AAV serotypes has the highest GFP transduction efficiency in different human tumor cell lines, although, in general, inferior to adenovirus. Quadruple mutant has superior transduction effectiveness in melanoma cells over the wild type AAV2 or other mutant variants

6. Infection of cells with adenovirus or AAV2 coding for *HLA-A2* gene modulates A2 expression in tumor cells resulting in either: 1) Recovery of lost HLA-A2 expression (in melanoma cells Ando-2, which permanently lacks A2 allele due

to a chromosomal loss); 2) Upregulation of endogenous genomic HLA-A2 in cells with low baseline A2 expression; or 3) Expression of a new HLA-A2 allele in addition to the existing HLA alleles in tumor cells with HLA genotype negative for A2.

7. Co-infection of tumor cells HLA-I-negative tumor cells with adenovirus vectors coding for *HLA-A2* or *β2m* genes demonstrated that the *de novo* expressed proteins can form a detectable HLA-I/β2m complex on the tumor cell surface.

# REFERENCES

Abdul, M., and N. Hoosein, 2000, Changes in beta-2 microglobulin expression in prostate cancer: Urol Oncol, v. 5, p. 168-172.

Akache, B., D. Grimm, K. Pandey, S. R. Yant, H. Xu, and M. A. Kay, 2006, The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9: J Virol, v. 80, p. 9831-6.

Algarra, I., A. García-Lora, T. Cabrera, F. Ruiz-Cabello, and F. Garrido, 2004, The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape: Cancer Immunol Immunother, v. 53, p. 904-10.

Altaner, C., 2008, Prodrug cancer gene therapy: Cancer Lett, v. 270, p. 191-201.

Alvarez, R. D., M. N. Barnes, J. Gomez-Navarro, M. Wang, T. V. Strong, W. Arafat, R. B. Arani, M. R. Johnson, B. L. Roberts, G. P. Siegal, and D. T. Curiel, 2000, A cancer gene therapy approach utilizing an anti-erbB-2 single-chain antibody-encoding adenovirus (AD21): a phase I trial: Clin Cancer Res, v. 6, p. 3081-7.

Androlewicz, M. J., K. S. Anderson, and P. Cresswell, 1993, Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner: Proc Natl Acad Sci U S A, v. 90, p. 9130-4.

Anisimov, A., A. Alitalo, P. Korpisalo, J. Soronen, S. Kaijalainen, V. M. Leppänen, M. Jeltsch, S. Ylä-Herttuala, and K. Alitalo, 2009, Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle: Circ Res, v. 104, p. 1302-12.

Aptsiauri, N., T. Cabrera, A. Garcia-Lora, M. A. Lopez-Nevot, F. Ruiz-Cabello, and F. Garrido, 2007a, MHC class I antigens and immune surveillance in transformed cells: Int Rev Cytol, v. 256, p. 139-89.

Aptsiauri, N., T. Cabrera, R. Mendez, A. Garcia-Lora, F. Ruiz-Cabello, and F. Garrido, 2007b, Role of altered expression of HLA class I molecules in cancer progression: Adv Exp Med Biol, v. 601, p. 123-31.

Aptsiauri, N., R. Carretero, A. Garcia-Lora, L. M. Real, T. Cabrera, and F. Garrido, 2008, Regressing and progressing metastatic lesions: resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations: Cancer Immunol Immunother, v. 57, p. 1727-33.

Aptsiauri, N., A. M. García-Lora, and F. Garrido, 2014, 'Hard' and 'soft' loss of MHC class I expression in cancer cells, *in* R. Rees, ed., Tumor Immunology and Immunotherapy: Oxford, UK, Oxford University Press, p. 63-78

Aptsiauri, N., M. García-Lora, and T. Cabrera, 2013, MHC Class I Antigens In Malignant Cells: Immune Escape and Response to Immunotherapy, v. 1, Springer.

Aruga, A., E. Aruga, K. Tanigawa, D. K. Bishop, V. K. Sondak, and A. E. Chang, 1997, Type 1 versus type 2 cytokine release by Vbeta T cell subpopulations determines in vivo antitumor reactivity: IL-10 mediates a suppressive role: J Immunol, v. 159, p. 664-73.

Asokan, A., J. B. Hamra, L. Govindasamy, M. Agbandje-McKenna, and R. J. Samulski, 2006, Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry: J Virol, v. 80, p. 8961-9.

Atchison, R. W., B. C. CASTO, and W. M. HAMMON, 1965, Adenovirus-associated defective virus particles: Science, v. 149, p. 754-6.

Bander, N. H., D. Yao, H. Liu, Y. T. Chen, M. Steiner, W. Zuccaro, and P. Moy, 1997, MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma: Prostate, v. 33, p. 233-9.

Bangari, D. S., and S. K. Mittal, 2006, Current strategies and future directions for eluding adenoviral vector immunity: Curr Gene Ther, v. 6, p. 215-26.

Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler, 1978, Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis: Cell, v. 14, p. 9-20.

Bartlett, J. S., R. Wilcher, and R. J. Samulski, 2000, Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors: J Virol, v. 74, p. 2777-85.

Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies, 1999, Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA: Science, v. 285, p. 727-9.

Benitez, R., D. Godelaine, M. A. Lopez-Nevot, F. Brasseur, P. Jiménez, M. Marchand, M. R. Oliva, N. van Baren, T. Cabrera, G. Andry, C. Landry, F. Ruiz-Cabello, T. Boon, and F. Garrido, 1998, Mutations of the beta2-microglobulin gene result in a lack of HLA class I molecules on melanoma cells of two patients immunized with MAGE peptides: Tissue Antigens, v. 52, p. 520-9.

Bennett, E. M., J. R. Bennink, J. W. Yewdell, and F. M. Brodsky, 1999, Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression: J Immunol, v. 162, p. 5049-52.

Bergelson, J. M., J. A. Cunningham, G. Droguett, E. A. Kurt-Jones, A. Krithivas, J. S. Hong, M. S. Horwitz, R. L. Crowell, and R. W. Finberg, 1997, Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5: Science, v. 275, p. 1320-3.

Bergmann-Leitner, E. S., and S. I. Abrams, 2001, Positive and negative consequences of soluble Fas ligand produced by an antigen-specific CD4(+) T cell response in human carcinoma immune interactions: Cell Immunol, v. 209, p. 49-62.

Bernal, M., F. Ruiz-Cabello, A. Concha, A. Paschen, and F. Garrido, 2012, Implication of the β2-microglobulin gene in the generation of tumor escape phenotypes: Cancer Immunol Immunother, v. 61, p. 1359-71.

Berns, K. I., and R. M. Linden, 1995, The cryptic life style of adeno-associated virus: Bioessays, v. 17, p. 237-45.

Berset, M., J. P. Cerottini, D. Guggisberg, P. Romero, F. Burri, D. Rimoldi, and R. G. Panizzon, 2001, Expression of Melan-A/MART-1 antigen as a prognostic factor in primary cutaneous melanoma: Int J Cancer, v. 95, p. 73-7.

Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham, 1994, An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3: Proc Natl Acad Sci U S A, v. 91, p. 8802-6.

Beyer, M., 2012, Interleukin-2 treatment of tumor patients can expand regulatory T cells: Oncoimmunology, v. 1, p. 1181-1182.

Birkeland, S. A., H. H. Storm, L. U. Lamm, L. Barlow, I. Blohmé, B. Forsberg, B. Eklund, O. Fjeldborg, M. Friedberg, and L. Frödin, 1995, Cancer risk after renal transplantation in the Nordic countries, 1964-1986: Int J Cancer, v. 60, p. 183-9.

Bjorkman, P. J., and P. Parham, 1990, Structure, function, and diversity of class I major histocompatibility complex molecules: Annu Rev Biochem, v. 59, p. 253-88.

Blades, R. A., P. J. Keating, L. J. McWilliam, N. J. George, and P. L. Stern, 1995, Loss of HLA class I expression in prostate cancer: implications for immunotherapy: Urology, v. 46, p. 681-6; discussion 686-7.

Boegel, S., M. Löwer, T. Bukur, U. Sahin, and J. C. Castle, 2014, A catalog of HLA type, HLA expression, and neo-epitope candidates in human cancer cell lines: Oncoimmunology, v. 3, p. e954893.

Boon, T., P. G. Coulie, and B. Van den Eynde, 1997, Tumor antigens recognized by T cells: Immunol Today, v. 18, p. 267-8.

Boorjian, S. A., J. A. Eastham, M. Graefen, B. Guillonneau, R. J. Karnes, J. W. Moul, E. M. Schaeffer, C. Stief, and K. C. Zorn, 2012, A critical analysis of the long-term impact of radical prostatectomy on cancer control and function outcomes: Eur Urol, v. 61, p. 664-75.

Brady, C. S., J. S. Bartholomew, D. J. Burt, M. F. Duggan-Keen, S. Glenville, N. Telford, A. M. Little, J. A. Davidson, P. Jimenez, F. Ruiz-Cabello, F. Garrido, and P. L. Stern, 2000,

Multiple mechanisms underlie HLA dysregulation in cervical cancer: Tissue Antigens, v. 55, p. 401-11.

Brahmer, J. R., S. S. Tykodi, L. Q. Chow, W. J. Hwu, S. L. Topalian, P. Hwu, C. G. Drake, L. H. Camacho, J. Kauh, K. Odunsi, H. C. Pitot, O. Hamid, S. Bhatia, R. Martins, K. Eaton, S. Chen, T. M. Salay, S. Alaparthy, J. F. Grosso, A. J. Korman, S. M. Parker, S. Agrawal, S. M. Goldberg, D. M. Pardoll, A. Gupta, and J. M. Wigginton, 2012, Safety and activity of anti-PD-L1 antibody in patients with advanced cancer: N Engl J Med, v. 366, p. 2455-65.

Brenner, T. L., and V. R. Adams, 1999, First MAb approved for treatment of metastatic breast cancer: J Am Pharm Assoc (Wash), v. 39, p. 236-8.

Browning, M., F. Petronzelli, D. Bicknell, P. Krausa, A. Rowan, S. Tonks, N. Murray, J. Bodmer, and W. Bodmer, 1996, Mechanisms of loss of HLA class I expression on colorectal tumor cells: Tissue Antigens, v. 47, p. 364-71.

Bukur, J., S. Jasinski, and B. Seliger, 2012, The role of classical and non-classical HLA class I antigens in human tumors: Semin Cancer Biol, v. 22, p. 350-8.

Buller, R. M., J. E. Janik, E. D. Sebring, and J. A. Rose, 1981, Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication: J Virol, v. 40, p. 241-7.

Burnet, F. M., 1970, The concept of immunological surveillance: Prog Exp Tumor Res, v. 13, p. 1-27.

Burnet, M., 1957, Cancer; a biological approach. I. The processes of control: Br Med J, v. 1, p. 779-86.

Burnet, M., 1964, Immunological factors in the process of carcinogenesis: Br Med Bull, v. 20, p. 154-8.

Cabrera, C. M., P. Jiménez, T. Cabrera, C. Esparza, F. Ruiz-Cabello, and F. Garrido, 2003a, Total loss of MHC class I in colorectal tumors can be explained by two molecular pathways: beta2-microglobulin inactivation in MSI-positive tumors and LMP7/TAP2 downregulation in MSI-negative tumors: Tissue Antigens, v. 61, p. 211-9.

Cabrera, C. M., M. A. López-Nevot, P. Jiménez, and F. Garrido, 2005, Involvement of the chaperone tapasin in HLA-B44 allelic losses in colorectal tumors: Int J Cancer, v. 113, p. 611-8.

Cabrera, T., M. Angustias Fernandez, A. Sierra, A. Garrido, A. Herruzo, A. Escobedo, A. Fabra, and F. Garrido, 1996, High frequency of altered HLA class I phenotypes in invasive breast carcinomas: Hum Immunol, v. 50, p. 127-34.

Cabrera, T., A. Collado, M. A. Fernandez, A. Ferron, J. Sancho, F. Ruiz-Cabello, and F. Garrido, 1998, High frequency of altered HLA class I phenotypes in invasive colorectal carcinomas: Tissue Antigens, v. 52, p. 114-23.

Cabrera, T., E. Lara, J. M. Romero, I. Maleno, L. M. Real, F. Ruiz-Cabello, P. Valero, F. M. Camacho, and F. Garrido, 2007, HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination: Cancer Immunol Immunother, v. 56, p. 709-17.

Cabrera, T., G. Pedrajas, J. M. Cozar, A. Garrido, J. Vicente, M. Tallada, and F. Garrido, 2003b, HLA class I expression in bladder carcinomas: Tissue Antigens, v. 62, p. 324-7.

Cabrera, T., J. Salinero, M. A. Fernandez, A. Garrido, J. Esquivias, and F. Garrido, 2000, High frequency of altered HLA class I phenotypes in laryngeal carcinomas: Hum Immunol, v. 61, p. 499-506.

Cafri, G., A. Margalit, E. Tzehoval, L. Eisenbach, and G. Gross, 2013, Development of novel genetic cancer vaccines based on membrane-attached β2 microglobulin: Ann N Y Acad Sci, v. 1283, p. 87-90.

Calne, R. Y., D. J. White, S. Thiru, D. B. Evans, P. McMaster, D. C. Dunn, G. N. Craddock, B. D. Pentlow, and K. Rolles, 1978, Cyclosporin A in patients receiving renal allografts from cadaver donors: Lancet, v. 2, p. 1323-7.

Campoli, M., C. C. Chang, and S. Ferrone, 2002, HLA class I antigen loss, tumor immune escape and immune selection: Vaccine, v. 20 Suppl 4, p. A40-5.

Cao, L., Q. Zeng, C. Xu, S. Shi, Z. Zhang, and X. Sun, 2013, Enhanced antitumor response mediated by the codelivery of paclitaxel and adenoviral vector expressing IL-12: Mol Pharm, v. 10, p. 1804-14.

Carlsson, B., T. H. Tötterman, and M. Essand, 2004, Generation of cytotoxic T lymphocytes specific for the prostate and breast tissue antigen TARP: Prostate, v. 61, p. 161-70.

Carosella, E. D., P. Moreau, J. Le Maoult, M. Le Discorde, J. Dausset, and N. Rouas-Freiss, 2003, HLA-G molecules: from maternal-fetal tolerance to tissue acceptance: Adv Immunol, v. 81, p. 199-252.

Carrel, S., S. Salvi, F. Hartmann, and D. Rimoldi, 1994, Recognition of HLA-A1 by murine monoclonal antibodies: Tissue Antigens, v. 43, p. 110-5.

Carretero, R., T. Cabrera, H. Gil, P. Saenz-Lopez, I. Maleno, N. Aptsiauri, J. M. Cozar, and F. Garrido, 2011, Bacillus Calmette-Guerin immunotherapy of bladder cancer induces selection of human leukocyte antigen class I-deficient tumor cells: Int J Cancer, v. 129, p. 839-46.

Carretero, R., J. M. Romero, F. Ruiz-Cabello, I. Maleno, F. Rodriguez, F. M. Camacho, L. M. Real, F. Garrido, and T. Cabrera, 2008, Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy: Immunogenetics, v. 60, p. 439-47.

Carretero, R., E. Wang, A. I. Rodriguez, J. Reinboth, M. L. Ascierto, A. M. Engle, H. Liu, F. M. Camacho, F. M. Marincola, F. Garrido, and T. Cabrera, 2012, Regression of melanoma metastases after immunotherapy is associated with activation of antigen presentation and interferon-mediated rejection genes: Int J Cancer, v. 131, p. 387-95.

Cascallo, M., M. M. Alonso, J. J. Rojas, A. Perez-Gimenez, J. Fueyo, and R. Alemany, 2007, Systemic toxicity-efficacy profile of ICOVIR-5, a potent and selective oncolytic adenovirus based on the pRB pathway: Mol Ther, v. 15, p. 1607-15.

Casto, B. C., R. W. Atchison, and W. M. Hammon, 1967, Studies on the relationship between adeno-associated virus type I (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus: Virology, v. 32, p. 52-9.

Catlett-Falcone, R., T. H. Landowski, M. M. Oshiro, J. Turkson, A. Levitzki, R. Savino, G. Ciliberto, L. Moscinski, J. L. Fernández-Luna, G. Nuñez, W. S. Dalton, and R. Jove, 1999, Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells: Immunity, v. 10, p. 105-15.

Cevher, E., A. D. Sezer, and E. S. Çaglar, 2012, Gene Delivery Systems: Recent Progress in VIral and Non-Viral Therapy, *in* A. D. Sezer, ed., Recent Advances in Novel Drug Carrier Systems, Intech.

Chambers, C. A., M. S. Kuhns, J. G. Egen, and J. P. Allison, 2001, CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy: Annu Rev Immunol, v. 19, p. 565-94.

Chang, C. C., M. Campoli, and S. Ferrone, 2003, HLA class I defects in malignant lesions: what have we learned?: Keio J Med, v. 52, p. 220-9.

Chang, C. C., M. Campoli, N. P. Restifo, X. Wang, and S. Ferrone, 2005, Immune selection of hot-spot beta 2-microglobulin gene mutations, HLA-A2 allospecificity loss, and antigen-processing machinery component down-regulation in melanoma cells derived from recurrent metastases following immunotherapy: J Immunol, v. 174, p. 1462-71.

Chang, C. C., and S. Ferrone, 2007, Immune selective pressure and HLA class I antigen defects in malignant lesions: Cancer Immunol Immunother, v. 56, p. 227-36.

Chatterjee-Kishore, M., F. van Den Akker, and G. R. Stark, 2000, Adenovirus E1A down-regulates LMP2 transcription by interfering with the binding of stat1 to IRF1: J Biol Chem, v. 275, p. 20406-11.

Chaux, P., M. Moutet, J. Faivre, F. Martin, and M. Martin, 1996, Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7-1 and B7-2 costimulatory molecules of the T-cell activation: Lab Invest, v. 74, p. 975-83.

Chen, C., V. Akerstrom, J. Baus, M. S. Lan, and M. B. Breslin, 2013, Comparative analysis of the transduction efficiency of five adeno associated virus serotypes and VSV-G pseudotype lentiviral vector in lung cancer cells: Virol J, v. 10, p. 86.

Chen, H. L., D. Gabrilovich, R. Tampé, K. R. Girgis, S. Nadaf, and D. P. Carbone, 1996, A functionally defective allele of TAP1 results in loss of MHC class I antigen presentation in a human lung cancer: Nat Genet, v. 13, p. 210-3.

Chen, Y., T. DeWeese, J. Dilley, Y. Zhang, Y. Li, N. Ramesh, J. Lee, R. Pennathur-Das, J. Radzyminski, J. Wypych, D. Brignetti, S. Scott, J. Stephens, D. B. Karpf, D. R. Henderson, and D. C. Yu, 2001, CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity: Cancer Res, v. 61, p. 5453-60.

Chiu, T. L., C. W. Peng, and M. J. Wang, 2011, Enhanced anti-glioblastoma activity of microglia by AAV2-mediated IL-12 through TRAIL and phagocytosis in vitro: Oncol Rep, v. 25, p. 1373-80.

Clark, R. E., I. A. Dodi, S. C. Hill, J. R. Lill, G. Aubert, A. R. Macintyre, J. Rojas, A. Bourdon, P. L. Bonner, L. Wang, S. E. Christmas, P. J. Travers, C. S. Creaser, R. C. Rees, and J. A. Madrigal, 2001, Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein: Blood, v. 98, p. 2887-93.

Clifford, G. M., J. Polesel, M. Rickenbach, L. Dal Maso, O. Keiser, A. Kofler, E. Rapiti, F. Levi, G. Jundt, T. Fisch, A. Bordoni, D. De Weck, S. Franceschi, and S. H. Cohort, 2005, Cancer risk in the Swiss HIV Cohort Study: associations with immunodeficiency, smoking, and highly active antiretroviral therapy: J Natl Cancer Inst, v. 97, p. 425-32.

Coley, W. B., 1891, II. Contribution to the Knowledge of Sarcoma: Ann Surg, v. 14, p. 199-220.

Conry, R. M., M. B. Khazaeli, M. N. Saleh, K. O. Allen, D. L. Barlow, S. E. Moore, D. Craig, R. B. Arani, J. Schlom, and A. F. LoBuglio, 1999, Phase I trial of a recombinant vaccinia virus encoding carcinoembryonic antigen in metastatic adenocarcinoma: comparison of intradermal versus subcutaneous administration: Clin Cancer Res, v. 5, p. 2330-7.

Cresswell, P., 1994, Assembly, transport, and function of MHC class II molecules: Annu Rev Immunol, v. 12, p. 259-93.

Cresswell, P., A. L. Ackerman, A. Giodini, D. R. Peaper, and P. A. Wearsch, 2005, Mechanisms of MHC class I-restricted antigen processing and cross-presentation: Immunol Rev, v. 207, p. 145-57.

Crowley, N. J., T. L. Darrow, M. A. Quinn-Allen, and H. F. Seigler, 1991, MHC-restricted recognition of autologous melanoma by tumor-specific cytotoxic T cells. Evidence for restriction by a dominant HLA-A allele: J Immunol, v. 146, p. 1692-9.

Curran, K. J., H. J. Pegram, and R. J. Brentjens, 2012, Chimeric antigen receptors for T cell immunotherapy: current understanding and future directions: J Gene Med, v. 14, p. 405-15.

D'Amico, A. V., R. Whittington, S. B. Malkowicz, D. Schultz, K. Blank, G. A. Broderick, J. E. Tomaszewski, A. A. Renshaw, I. Kaplan, C. J. Beard, and A. Wein, 1998, Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer: Jama-Journal of the American Medical Association, v. 280, p. 969-974.

David, V., F. Hochstenbach, S. Rajagopalan, and M. B. Brenner, 1993, Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin): J Biol Chem, v. 268, p. 9585-92.

Daya, S., and K. I. Berns, 2008, Gene therapy using adeno-associated virus vectors: Clin Microbiol Rev, v. 21, p. 583-93.

del Campo, A. B., N. Aptsiauri, R. Méndez, S. Zinchenko, A. Vales, A. Paschen, S. Ward, F. Ruiz-Cabello, G. González-Aseguinolaza, and F. Garrido, 2009, Efficient recovery of HLA class I expression in human tumor cells after beta2-microglobulin gene transfer using adenoviral vector: implications for cancer immunotherapy: Scand J Immunol, v. 70, p. 125-35.

del Campo, A. B., J. A. Kyte, J. Carretero, S. Zinchencko, R. Méndez, G. González-Aseguinolaza, F. Ruiz-Cabello, S. Aamdal, G. Gaudernack, F. Garrido, and N. Aptsiauri, 2014, Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma: Int J Cancer, v. 134, p. 102-13.

Derré, L., M. Corvaisier, B. Charreau, A. Moreau, E. Godefroy, A. Moreau-Aubry, F. Jotereau, and N. Gervois, 2006, Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells: J Immunol, v. 177, p. 3100-7.

DeWeese, T. L., H. van der Poel, S. Li, B. Mikhak, R. Drew, M. Goemann, U. Hamper, R. DeJong, N. Detorie, R. Rodriguez, T. Haulk, A. M. DeMarzo, S. Piantadosi, D. C. Yu, Y. Chen, D. R. Henderson, M. A. Carducci, W. G. Nelson, and J. W. Simons, 2001, A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy: Cancer Res, v. 61, p. 7464-72.

Dighe, A. S., E. Richards, L. J. Old, and R. D. Schreiber, 1994, Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors: Immunity, v. 1, p. 447-56.

Doherty, G. J., and H. T. McMahon, 2009, Mechanisms of endocytosis: Annu Rev Biochem, v. 78, p. 857-902.

Dong, H., S. E. Strome, D. R. Salomao, H. Tamura, F. Hirano, D. B. Flies, P. C. Roche, J. Lu, G. Zhu, K. Tamada, V. A. Lennon, E. Celis, and L. Chen, 2002, Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion: Nat Med, v. 8, p. 793-800.

Dormond, E., M. Perrier, and A. Kamen, 2009, From the first to the third generation adenoviral vector: what parameters are governing the production yield?: Biotechnol Adv, v. 27, p. 133-44.

Doronin, K., M. Kuppuswamy, K. Toth, A. E. Tollefson, P. Krajcsi, V. Krougliak, and W. S. Wold, 2001, Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy: J Virol, v. 75, p. 3314-24.

Douar, A. M., K. Poulard, D. Stockholm, and O. Danos, 2001, Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation: J Virol, v. 75, p. 1824-33.

Doukas, J., and A. Rolland, 2012, Mechanisms of action underlying the immunotherapeutic activity of Allovectin in advanced melanoma: Cancer Gene Ther, v. 19, p. 811-7.

Dudley, M. E., J. C. Yang, R. Sherry, M. S. Hughes, R. Royal, U. Kammula, P. F. Robbins, J. Huang, D. E. Citrin, S. F. Leitman, J. Wunderlich, N. P. Restifo, A. Thomasian, S. G. Downey, F. O. Smith, J. Klapper, K. Morton, C. Laurencot, D. E. White, and S. A. Rosenberg, 2008, Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens: J Clin Oncol, v. 26, p. 5233-9.

Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber, 2002, Cancer immunoediting: from immunosurveillance to tumor escape: Nat Immunol, v. 3, p. 991-8.

Dunn, G. P., L. J. Old, and R. D. Schreiber, 2004, The three Es of cancer immunoediting: Annu Rev Immunol, v. 22, p. 329-60.

Dunn, G. P., K. C. Sheehan, L. J. Old, and R. D. Schreiber, 2005, IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression: Cancer Res, v. 65, p. 3447-53.

Echchakir, H., F. Mami-Chouaib, I. Vergnon, J. F. Baurain, V. Karanikas, S. Chouaib, and P. G. Coulie, 2001, A point mutation in the alpha-actinin-4 gene generates an antigenic peptide recognized by autologous cytolytic T lymphocytes on a human lung carcinoma: Cancer Res, v. 61, p. 4078-83.

Ehrlich, P., 1909, Ueberden jetziegen stand der karzinomforshung: Ned Tijdschr Geneeskd, v. 5, p. 273-290.

Elhag, O. A., X. J. Hu, Z. Wen-Ying, X. Li, Y. Z. Yuan, L. F. Deng, D. L. Liu, Y. L. Liu, and G. Hui, 2012, Reconstructed adeno-associated virus with the extracellular domain of murine PD-1 induces antitumor immunity: Asian Pac J Cancer Prev, v. 13, p. 4031-6.

Engel, A. M., I. M. Svane, J. Rygaard, and O. Werdelin, 1997, MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice: Scand J Immunol, v. 45, p. 463-70.

Engelhardt, J. F., L. Litzky, and J. M. Wilson, 1994, Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a: Hum Gene Ther, v. 5, p. 1217-29.

Evans, C. H., 2012, Gene delivery to bone: Adv Drug Deliv Rev, v. 64, p. 1331-40.

Evans, C. H., E. Gouze, J. N. Gouze, P. D. Robbins, and S. C. Ghivizzani, 2006, Gene therapeutic approaches-transfer in vivo: Adv Drug Deliv Rev, v. 58, p. 243-58.

Everts, B., and H. G. van der Poel, 2005, Replication-selective oncolytic viruses in the treatment of cancer: Cancer Gene Ther, v. 12, p. 141-61.

Feenstra, M., E. Rozemuller, K. Duran, I. Stuy, J. van den Tweel, P. Slootweg, R. de Weger, and M. Tilanus, 1999, Mutation in the beta 2m gene is not a frequent event in head and neck squamous cell carcinomas: Hum Immunol, v. 60, p. 697-706.

Ferrara, N., K. J. Hillan, H. P. Gerber, and W. Novotny, 2004, Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer: Nat Rev Drug Discov, v. 3, p. 391-400.

Festenstein, H., 1987, The biological consequences of altered MHC expression on tumours: Br Med Bull, v. 43, p. 217-27.

Flotte, T., B. Carter, C. Conrad, W. Guggino, T. Reynolds, B. Rosenstein, G. Taylor, S. Walden, and R. Wetzel, 1996, A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease: Hum Gene Ther, v. 7, p. 1145-59.

Frank, K. M., D. K. Hogarth, J. L. Miller, S. Mandal, P. J. Mease, R. J. Samulski, G. A. Weisgerber, and J. Hart, 2009, Investigation of the cause of death in a gene-therapy trial: N Engl J Med, v. 361, p. 161-9.

Fridman, W. H., F. Pagès, C. Sautès-Fridman, and J. Galon, 2012, The immune contexture in human tumours: impact on clinical outcome: Nat Rev Cancer, v. 12, p. 298-306.

Fujihara, T., M. Yashiro, T. Inoue, T. Sawada, Y. Kato, M. Ohira, Y. Nishiguchi, T. Ishikawa, M. Sowa, and K. H. Chung, 1999, Decrease in ICAM-1 expression on gastric cancer cells is correlated with lymph node metastasis: Gastric Cancer, v. 2, p. 221-225.

Gabrilovich, D., T. Ishida, T. Oyama, S. Ran, V. Kravtsov, S. Nadaf, and D. P. Carbone, 1998, Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo: Blood, v. 92, p. 4150-66.

Gaczynska, M., K. L. Rock, and A. L. Goldberg, 1993, Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes: Nature, v. 365, p. 264-7.

Garbe, C., P. Terheyden, U. Keilholz, O. Kölbl, and A. Hauschild, 2008, Treatment of melanoma: Dtsch Arztebl Int, v. 105, p. 845-51.

Garcia-Lora, A., I. Algarra, and F. Garrido, 2003a, MHC class I antigens, immune surveillance, and tumor immune escape: J Cell Physiol, v. 195, p. 346-55.

Garcia-Lora, A., M. Martinez, I. Algarra, J. J. Gaforio, and F. Garrido, 2003b, MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components: Int J Cancer, v. 106, p. 521-7.

Garrido, F., and I. Algarra, 2001, MHC antigens and tumor escape from immune surveillance: Advances in Cancer Research, Vol 83, v. 83, p. 117-158.

Garrido, F., I. Algarra, and A. M. García-Lora, 2010a, The escape of cancer from T lymphocytes: immunoselection of MHC class I loss variants harboring structural-irreversible "hard" lesions: Cancer Immunol Immunother, v. 59, p. 1601-6.

Garrido, F., T. Cabrera, and N. Aptsiauri, 2010b, "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy: Int J Cancer, v. 127, p. 249-56.

Garrido F, C. T., Accolla RS, Bensa JC, Bodmer W, Dohr G, Drouet M,, F. G. Fauchet R, Ferrone S, Giacomini P, Kageshita T, Koopman L,, and M. F. Maio M, Mazzilli C, Morel PA, Murray A, Papasteriades Crh, Salvaneschi L, Stern PL, Ziegler A, 1996, 12th International Histocompatibility Conference. Genetic diversity of HLA: functional and medical implications. Paris, France, June 9-12, 1996. Abstracts: Hum Immunol, v. 47, p. 1-184.

Garrido, F., F. Ruiz-Cabello, T. Cabrera, J. J. Pérez-Villar, M. López-Botet, M. Duggan-Keen, and P. L. Stern, 1997, Implications for immunosurveillance of altered HLA class I phenotypes in human tumours: Immunol Today, v. 18, p. 89-95.

Gasser, S., S. Orsulic, E. J. Brown, and D. H. Raulet, 2005, The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor: Nature, v. 436, p. 1186-90.

Gatti, R. A., and R. A. Good, 1971, Occurrence of malignancy in immunodeficiency diseases. A literature review: Cancer, v. 28, p. 89-98.

Geertsen, R., R. Böni, R. Blasczyk, P. Romero, D. Betts, D. Rimoldi, X. Hong, E. Laine, J. Willers, and R. Dummer, 2002, Loss of single HLA class I allospecificities in melanoma cells due to selective genomic abbreviations: Int J Cancer, v. 99, p. 82-7.

Georges, R. N., T. Mukhopadhyay, Y. Zhang, N. Yen, and J. A. Roth, 1993, Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct: Cancer Res, v. 53, p. 1743-6.

Ginn, S. L., I. E. Alexander, M. L. Edelstein, M. R. Abedi, and J. Wixon, 2013, Gene therapy clinical trials worldwide to 2012 - an update: J Gene Med, v. 15, p. 65-77.

Giovannucci, E., Y. Liu, E. A. Platz, M. J. Stampfer, and W. C. Willett, 2007, Risk factors for prostate cancer incidence and progression in the health professionals follow-up study: Int J Cancer, v. 121, p. 1571-8.

Glas, R., K. Sturmhöfel, G. J. Hämmerling, K. Kärre, and H. G. Ljunggren, 1992, Restoration of a tumorigenic phenotype by beta 2-microglobulin transfection to EL-4 mutant cells: J Exp Med, v. 175, p. 843-6.

Glushakova, L. G., M. J. Lisankie, E. B. Eruslanov, C. Ojano-Dirain, I. Zolotukhin, C. Liu, A. Srivastava, and P. W. Stacpoole, 2009, AAV3-mediated transfer and expression of the pyruvate dehydrogenase E1 alpha subunit gene causes metabolic remodeling and apoptosis of human liver cancer cells: Mol Genet Metab, v. 98, p. 289-99.

Godelaine, D., J. Carrasco, S. Lucas, V. Karanikas, B. Schuler-Thurner, P. G. Coulie, G. Schuler, T. Boon, and A. Van Pel, 2003, Polyclonal CTL responses observed in melanoma patients vaccinated with dendritic cells pulsed with a MAGE-3.A1 peptide: J Immunol, v. 171, p. 4893-7.

Gordon, E. M., G. H. Cornelio, C. C. Lorenzo, J. P. Levy, R. A. Reed, L. Liu, and F. L. Hall, 2004, First clinical experience using a 'pathotropic' injectable retroviral vector (Rexin-G) as intervention for stage IV pancreatic cancer: Int J Oncol, v. 24, p. 177-85.

Gordon, E. M., P. X. Liu, Z. H. Chen, L. Liu, M. D. Whitley, C. Gee, S. Groshen, D. R. Hinton, R. W. Beart, and F. L. Hall, 2000, Inhibition of metastatic tumor growth in nude mice by portal vein infusions of matrix-targeted retroviral vectors bearing a cytocidal cyclin G1 construct: Cancer Res, v. 60, p. 3343-7.

Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn, 1977, Characteristics of a human cell line transformed by DNA from human adenovirus type 5: J Gen Virol, v. 36, p. 59-74.

Grant, G. A., and J. F. Miller, 1965, Effect of neonatal thymectomy on the induction of sarcomata in C57 BL mice: Nature, v. 205, p. 1124-5.

Griscelli, F., P. Opolon, P. Saulnier, F. Mami-Chouaib, E. Gautier, H. Echchakir, E. Angevin, T. Le Chevalier, V. Bataille, P. Squiban, T. Tursz, and B. Escudier, 2003, Recombinant adenovirus shedding after intratumoral gene transfer in lung cancer patients: Gene Ther, v. 10, p. 386-95.

Groh, V., S. Bahram, S. Bauer, A. Herman, M. Beauchamp, and T. Spies, 1996, Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium: Proc Natl Acad Sci U S A, v. 93, p. 12445-50.

Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies, 1999, Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB: Proc Natl Acad Sci U S A, v. 96, p. 6879-84.

Groh, V., J. Wu, C. Yee, and T. Spies, 2002, Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation: Nature, v. 419, p. 734-8.

Groothuis, T. A., A. C. Griekspoor, J. J. Neijssen, C. A. Herberts, and J. J. Neefjes, 2005, MHC class I alleles and their exploration of the antigen-processing machinery: Immunol Rev, v. 207, p. 60-76.

Grothey, A., P. Heistermann, S. Philippou, and R. Voigtmann, 1998, Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1, CD54) in patients with non-small-cell lung cancer: correlation with histological expression of ICAM-1 and tumour stage: Br J Cancer, v. 77, p. 801-7.

Guiguet, M., F. Boué, J. Cadranel, J. M. Lang, E. Rosenthal, D. Costagliola, and C. E. G. o. t. F.-A. C. cohort, 2009, Effect of immunodeficiency, HIV viral load, and antiretroviral therapy on the risk of individual malignancies (FHDH-ANRS CO4): a prospective cohort study: Lancet Oncol, v. 10, p. 1152-9.

Gulley, J. L., R. A. Madan, K. Y. Tsang, C. Jochems, J. L. Marté, B. Farsaci, J. A. Tucker, J. W. Hodge, D. J. Liewehr, S. M. Steinberg, C. R. Heery, and J. Schlom, 2014, Immune impact induced by PROSTVAC (PSA-TRICOM), a therapeutic vaccine for prostate cancer: Cancer Immunol Res, v. 2, p. 133-41.

Han, T., U. M. Abdel-Motal, D. K. Chang, J. Sui, A. Muvaffak, J. Campbell, Q. Zhu, T. S. Kupper, and W. A. Marasco, 2012, Human anti-CCR4 minibody gene transfer for the treatment of cutaneous T-cell lymphoma: PLoS One, v. 7, p. e44455.

Hanagiri, T., Y. Shigematsu, K. Kuroda, T. Baba, H. Shiota, Y. Ichiki, Y. Nagata, M. Yasuda, H. Uramoto, T. So, M. Takenoyama, and F. Tanaka, 2013, Prognostic implications of human leukocyte antigen class I expression in patients who underwent surgical resection for non-small-cell lung cancer: J Surg Res, v. 181, p. e57-63.

Hardy, S., M. Kitamura, T. Harris-Stansil, Y. Dai, and M. L. Phipps, 1997, Construction of adenovirus vectors through Cre-lox recombination: J Virol, v. 71, p. 1842-9.

Hartman, Z. C., D. M. Appledorn, and A. Amalfitano, 2008, Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications: Virus Res, v. 132, p. 1-14.

Hayday, A. C., 2000, [gamma][delta] cells: a right time and a right place for a conserved third way of protection: Annu Rev Immunol, v. 18, p. 975-1026.

He, S. S., H. S. Shi, T. Yin, Y. X. Li, S. T. Luo, Q. J. Wu, L. Lu, Y. Q. Wei, and L. Yang, 2012, AAV-mediated gene transfer of human pigment epithelium-derived factor inhibits Lewis lung carcinoma growth in mice: Oncol Rep, v. 27, p. 1142-8.

He, Z. H., Z. Lei, Y. Zhen, W. Gong, B. Huang, Y. Yuan, G. M. Zhang, X. J. Wang, and Z. H. Feng, 2014, Adeno-associated virus-mediated expression of recombinant CBD-HepII polypeptide of human fibronectin inhibits metastasis of breast cancer: Breast Cancer Res Treat, v. 143, p. 33-45.

Hinz, S., A. Trauzold, L. Boenicke, C. Sandberg, S. Beckmann, E. Bayer, H. Walczak, H. Kalthoff, and H. Ungefroren, 2000, Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis: Oncogene, v. 19, p. 5477-86.

Hitt, M. M., and F. L. Graham, 2000, Adenovirus vectors for human gene therapy: Adv Virus Res, v. 55, p. 479-505.

Horwitz, M. S., 1996, Adenoviruses, Raven publishers.

Hu, J., X. Yuan, M. L. Belladonna, J. M. Ong, S. Wachsmann-Hogiu, D. L. Farkas, K. L. Black, and J. S. Yu, 2006, Induction of potent antitumor immunity by intratumoral injection of interleukin 23-transduced dendritic cells: Cancer Res, v. 66, p. 8887-96.

Huang, W. C., D. Wu, Z. Xie, H. E. Zhau, T. Nomura, M. Zayzafoon, J. Pohl, C. L. Hsieh, M. N. Weitzmann, M. C. Farach-Carson, and L. W. Chung, 2006, beta2-microglobulin is a signaling and growth-promoting factor for human prostate cancer bone metastasis: Cancer Res, v. 66, p. 9108-16.

Huebner, R. J., W. P. Rowe, W. E. Schatten, R. R. Smith, and L. B. Thomas, 1956, Studies on the use of viruses in the treatment of carcinoma of the cervix: Cancer, v. 9, p. 1211-8.

Hui, K., F. Grosveld, and H. Festenstein, 1984, Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation: Nature, v. 311, p. 750-2.

Hutter, H., A. Hammer, A. Blaschitz, M. Hartmann, P. Ebbesen, G. Dohr, A. Ziegler, and B. Uchanska-Ziegler, 1996, Expression of HLA class I molecules in human first trimester and term placenta trophoblast: Cell Tissue Res, v. 286, p. 439-47.

Im, S. A., C. Gomez-Manzano, J. Fueyo, T. J. Liu, L. D. Ke, J. S. Kim, H. Y. Lee, P. A. Steck, A. P. Kyritsis, and W. K. Yung, 1999, Antiangiogenesis treatment for gliomas: transfer of antisense-vascular endothelial growth factor inhibits tumor growth in vivo: Cancer Res, v. 59, p. 895-900.

Imperiale, M. J., and S. Kochanek, 2004, Adenovirus vectors: biology, design, and production: Curr Top Microbiol Immunol, v. 273, p. 335-57.

Jager, L., and A. Ehrhardt, 2007, Emerging adenoviral vectors for stable correction of genetic disorders: Curr Gene Ther, v. 7, p. 272-83.

Jansen, B., V. Wacheck, E. Heere-Ress, H. Schlagbauer-Wadl, C. Hoeller, T. Lucas, M. Hoermann, U. Hollenstein, K. Wolff, and H. Pehamberger, 2000, Chemosensitisation of malignant melanoma by BCL2 antisense therapy: Lancet, v. 356, p. 1728-33.

Jiménez, P., T. Cabrera, R. Méndez, C. Esparza, J. M. Cozar, M. Tallada, M. A. López-Nevot, F. Ruiz-Cabello, and F. Garrido, 2001, A nucleotide insertion in exon 4 is responsible for the absence of expression of an HLA-A*0301 allele in a prostate carcinoma cell line: Immunogenetics, v. 53, p. 606-10.

Jinushi, M., T. Takehara, T. Tatsumi, T. Kanto, V. Groh, T. Spies, R. Kimura, T. Miyagi, K. Mochizuki, Y. Sasaki, and N. Hayashi, 2003, Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid: Int J Cancer, v. 104, p. 354-61.

Johnsen, A. K., D. J. Templeton, M. Sy, and C. V. Harding, 1999, Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis: J Immunol, v. 163, p. 4224-31.

Johnson, J. S., and R. J. Samulski, 2009, Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleolus: J Virol, v. 83, p. 2632-44.

Jäger, E., M. Ringhoffer, J. Karbach, M. Arand, F. Oesch, and A. Knuth, 1996, Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8+ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo: Int J Cancer, v. 66, p. 470-6.

Kageshita, T., T. Ishihara, M. Campoli, and S. Ferrone, 2005, Selective monomorphic and polymorphic HLA class I antigenic determinant loss in surgically removed melanoma lesions: Tissue Antigens, v. 65, p. 419-28.

Kaneko, K., S. Ishigami, Y. Kijima, Y. Funasako, M. Hirata, H. Okumura, H. Shinchi, C. Koriyama, S. Ueno, H. Yoshinaka, and S. Natsugoe, 2011, Clinical implication of HLA class I expression in breast cancer: BMC Cancer, v. 11, p. 454.

Kanerva, A., P. Nokisalmi, I. Diaconu, A. Koski, V. Cerullo, I. Liikanen, S. Tähtinen, M. Oksanen, R. Heiskanen, S. Pesonen, T. Joensuu, T. Alanko, K. Partanen, L. Laasonen, K. Kairemo, L. Kangasniemi, and A. Hemminki, 2013, Antiviral and antitumor T-cell immunity in patients treated with GM-CSF-coding oncolytic adenovirus: Clin Cancer Res, v. 19, p. 2734-44.

Kantoff, P. W., C. S. Higano, N. D. Shore, E. R. Berger, E. J. Small, D. F. Penson, C. H. Redfern, A. C. Ferrari, R. Dreicer, R. B. Sims, Y. Xu, M. W. Frohlich, P. F. Schellhammer, and I. S. Investigators, 2010a, Sipuleucel-T immunotherapy for castration-resistant prostate cancer: N Engl J Med, v. 363, p. 411-22.

Kantoff, P. W., T. J. Schuetz, B. A. Blumenstein, L. M. Glode, D. L. Bilhartz, M. Wyand, K. Manson, D. L. Panicali, R. Laus, J. Schlom, W. L. Dahut, P. M. Arlen, J. L. Gulley, and W. R. Godfrey, 2010b, Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer: J Clin Oncol, v. 28, p. 1099-105.

Kaplan, D. H., V. Shankaran, A. S. Dighe, E. Stockert, M. Aguet, L. J. Old, and R. D. Schreiber, 1998, Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice: Proc Natl Acad Sci U S A, v. 95, p. 7556-61.

Kashiwakura, Y., K. Tamayose, K. Iwabuchi, Y. Hirai, T. Shimada, K. Matsumoto, T. Nakamura, M. Watanabe, K. Oshimi, and H. Daida, 2005, Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection: J Virol, v. 79, p. 609-14.

Kass, E., D. L. Panicali, G. Mazzara, J. Schlom, and J. W. Greiner, 2001, Granulocyte/macrophage-colony stimulating factor produced by recombinant avian poxviruses enriches the regional lymph nodes with antigen-presenting cells and acts as an immunoadjuvant: Cancer Res, v. 61, p. 206-14.

Kataoka, T., M. Schröter, M. Hahne, P. Schneider, M. Irmler, M. Thome, C. J. Froelich, and J. Tschopp, 1998, FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation: J Immunol, v. 161, p. 3936-42.

Kaufman, H. L., G. Deraffele, J. Mitcham, D. Moroziewicz, S. M. Cohen, K. S. Hurst-Wicker, K. Cheung, D. S. Lee, J. Divito, M. Voulo, J. Donovan, K. Dolan, K. Manson, D. Panicali, E. Wang, H. Hörig, and F. M. Marincola, 2005, Targeting the local tumor microenvironment with vaccinia virus expressing B7.1 for the treatment of melanoma: J Clin Invest, v. 115, p. 1903-12.

Kay, M. A., A. X. Holterman, L. Meuse, A. Gown, H. D. Ochs, P. S. Linsley, and C. B. Wilson, 1995, Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration: Nat Genet, v. 11, p. 191-7.

Khong, H. T., and N. P. Restifo, 2002, Natural selection of tumor variants in the generation of "tumor escape" phenotypes: Nat Immunol, v. 3, p. 999-1005.

Khong, H. T., Q. J. Wang, and S. A. Rosenberg, 2004, Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression: J Immunother, v. 27, p. 184-90.

Kikuchi, E., K. Yamazaki, T. Torigoe, Y. Cho, M. Miyamoto, S. Oizumi, F. Hommura, H. Dosaka-Akita, and M. Nishimura, 2007, HLA class I antigen expression is associated with a favorable prognosis in early stage non-small cell lung cancer: Cancer Sci, v. 98, p. 1424-30.

Kim, D. W., V. Krishnamurthy, S. D. Bines, and H. L. Kaufman, 2010, TroVax, a recombinant modified vaccinia Ankara virus encoding 5T4: lessons learned and future development: Hum Vaccin, v. 6, p. 784-91.

Kim, J. Y., J. H. Kim, M. Khim, H. S. Lee, J. H. Jung, D. H. Moon, S. Jeong, and H. Lee, 2011, Persistent anti-tumor effects via recombinant adeno-associated virus encoding herpes thymidine kinase gene monitored by PET-imaging: Oncol Rep, v. 25, p. 1263-9.

King, J., J. Waxman, and H. Stauss, 2008, Advances in tumour immunotherapy: QJM, v. 101, p. 675-83.

Kinlen, L. J., A. D. Webster, A. G. Bird, R. Haile, J. Peto, J. F. Soothill, and R. A. Thompson, 1985, Prospective study of cancer in patients with hypogammaglobulinaemia: Lancet, v. 1, p. 263-6.

Kitamura, H., T. Torigoe, H. Asanuma, I. Honma, N. Sato, and T. Tsukamoto, 2007, Down-regulation of HLA class I antigens in prostate cancer tissues and up-regulation by histone deacetylase inhibition: J Urol, v. 178, p. 692-6.

Klatzmann, D., P. Chérin, G. Bensimon, O. Boyer, A. Coutellier, F. Charlotte, C. Boccaccio, J. L. Salzmann, and S. Herson, 1998, A phase I/II dose-escalation study of herpes simplex virus type 1 thymidine kinase "suicide" gene therapy for metastatic melanoma. Study Group on Gene Therapy of Metastatic Melanoma: Hum Gene Ther, v. 9, p. 2585-94.

Klebanoff, C. A., N. Acquavella, Z. Yu, and N. P. Restifo, 2011, Therapeutic cancer vaccines: are we there yet?: Immunol Rev, v. 239, p. 27-44.

Kloor, M., S. Michel, B. Buckowitz, J. Rüschoff, R. Büttner, E. Holinski-Feder, W. Dippold, R. Wagner, M. Tariverdian, A. Benner, Y. Schwitalle, B. Kuchenbuch, and M. von Knebel Doeberitz, 2007, Beta2-microglobulin mutations in microsatellite unstable colorectal tumors: Int J Cancer, v. 121, p. 454-8.

Kobayashi, K. S., and P. J. van den Elsen, 2012, NLRC5: a key regulator of MHC class I-dependent immune responses: Nat Rev Immunol, v. 12, p. 813-20.

Kochanek, S., P. R. Clemens, K. Mitani, H. H. Chen, S. Chan, and C. T. Caskey, 1996, A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase: Proc Natl Acad Sci U S A, v. 93, p. 5731-6.

Koebel, C. M., W. Vermi, J. B. Swann, N. Zerafa, S. J. Rodig, L. J. Old, M. J. Smyth, and R. D. Schreiber, 2007, Adaptive immunity maintains occult cancer in an equilibrium state: Nature, v. 450, p. 903-7.

Koopman, L. A., W. E. Corver, A. R. van der Slik, M. J. Giphart, and G. J. Fleuren, 2000, Multiple genetic alterations cause frequent and heterogeneous human histocompatibility leukocyte antigen class I loss in cervical cancer: J Exp Med, v. 191, p. 961-76.

Kotin, R. M., M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns, 1990, Site-specific integration by adeno-associated virus: Proc Natl Acad Sci U S A, v. 87, p. 2211-5.

Kou, J., J. Yang, J. E. Lim, A. Pattanayak, M. Song, S. Planque, S. Paul, and K. Fukuchi, 2015, Catalytic immunoglobulin gene delivery in a mouse model of Alzheimer's disease: prophylactic and therapeutic applications: Mol Neurobiol, v. 51, p. 43-56.

Kresowik, T. P., and T. S. Griffith, 2009, Bacillus Calmette-Guerin immunotherapy for urothelial carcinoma of the bladder: Immunotherapy, v. 1, p. 281-8.

Lamont, J. P., J. Nemunaitis, J. A. Kuhn, S. A. Landers, and T. M. McCarty, 2000, A prospective phase II trial of ONYX-015 adenovirus and chemotherapy in recurrent squamous cell carcinoma of the head and neck (the Baylor experience): Ann Surg Oncol, v. 7, p. 588-92.

Lampen, M. H., and T. van Hall, 2011, Strategies to counteract MHC-I defects in tumors: Curr Opin Immunol, v. 23, p. 293-8.

Lampson, L. A., C. A. Fisher, and J. P. Whelan, 1983, Striking paucity of HLA-A, B, C and beta 2-microglobulin on human neuroblastoma cell lines: J Immunol, v. 130, p. 2471-8.

Langeberg, W. J., W. B. Isaacs, and J. L. Stanford, 2007, Genetic etiology of hereditary prostate cancer: Front Biosci, v. 12, p. 4101-10.

Le, H. T., Q. C. Yu, J. M. Wilson, and M. A. Croyle, 2005, Utility of PEGylated recombinant adeno-associated viruses for gene transfer: J Control Release, v. 108, p. 161-77.

Leblond, V., F. Davi, F. Charlotte, R. Dorent, M. O. Bitker, L. Sutton, I. Gandjbakhch, J. L. Binet, and M. Raphael, 1998, Posttransplant lymphoproliferative disorders not associated with Epstein-Barr virus: a distinct entity?: J Clin Oncol, v. 16, p. 2052-9.

Lee, G. K., N. Maheshri, B. Kaspar, and D. V. Schaffer, 2005, PEG conjugation moderately protects adeno-associated viral vectors against antibody neutralization: Biotechnol Bioeng, v. 92, p. 24-34.

Lehmann, F., M. Marchand, P. Hainaut, P. Pouillart, X. Sastre, H. Ikeda, T. Boon, and P. G. Coulie, 1995, Differences in the antigens recognized by cytolytic T cells on two successive metastases of a melanoma patient are consistent with immune selection: Eur J Immunol, v. 25, p. 340-7.

Li, H., F. Griscelli, F. Lindenmeyer, P. Opolon, L. Q. Sun, E. Connault, J. Soria, C. Soria, M. Perricaudet, P. Yeh, and H. Lu, 1999, Systemic delivery of antiangiogenic adenovirus AdmATF induces liver resistance to metastasis and prolongs survival of mice: Hum Gene Ther, v. 10, p. 3045-53.

Li, H., H. Lu, F. Griscelli, P. Opolon, L. Q. Sun, T. Ragot, Y. Legrand, D. Belin, J. Soria, C. Soria, M. Perricaudet, and P. Yeh, 1998, Adenovirus-mediated delivery of a uPA/uPAR antagonist suppresses angiogenesis-dependent tumor growth and dissemination in mice: Gene Ther, v. 5, p. 1105-13.

Li, H. L., S. Li, J. Y. Shao, X. B. Lin, Y. Cao, W. Q. Jiang, R. Y. Liu, P. Zhao, X. F. Zhu, M. S. Zeng, Z. Z. Guan, and W. Huang, 2008, Pharmacokinetic and pharmacodynamic study of intratumoral injection of an adenovirus encoding endostatin in patients with advanced tumors: Gene Ther, v. 15, p. 247-56.

Lochrie, M. A., G. P. Tatsuno, B. Christie, J. W. McDonnell, S. Zhou, R. Surosky, G. F. Pierce, and P. Colosi, 2006, Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization: J Virol, v. 80, p. 821-34.

Lopez-Nevot, M. A., T. Cabrera, B. de la Higuera, F. Ruiz-Cabello, and F. Garrido, 1986, Production and characterization of monoclonal antibodies against leukemic cells, Inmunología, p. 51.

Loskog, A., H. Dzojic, S. Vikman, C. Ninalga, M. Essand, O. Korsgren, and T. H. Totterman, 2004, Adenovirus CD40 ligand gene therapy counteracts immune escape mechanisms in the tumor Microenvironment: J Immunol, v. 172, p. 7200-5.

Loskog, A. S., M. E. Fransson, and T. T. Totterman, 2005, AdCD40L gene therapy counteracts T regulatory cells and cures aggressive tumors in an orthotopic bladder cancer model: Clin Cancer Res, v. 11, p. 8816-21.

Lou, Y., R. P. Seipp, B. Cai, S. S. Chen, T. Z. Vitalis, K. B. Choi, A. P. Jeffries, R. S. Gopaul, X. L. Li, B. Seliger, T. W. Pearson, and W. A. Jefferies, 2007, Tumour immunity and T cell memory are induced by low dose inoculation with a non-replicating adenovirus encoding TAP1: Vaccine, v. 25, p. 2331-9.

Lozano, F., L. Borche, L. Places, J. Alberola-Ila, A. Gayá, R. Vilella, and J. Vives, 1990, Biochemical and serological characterization of a public antigenic determinant present on HLA-B molecules: Tissue Antigens, v. 35, p. 193-5.

Lozano, F., J. Santos-Aguado, L. Borche, L. Places, N. Doménech, A. Gayá, R. Vilella, and J. Vives, 1989, Identification of the amino acid residues defining an intralocus determinant in the alpha 1 domain of HLA-A molecules: Immunogenetics, v. 30, p. 50-3.

Lu, L., S. T. Luo, H. S. Shi, M. Li, H. L. Zhang, S. S. He, Y. Liu, Y. Pan, and L. Yang, 2012, AAV2-mediated gene transfer of VEGF-Trap with potent suppression of primary breast tumor growth and spontaneous pulmonary metastases by long-term expression: Oncol Rep, v. 28, p. 1332-8.

Lu, Q. L., P. Abel, S. Mitchell, C. Foster, and E. N. Lalani, 2000, Decreased HLA-A expression in prostate cancer is associated with normal allele dosage in the majority of cases: J Pathol, v. 190, p. 169-76.

Lubaroff, D. M., B. R. Konety, B. Link, J. Gerstbrein, T. Madsen, M. Shannon, J. Howard, J. Paisley, D. Boeglin, T. L. Ratliff, and R. D. Williams, 2009, Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: safety and immunologic results: Clin Cancer Res, v. 15, p. 7375-80.

Lusky, M., M. Christ, K. Rittner, A. Dieterle, D. Dreyer, B. Mourot, H. Schultz, F. Stoeckel, A. Pavirani, and M. Mehtali, 1998, In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted: J Virol, v. 72, p. 2022-32.

López-Botet, M., M. Llano, F. Navarro, and T. Bellón, 2000, NK cell recognition of non-classical HLA class I molecules: Semin Immunol, v. 12, p. 109-19.

Maass, G., C. Bogedain, U. Scheer, D. Michl, M. Hörer, M. Braun-Falco, M. Volkenandt, D. Schadendorf, C. M. Wendtner, E. L. Winnacker, R. M. Kotin, and M. Hallek, 1998, Recombinant adeno-associated virus for the generation of autologous, gene-modified tumor vaccines: evidence for a high transduction efficiency into primary epithelial cancer cells: Hum Gene Ther, v. 9, p. 1049-59.

Mackensen, A., N. Meidenbauer, S. Vogl, M. Laumer, J. Berger, and R. Andreesen, 2006, Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma: J Clin Oncol, v. 24, p. 5060-9.

Madan, R. A., M. Bilusic, C. Heery, J. Schlom, and J. L. Gulley, 2012, Clinical evaluation of TRICOM vector therapeutic cancer vaccines: Semin Oncol, v. 39, p. 296-304.

Madjd, Z., I. Spendlove, S. E. Pinder, I. O. Ellis, and L. G. Durrant, 2005, Total loss of MHC class I is an independent indicator of good prognosis in breast cancer: Int J Cancer, v. 117, p. 248-55.

Maecker, B., M. S. von Bergwelt-Baildon, D. H. Sherr, L. M. Nadler, and J. L. Schultze, 2005, Identification of a new HLA-A*0201-restricted cryptic epitope from CYP1B1: Int J Cancer, v. 115, p. 333-6.

Maeda, H., R. Hirata, A. Thompson, and R. Mukai, 1986, Molecular characterization of three HLA class II molecules on DR4 and DRw9 haplotypes: serologic and structural relationships at the polypeptides level: Hum Immunol, v. 15, p. 1-15.

Mahadevan, M., Y. Liu, C. You, R. Luo, H. You, J. L. Mehta, and P. L. Hermonat, 2007, Generation of robust cytotoxic T lymphocytes against prostate specific antigen by transduction of dendritic cells using protein and recombinant adeno-associated virus: Cancer Immunol Immunother, v. 56, p. 1615-24.

Maleckar, J. R., and L. A. Sherman, 1987, The composition of the T cell receptor repertoire in nude mice: J Immunol, v. 138, p. 3873-6.

Maleno, I., N. Aptsiauri, T. Cabrera, A. Gallego, A. Paschen, M. A. López-Nevot, and F. Garrido, 2011, Frequent loss of heterozygosity in the β2-microglobulin region of chromosome 15 in primary human tumors: Immunogenetics, v. 63, p. 65-71.

Maleno, I., C. M. Cabrera, T. Cabrera, L. Paco, M. A. López-Nevot, A. Collado, A. Ferrón, and F. Garrido, 2004, Distribution of HLA class I altered phenotypes in colorectal carcinomas: high frequency of HLA haplotype loss associated with loss of heterozygosity in chromosome region 6p21: Immunogenetics, v. 56, p. 244-53.

Maleno, I., M. A. López-Nevot, T. Cabrera, J. Salinero, and F. Garrido, 2002, Multiple mechanisms generate HLA class I altered phenotypes in laryngeal carcinomas: high frequency of HLA haplotype loss associated with loss of heterozygosity in chromosome region 6p21: Cancer Immunol Immunother, v. 51, p. 389-96.

Maleno, I., J. M. Romero, T. Cabrera, L. Paco, N. Aptsiauri, J. M. Cozar, M. Tallada, M. A. López-Nevot, and F. Garrido, 2006, LOH at 6p21.3 region and HLA class I altered phenotypes in bladder carcinomas: Immunogenetics, v. 58, p. 503-10.

Malmström, P. U., A. S. Loskog, C. A. Lindqvist, S. M. Mangsbo, M. Fransson, A. Wanders, T. Gårdmark, and T. H. Tötterman, 2010, AdCD40L immunogene therapy for bladder carcinoma--the first phase I/IIa trial: Clin Cancer Res, v. 16, p. 3279-87.

Mantovani, A., T. Schioppa, C. Porta, P. Allavena, and A. Sica, 2006, Role of tumor-associated macrophages in tumor progression and invasion: Cancer Metastasis Rev, v. 25, p. 315-22.

Marchand, M., P. Weynants, E. Rankin, F. Arienti, F. Belli, G. Parmiani, N. Cascinelli, A. Bourlond, R. Vanwijck, and Y. Humblet, 1995, Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3: Int J Cancer, v. 63, p. 883-5.

Margolin, K., 2012, Ipilimumab in a Phase II trial of melanoma patients with brain metastases: Oncoimmunology, v. 1, p. 1197-1199.

Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone, 2000, Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance: Adv Immunol, v. 74, p. 181-273.

Martayan, A., L. Sibilio, E. Tremante, E. Lo Monaco, A. Mulder, D. Fruci, A. Cova, L. Rivoltini, and P. Giacomini, 2009, Class I HLA folding and antigen presentation in beta 2-microglobulin-defective Daudi cells: J Immunol, v. 182, p. 3609-17.

Martini, M., M. G. Testi, M. Pasetto, M. C. Picchio, G. Innamorati, M. Mazzocco, S. Ugel, S. Cingarlini, V. Bronte, P. Zanovello, M. Krampera, F. Mosna, T. Cestari, A. P. Riviera, N. Brutti, O. Barbieri, L. Matera, G. Tridente, M. Colombatti, and S. Sartoris, 2010, IFN-gamma-mediated upmodulation of MHC class I expression activates tumor-specific immune response in a mouse model of prostate cancer: Vaccine, v. 28, p. 3548-57.

Masuda, K., A. Hiraki, N. Fujii, T. Watanabe, M. Tanaka, K. Matsue, Y. Ogama, M. Ouchida, K. Shimizu, K. Ikeda, and M. Tanimoto, 2007, Loss or down-regulation of HLA class I expression at the allelic level in freshly isolated leukemic blasts: Cancer Sci, v. 98, p. 102-8.

Matsushita, T., S. Elliger, C. Elliger, G. Podsakoff, L. Villarreal, G. J. Kurtzman, Y. Iwaki, and P. Colosi, 1998, Adeno-associated virus vectors can be efficiently produced without helper virus: Gene Ther, v. 5, p. 938-45.

Mayor, S., R. G. Parton, and J. G. Donaldson, 2014, Clathrin-independent pathways of endocytosis: Cold Spring Harb Perspect Biol, v. 6.

Mazzolini, G., C. Alfaro, B. Sangro, E. Feijoó, J. Ruiz, A. Benito, I. Tirapu, A. Arina, J. Sola, M. Herraiz, F. Lucena, C. Olagüe, J. Subtil, J. Quiroga, I. Herrero, B. Sádaba, M. Bendandi, C. Qian, J. Prieto, and I. Melero, 2005, Intratumoral injection of dendritic cells engineered to secrete interleukin-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas: J Clin Oncol, v. 23, p. 999-1010.

McCarty, D. M., P. E. Monahan, and R. J. Samulski, 2001, Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis: Gene Ther, v. 8, p. 1248-54.

McDonald, D., L. Stockwin, T. Matzow, M. E. Blair Zajdel, and G. E. Blair, 1999, Coxsackie and adenovirus receptor (CAR)-dependent and major histocompatibility complex (MHC) class I-independent uptake of recombinant adenoviruses into human tumour cells: Gene Ther, v. 6, p. 1512-9.

McLaughlin, P., A. J. Grillo-López, B. K. Link, R. Levy, M. S. Czuczman, M. E. Williams, M. R. Heyman, I. Bence-Bruckler, C. A. White, F. Cabanillas, V. Jain, A. D. Ho, J. Lister, K. Wey, D. Shen, and B. K. Dallaire, 1998, Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program: J Clin Oncol, v. 16, p. 2825-33.

Meissner, M., T. E. Reichert, M. Kunkel, W. Gooding, T. L. Whiteside, S. Ferrone, and B. Seliger, 2005, Defects in the human leukocyte antigen class I antigen processing machinery in

head and neck squamous cell carcinoma: association with clinical outcome: Clin Cancer Res, v. 11, p. 2552-60.

Menon, A. G., H. Morreau, R. A. Tollenaar, E. Alphenaar, M. Van Puijenbroek, H. Putter, C. M. Janssen-Van Rhijn, C. J. Van De Velde, G. J. Fleuren, and P. J. Kuppen, 2002, Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients: Lab Invest, v. 82, p. 1725-33.

Mingozzi, F., and K. A. High, 2011, Immune responses to AAV in clinical trials: Curr Gene Ther, v. 11, p. 321-30.

Mink, S. R., A. Hodge, D. B. Agus, A. Jain, and M. E. Gross, 2010, Beta-2-microglobulin expression correlates with high-grade prostate cancer and specific defects in androgen signaling: Prostate, v. 70, p. 1201-10.

Miyagi, T., T. Tatsumi, T. Takehara, T. Kanto, N. Kuzushita, Y. Sugimoto, M. Jinushi, A. Kasahara, Y. Sasaki, M. Hori, and N. Hayashi, 2003, Impaired expression of proteasome subunits and human leukocyte antigens class I in human colon cancer cells: J Gastroenterol Hepatol, v. 18, p. 32-40.

Morabito, A., B. Dozin, S. Salvi, G. Pasciucco, G. Balbi, S. Laurent, S. Pastorino, F. Carli, M. Truini, P. Bruzzi, L. Del Mastro, and M. P. Pistillo, 2009, Analysis and clinical relevance of human leukocyte antigen class I, heavy chain, and beta2-microglobulin downregulation in breast cancer: Hum Immunol, v. 70, p. 492-5.

Moretta, A., C. Bottino, M. Vitale, D. Pende, R. Biassoni, M. C. Mingari, and L. Moretta, 1996, Receptors for HLA class-I molecules in human natural killer cells: Annu Rev Immunol, v. 14, p. 619-48.

Moretti, S., C. Pinzi, E. Berti, A. Spallanzani, A. Chiarugi, V. Boddi, U. M. Reali, and B. Giannotti, 1997, In situ expression of transforming growth factor beta is associated with melanoma progression and correlates with Ki67, HLA-DR and beta 3 integrin expression: Melanoma Res, v. 7, p. 313-21.

Morgan, R. A., M. E. Dudley, J. R. Wunderlich, M. S. Hughes, J. C. Yang, R. M. Sherry, R. E. Royal, S. L. Topalian, U. S. Kammula, N. P. Restifo, Z. Zheng, A. Nahvi, C. R. de Vries, L. J. Rogers-Freezer, S. A. Mavroukakis, and S. A. Rosenberg, 2006, Cancer regression in patients after transfer of genetically engineered lymphocytes: Science, v. 314, p. 126-9.

Morrice, N. A., and S. J. Powis, 1998, A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules: Curr Biol, v. 8, p. 713-6.

Mueller, C., and T. R. Flotte, 2008, Clinical gene therapy using recombinant adeno-associated virus vectors: Gene Ther, v. 15, p. 858-63.

Mumberg, D., M. Wick, and H. Schreiber, 1996, Unique tumor antigens redefined as mutant tumor-specific antigens: Semin Immunol, v. 8, p. 289-93.

Méndez, R., T. Rodríguez, A. Del Campo, E. Monge, I. Maleno, N. Aptsiauri, P. Jiménez, S. Pedrinaci, G. Pawelec, F. Ruiz-Cabello, and F. Garrido, 2008, Characterization of HLA class I altered phenotypes in a panel of human melanoma cell lines: Cancer Immunol Immunother, v. 57, p. 719-29.

Nakai, H., T. A. Storm, and M. A. Kay, 2000, Increasing the size of rAAV-mediated expression cassettes in vivo by intermolecular joining of two complementary vectors: Nat Biotechnol, v. 18, p. 527-32.

Nanda, N. K., L. Birch, N. M. Greenberg, and G. S. Prins, 2006, MHC class I and class II molecules are expressed in both human and mouse prostate tumor microenvironment: Prostate, v. 66, p. 1275-84.

Nathwani, A. C., J. T. Gray, C. Y. Ng, J. Zhou, Y. Spence, S. N. Waddington, E. G. Tuddenham, G. Kemball-Cook, J. McIntosh, M. Boon-Spijker, K. Mertens, and A. M. Davidoff, 2006, Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver: Blood, v. 107, p. 2653-61.

Naumer, M., F. Sonntag, K. Schmidt, K. Nieto, C. Panke, N. E. Davey, R. Popa-Wagner, and J. A. Kleinschmidt, 2012, Properties of the adeno-associated virus assembly-activating protein: J Virol, v. 86, p. 13038-48.

Nayak, S., and R. W. Herzog, 2010, Progress and prospects: immune responses to viral vectors: Gene Ther, v. 17, p. 295-304.

Neefjes, J. J., F. Momburg, and G. J. Hämmerling, 1993, Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter: Science, v. 261, p. 769-71.

Neerincx, A., G. M. Rodriguez, V. Steimle, and T. A. Kufer, 2012, NLRC5 controls basal MHC class I gene expression in an MHC enhanceosome-dependent manner: J Immunol, v. 188, p. 4940-50.

Nemunaitis, J., T. Fong, F. Burrows, J. Bruce, G. Peters, N. Ognoskie, W. Meyer, D. Wynne, R. Kerr, J. Pippen, F. Oldham, and D. Ando, 1999a, Phase I trial of interferon gamma retroviral vector administered intratumorally with multiple courses in patients with metastatic melanoma: Hum Gene Ther, v. 10, p. 1289-98.

Nemunaitis, J., T. Fong, J. M. Robbins, G. Edelman, W. Edwards, R. S. Paulson, J. Bruce, N. Ognoskie, D. Wynne, M. Pike, K. Kowal, J. Merritt, and D. Ando, 1999b, Phase I trial of interferon-gamma (IFN-gamma) retroviral vector administered intratumorally to patients with metastatic melanoma: Cancer Gene Ther, v. 6, p. 322-30.

Nemunaitis, J., F. Khuri, I. Ganly, J. Arseneau, M. Posner, E. Vokes, J. Kuhn, T. McCarty, S. Landers, A. Blackburn, L. Romel, B. Randlev, S. Kaye, and D. Kirn, 2001, Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer: J Clin Oncol, v. 19, p. 289-98.

Nieto, K., A. Kern, B. Leuchs, L. Gissmann, M. Müller, and J. A. Kleinschmidt, 2009, Combined prophylactic and therapeutic intranasal vaccination against human papillomavirus type-16 using different adeno-associated virus serotype vectors: Antivir Ther, v. 14, p. 1125-37.

Nonnenmacher, M., and T. Weber, 2012, Intracellular transport of recombinant adeno-associated virus vectors: Gene Ther, v. 19, p. 649-58.

Norell, H., M. Carlsten, T. Ohlum, K. J. Malmberg, G. Masucci, K. Schedvins, W. Altermann, D. Handke, D. Atkins, B. Seliger, and R. Kiessling, 2006, Frequent loss of HLA-A2 expression in metastasizing ovarian carcinomas associated with genomic haplotype loss and HLA-A2-restricted HER-2/neu-specific immunity: Cancer Res, v. 66, p. 6387-94.

Opelz, G., and B. Döhler, 2004, Lymphomas after solid organ transplantation: a collaborative transplant study report: Am J Transplant, v. 4, p. 222-30.

Ostrander, E. A., and J. L. Stanford, 2000, Genetics of prostate cancer: too many loci, too few genes: Am J Hum Genet, v. 67, p. 1367-75.

Paco, L., A. M. Garcia-Lora, C. Casares, C. Cabrera, I. Algarra, A. Collado, I. Maleno, F. Garrido, and M. A. Lopez-Nevot, 2007, Total loss of HLA class I expression on a melanoma cell line after growth in nude mice in absence of autologous antitumor immune response: Int J Cancer, v. 121, p. 2023-30.

Pan, J., Q. Zhang, J. Zhou, D. Ma, X. Xiao, and D. W. Wang, 2009, Recombinant adeno-associated virus encoding Epstein-Barr virus latent membrane proteins fused with heat shock protein as a potential vaccine for nasopharyngeal carcinoma: Mol Cancer Ther, v. 8, p. 2754-61.

Pan, J. G., X. Zhou, R. Luo, and R. F. Han, 2012, The adeno-associated virus-mediated HSV-TK/GCV suicide system: a potential strategy for the treatment of bladder carcinoma: Med Oncol, v. 29, p. 1938-47.

Pardoll, D., 2003, Does the immune system see tumors as foreign or self?: Annu Rev Immunol, v. 21, p. 807-39.

Pardoll, D. M., 2012, The blockade of immune checkpoints in cancer immunotherapy: Nat Rev Cancer, v. 12, p. 252-64.

Park, T. S., S. A. Rosenberg, and R. A. Morgan, 2011, Treating cancer with genetically engineered T cells: Trends Biotechnol, v. 29, p. 550-7.

Paschen, A., N. Arens, A. Sucker, K. M. Greulich-Bode, E. Fonsatti, A. Gloghini, S. Striegel, N. Schwinn, A. Carbone, R. Hildenbrand, A. Cerwenka, M. Maio, and D. Schadendorf, 2006, The coincidence of chromosome 15 aberrations and beta2-microglobulin gene mutations is causative for the total loss of human leukocyte antigen class I expression in melanoma: Clin Cancer Res, v. 12, p. 3297-305.

Paschen, A., R. M. Méndez, P. Jimenez, A. Sucker, F. Ruiz-Cabello, M. Song, F. Garrido, and D. Schadendorf, 2003, Complete loss of HLA class I antigen expression on melanoma cells: a result of successive mutational events: Int J Cancer, v. 103, p. 759-67.

Paul, S., D. Weiskopf, M. A. Angelo, J. Sidney, B. Peters, and A. Sette, 2013, HLA class I alleles are associated with peptide-binding repertoires of different size, affinity, and immunogenicity: J Immunol, v. 191, p. 5831-9.

Pawelec, G., and S. G. Marsh, 2006, ESTDAB: a collection of immunologically characterised melanoma cell lines and searchable databank: Cancer Immunol Immunother, v. 55, p. 623-7.

Persano, L., M. Crescenzi, and S. Indraccolo, 2007, Anti-angiogenic gene therapy of cancer: current status and future prospects: Mol Aspects Med, v. 28, p. 87-114.

Pesonen, S., I. Diaconu, V. Cerullo, S. Escutenaire, M. Raki, L. Kangasniemi, P. Nokisalmi, G. Dotti, K. Guse, L. Laasonen, K. Partanen, E. Karli, E. Haavisto, M. Oksanen, A. Karioja-Kallio, P. Hannuksela, S. L. Holm, S. Kauppinen, T. Joensuu, A. Kanerva, and A. Hemminki, 2012, Integrin targeted oncolytic adenoviruses Ad5-D24-RGD and Ad5-RGD-D24-GMCSF for treatment of patients with advanced chemotherapy refractory solid tumors: Int J Cancer, v. 130, p. 1937-47.

Pezaro, C., A. Omlin, D. Lorente, and J. de Bono, 2013, Management of patients with castration-resistant disease: Hematol Oncol Clin North Am, v. 27, p. 1243-60, ix.

Pfeifer, A., and I. M. Verma, 2001, Gene therapy: promises and problems: Annu Rev Genomics Hum Genet, v. 2, p. 177-211.

Ploegh, H. L., 1998, Viral strategies of immune evasion: Science, v. 280, p. 248-53.

Ploegh, H. L., L. E. Cannon, and J. L. Strominger, 1979, Cell-free translation of the mRNAs for the heavy and light chains of HLA-A and HLA-B antigens: Proc Natl Acad Sci U S A, v. 76, p. 2273-7.

Powell, A. G., P. G. Horgan, and J. Edwards, 2012, The bodies fight against cancer: is human leucocyte antigen (HLA) class 1 the key?: J Cancer Res Clin Oncol, v. 138, p. 723-8.

Pérez, B., R. Benitez, M. A. Fernández, M. R. Oliva, J. L. Soto, S. Serrano, M. A. López Nevot, and F. Garrido, 1999, A new beta 2 microglobulin mutation found in a melanoma tumor cell line: Tissue Antigens, v. 53, p. 569-72.

Qaseem, A., M. J. Barry, T. D. Denberg, D. K. Owens, P. Shekelle, and C. G. C. o. t. A. C. o. Physicians, 2013, Screening for prostate cancer: a guidance statement from the Clinical Guidelines Committee of the American College of Physicians: Ann Intern Med, v. 158, p. 761-9.

Qing, K., C. Mah, J. Hansen, S. Zhou, V. Dwarki, and A. Srivastava, 1999, Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2: Nat Med, v. 5, p. 71-7.

Quoix, E., R. Ramlau, V. Westeel, Z. Papai, A. Madroszyk, A. Riviere, P. Koralewski, J. L. Breton, E. Stoelben, D. Braun, D. Debieuvre, H. Lena, M. Buyse, M. P. Chenard, B. Acres, G. Lacoste, B. Bastien, A. Tavernaro, N. Bizouarne, J. Y. Bonnefoy, and J. M. Limacher, 2011, Therapeutic vaccination with TG4010 and first-line chemotherapy in advanced non-small-cell lung cancer: a controlled phase 2B trial: Lancet Oncol, v. 12, p. 1125-33.

Rabinowitz, J. E., F. Rolling, C. Li, H. Conrath, W. Xiao, X. Xiao, and R. J. Samulski, 2002, Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity: J Virol, v. 76, p. 791-801.

Ramnath, N., D. Tan, Q. Li, B. L. Hylander, P. Bogner, L. Ryes, and S. Ferrone, 2006, Is downregulation of MHC class I antigen expression in human non-small cell lung cancer associated with prolonged survival?: Cancer Immunol Immunother, v. 55, p. 891-9.

Rehm, A., A. Rohr, C. Seitz, K. Wonigeit, A. Ziegler, and B. Uchanska-Ziegler, 2000, Structurally diverse forms of HLA-B27 molecules are displayed in vivo in a cell type-dependent manner: Hum Immunol, v. 61, p. 408-18.

Rein, R. S., G. H. Seemann, J. J. Neefjes, F. M. Hochstenbach, N. J. Stam, and H. L. Ploegh, 1987, Association with beta 2-microglobulin controls the expression of transfected human class I genes: J Immunol, v. 138, p. 1178-83.

Respa, A., J. Bukur, S. Ferrone, G. Pawelec, Y. Zhao, E. Wang, F. M. Marincola, and B. Seliger, 2011, Association of IFN-gamma signal transduction defects with impaired HLA class I antigen processing in melanoma cell lines: Clin Cancer Res, v. 17, p. 2668-78.

Restifo, N. P., P. A. Antony, S. E. Finkelstein, W. W. Leitner, D. P. Surman, M. R. Theoret, and C. E. Touloukian, 2002, Assumptions of the tumor 'escape' hypothesis: Semin Cancer Biol, v. 12, p. 81-6.

Restifo, N. P., F. M. Marincola, Y. Kawakami, J. Taubenberger, J. R. Yannelli, and S. A. Rosenberg, 1996, Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy: J Natl Cancer Inst, v. 88, p. 100-8.

Rodriguez, T., R. Méndez, C. H. Roberts, F. Ruiz-Cabello, I. A. Dodi, M. A. López Nevot, L. Paco, I. Maleno, S. G. Marsh, G. Pawelec, and F. Garrido, 2005, High frequency of homozygosity of the HLA region in melanoma cell lines reveals a pattern compatible with extensive loss of heterozygosity: Cancer Immunol Immunother, v. 54, p. 141-8.

Rodríguez, T., R. Méndez, A. Del Campo, N. Aptsiauri, J. Martín, G. Orozco, G. Pawelec, D. Schadendorf, F. Ruiz-Cabello, and F. Garrido, 2007a, Patterns of constitutive and IFN-gamma inducible expression of HLA class II molecules in human melanoma cell lines: Immunogenetics, v. 59, p. 123-33.

Rodríguez, T., R. Méndez, A. Del Campo, P. Jiménez, N. Aptsiauri, F. Garrido, and F. Ruiz-Cabello, 2007b, Distinct mechanisms of loss of IFN-gamma mediated HLA class I inducibility in two melanoma cell lines: BMC Cancer, v. 7, p. 34.

Romero, J. M., P. Jimenez, T. Cabrera, J. M. Cozar, S. Pedrinaci, M. Tallada, F. Garrido, and F. Ruiz-Cabello, 2005, Coordinated downregulation of the antigen presentation machinery and HLA class I/beta 2-microglobulin complex is responsible for HLA-ABC loss in bladder cancer: International Journal of Cancer, v. 113, p. 605-610.

Rosenberg, S. A., 2011, Cell transfer immunotherapy for metastatic solid cancer--what clinicians need to know: Nat Rev Clin Oncol, v. 8, p. 577-85.

Rosenberg, S. A., and N. P. Restifo, 2015, Adoptive cell transfer as personalized immunotherapy for human cancer: Science, v. 348, p. 62-8.

Rosenberg, S. A., J. C. Yang, and N. P. Restifo, 2004, Cancer immunotherapy: moving beyond current vaccines: Nat Med, v. 10, p. 909-15.

Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward, 1953, Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture: Proc Soc Exp Biol Med, v. 84, p. 570-3.

Russell, J. S., F. F. Lang, T. Huet, M. Janicot, S. Chada, D. R. Wilson, and P. J. Tofilon, 1999, Radiosensitization of human tumor cell lines induced by the adenovirus-mediated expression of an anti-Ras single-chain antibody fragment: Cancer Res, v. 59, p. 5239-44.

Russo, C., A. K. Ng, M. A. Pellegrino, and S. Ferrone, 1983, The monoclonal antibody CR11-351 discriminates HLA-A2 variants identified by T cells: Immunogenetics, v. 18, p. 23-35.

Régulier, E., S. Paul, M. Marigliano, J. Kintz, Y. Poitevin, C. Ledoux, D. Roecklin, G. Cauet, V. Calenda, and H. E. Homann, 2001, Adenovirus-mediated delivery of antiangiogenic genes as an antitumor approach: Cancer Gene Ther, v. 8, p. 45-54.

Sadasivan, B., P. J. Lehner, B. Ortmann, T. Spies, and P. Cresswell, 1996, Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP: Immunity, v. 5, p. 103-14.

Sadasivan, B. K., A. Cariappa, G. L. Waneck, and P. Cresswell, 1995, Assembly, peptide loading, and transport of MHC class I molecules in a calnexin-negative cell line: Cold Spring Harb Symp Quant Biol, v. 60, p. 267-75.

Sadelain, M., I. Rivière, and R. Brentjens, 2003, Targeting tumours with genetically enhanced T lymphocytes: Nat Rev Cancer, v. 3, p. 35-45.

Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi, 2001, Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance: Immunol Rev, v. 182, p. 18-32.

Salavoura, K., A. Kolialexi, G. Tsangaris, and A. Mavrou, 2008, Development of cancer in patients with primary immunodeficiencies: Anticancer Res, v. 28, p. 1263-9.

Sanda, M. G., N. P. Restifo, J. C. Walsh, Y. Kawakami, W. G. Nelson, D. M. Pardoll, and J. W. Simons, 1995, Molecular characterization of defective antigen processing in human prostate cancer: Journal of the National Cancer Institute, v. 87, p. 280-285.

Sanford, B. H., H. I. Kohn, J. J. Daly, and S. F. Soo, 1973, Long-term spontaneous tumor incidence in neonatally thymectomized mice: J Immunol, v. 110, p. 1437-9.

Schiedner, G., N. Morral, R. J. Parks, Y. Wu, S. C. Koopmans, C. Langston, F. L. Graham, A. L. Beaudet, and S. Kochanek, 1998, Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity: Nat Genet, v. 18, p. 180-3.

Schlom, J., 2012, Recent advances in therapeutic cancer vaccines: Cancer Biother Radiopharm, v. 27, p. 2-5.

Schreiber, R. D., L. J. Old, and M. J. Smyth, 2011, Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion: Science, v. 331, p. 1565-70.

Schultze, J., and L. Nadler, 2003, Mechanisms of tumor escape mechanism: implications for immunotherapy.: London, Taylor & Francis.

Schwartzentruber, D. J., D. H. Lawson, J. M. Richards, R. M. Conry, D. M. Miller, J. Treisman, F. Gailani, L. Riley, K. Conlon, B. Pockaj, K. L. Kendra, R. L. White, R. Gonzalez, T. M. Kuzel, B. Curti, P. D. Leming, E. D. Whitman, J. Balkissoon, D. S. Reintgen, H. Kaufman, F. M. Marincola, M. J. Merino, S. A. Rosenberg, P. Choyke, D. Vena, and P. Hwu, 2011, gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma: N Engl J Med, v. 364, p. 2119-27.

Sconocchia, G., S. Eppenberger-Castori, I. Zlobec, E. Karamitopoulou, R. Arriga, A. Coppola, S. Caratelli, G. C. Spagnoli, D. Lauro, A. Lugli, J. Han, G. Iezzi, C. Ferrone, A. Ferlosio, L. Tornillo, R. Droeser, P. Rossi, A. Attanasio, S. Ferrone, and L. Terracciano, 2014, HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker: Neoplasia, v. 16, p. 31-42.

Seliger, B., T. Cabrera, F. Garrido, and S. Ferrone, 2002, HLA class I antigen abnormalities and immune escape by malignant cells: Semin Cancer Biol, v. 12, p. 3-13.

Seliger, B., M. J. Maeurer, and S. Ferrone, 2000a, Antigen-processing machinery breakdown and tumor growth: Immunol Today, v. 21, p. 455-64.

Seliger, B., U. Ritz, R. Abele, M. Bock, R. Tampé, G. Sutter, I. Drexler, C. Huber, and S. Ferrone, 2001, Immune escape of melanoma: first evidence of structural alterations in two distinct components of the MHC class I antigen processing pathway: Cancer Res, v. 61, p. 8647-50.

Seliger, B., F. Ruiz-Cabello, and F. Garrido, 2008, IFN inducibility of major histocompatibility antigens in tumors: Adv Cancer Res, v. 101, p. 249-76.

Seliger, B., R. Stoehr, D. Handke, A. Mueller, S. Ferrone, B. Wullich, A. Tannapfel, F. Hofstaedter, and A. Hartmann, 2010, Association of HLA class I antigen abnormalities

with disease progression and early recurrence in prostate cancer: Cancer Immunology Immunotherapy, v. 59, p. 529-540.
Seliger, B., U. Wollscheid, F. Momburg, T. Blankenstein, and C. Huber, 2000b, Coordinate downregulation of multiple MHC class I antigen processing genes in chemical-induced murine tumor cell lines of distinct origin: Tissue Antigens, v. 56, p. 327-36.
Senzer, N., and J. Nemunaitis, 2009, A review of contusugene ladenovec (Advexin) p53 therapy: Curr Opin Mol Ther, v. 11, p. 54-61.
Seong, R. H., C. A. Clayberger, A. M. Krensky, and J. R. Parnes, 1988, Rescue of Daudi cell HLA expression by transfection of the mouse beta 2-microglobulin gene: J Exp Med, v. 167, p. 288-99.
Serrano, A., C. S. Brady, P. Jimenez, M. F. Duggan-Keen, R. Mendez, P. Stern, F. Garrido, and F. Ruiz-Cabello, 2000, A mutation determining the loss of HLA-A2 antigen expression in a cervical carcinoma reveals novel splicing of human MHC class I classical transcripts in both tumoral and normal cells: Immunogenetics, v. 51, p. 1047-52.
Serrano, A., S. Tanzarella, I. Lionello, R. Mendez, C. Traversari, F. Ruiz-Cabello, and F. Garrido, 2001, Rexpression of HLA class I antigens and restoration of antigen-specific CTL response in melanoma cells following 5-aza-2'-deoxycytidine treatment: Int J Cancer, v. 94, p. 243-51.
Seth, P., 2000, Adenoviral vectors: Adv Exp Med Biol, v. 465, p. 13-22.
Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber, 2001, IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity: Nature, v. 410, p. 1107-11.
Shawar, S. M., J. M. Vyas, J. R. Rodgers, and R. R. Rich, 1994, Antigen presentation by major histocompatibility complex class I-B molecules: Annu Rev Immunol, v. 12, p. 839-80.
Shenk, T. E., 1996, Adenoviridae, the viruses and their replication, *in* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. S.E., eds., Fields Virology: Lippencott, Philadelphia, PA., Raven Publishers.
Shiels, M. S., S. R. Cole, G. D. Kirk, and C. Poole, 2009, A meta-analysis of the incidence of non-AIDS cancers in HIV-infected individuals: J Acquir Immune Defic Syndr, v. 52, p. 611-22.
Shin, E. C., J. M. Ahn, C. H. Kim, Y. Choi, Y. S. Ahn, H. Kim, S. J. Kim, and J. H. Park, 2001, IFN-gamma induces cell death in human hepatoma cells through a TRAIL/death receptor-mediated apoptotic pathway: Int J Cancer, v. 93, p. 262-8.
Singh, A. A., L. W. Jones, J. A. Antonelli, L. Gerber, E. E. Calloway, K. H. Shuler, S. J. Freedland, D. J. Grant, C. Hoyo, and L. L. Bañez, 2013, Association between exercise and primary incidence of prostate cancer: does race matter?: Cancer, v. 119, p. 1338-43.
Small, E. J., N. Sacks, J. Nemunaitis, W. J. Urba, E. Dula, A. S. Centeno, W. G. Nelson, D. Ando, C. Howard, F. Borellini, M. Nguyen, K. Hege, and J. W. Simons, 2007, Granulocyte macrophage colony-stimulating factor--secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer: Clin Cancer Res, v. 13, p. 3883-91.
Smyth, M. J., G. P. Dunn, and R. D. Schreiber, 2006, Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity: Adv Immunol, v. 90, p. 1-50.
Sonntag, F., S. Bleker, B. Leuchs, R. Fischer, and J. A. Kleinschmidt, 2006, Adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus: J Virol, v. 80, p. 11040-54.
Speetjens, F. M., E. C. de Bruin, H. Morreau, E. C. Zeestraten, H. Putter, J. H. van Krieken, M. M. van Buren, M. van Velzen, N. G. Dekker-Ensink, C. J. van de Velde, and P. J. Kuppen, 2008, Clinical impact of HLA class I expression in rectal cancer: Cancer Immunol Immunother, v. 57, p. 601-9.
Stam, N. J., T. M. Vroom, P. J. Peters, E. B. Pastoors, and H. L. Ploegh, 1990, HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in western blots, in

formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy: Int Immunol, v. 2, p. 113-25.
Stern, L. J., and D. C. Wiley, 1994, Antigenic peptide binding by class I and class II histocompatibility proteins: Structure, v. 2, p. 245-51.
Stewart, A. K., N. J. Lassam, I. C. Quirt, D. J. Bailey, L. E. Rotstein, M. Krajden, S. Dessureault, S. Gallinger, D. Cappe, Y. Wan, C. L. Addison, R. C. Moen, J. Gauldie, and F. L. Graham, 1999, Adenovector-mediated gene delivery of interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial: Gene Ther, v. 6, p. 350-63.
Stone, D., 2010, Novel viral vector systems for gene therapy: Viruses, v. 2, p. 1002-7.
Stone, D., and A. Lieber, 2006, New serotypes of adenoviral vectors: Curr Opin Mol Ther, v. 8, p. 423-31.
Stutman, O., 1974, Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice: Science, v. 183, p. 534-6.
Summerford, C., J. S. Bartlett, and R. J. Samulski, 1999, AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection: Nat Med, v. 5, p. 78-82.
Summerford, C., and R. J. Samulski, 1998, Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions: J Virol, v. 72, p. 1438-45.
Sun, H., Y. Liu, D. Bu, X. Liu, J. S. Norris, and S. Xiao, 2012, Efficient growth suppression and apoptosis in human laryngeal carcinoma cell line HEP-2 induced by an adeno-associated virus expressing human FAS ligand: Head Neck, v. 34, p. 1628-33.
Sáenz-López, P., C. Gouttefangeas, J. Hennenlotter, A. Concha, I. Maleno, F. Ruiz-Cabello, J. M. Cózar, M. Tallada, A. Stenzl, H. G. Rammensee, F. Garrido, and T. Cabrera, 2010, Higher HLA class I expression in renal cell carcinoma than in autologous normal tissue: Tissue Antigens, v. 75, p. 110-8.
Takahashi, H., F. Feuerhake, J. L. Kutok, S. Monti, P. Dal Cin, D. Neuberg, J. C. Aster, and M. A. Shipp, 2006, FAS death domain deletions and cellular FADD-like interleukin 1beta converting enzyme inhibitory protein (long) overexpression: alternative mechanisms for deregulating the extrinsic apoptotic pathway in diffuse large B-cell lymphoma subtypes: Clin Cancer Res, v. 12, p. 3265-71.
Takayama, K., H. Ueno, Y. Nakanishi, T. Sakamoto, K. Inoue, K. Shimizu, H. Oohashi, and N. Hara, 2000, Suppression of tumor angiogenesis and growth by gene transfer of a soluble form of vascular endothelial growth factor receptor into a remote organ: Cancer Res, v. 60, p. 2169-77.
Takeda, K., M. J. Smyth, E. Cretney, Y. Hayakawa, N. Kayagaki, H. Yagita, and K. Okumura, 2002, Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development: J Exp Med, v. 195, p. 161-9.
Tanaka, K., K. J. Isselbacher, G. Khoury, and G. Jay, 1985, Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene: Science, v. 228, p. 26-30.
Tartaglia, J., M. C. Bonnet, N. Berinstein, B. Barber, M. Klein, and P. Moingeon, 2001, Therapeutic vaccines against melanoma and colorectal cancer: Vaccine, v. 19, p. 2571-5.
Tazawa, H., S. Kagawa, and T. Fujiwara, 2013, Advances in adenovirus-mediated p53 cancer gene therapy: Expert Opin Biol Ther, v. 13, p. 1569-83.
Teng, M. W., J. B. Swann, C. M. Koebel, R. D. Schreiber, and M. J. Smyth, 2008, Immune-mediated dormancy: an equilibrium with cancer: J Leukoc Biol, v. 84, p. 988-93.
Terabe, M., and J. A. Berzofsky, 2004, Immunoregulatory T cells in tumor immunity: Curr Opin Immunol, v. 16, p. 157-62.
Teschendorf, C., B. Emons, N. Muzyczka, U. Graeven, and W. Schmiegel, 2010, Efficacy of recombinant adeno-associated viral vectors serotypes 1, 2, and 5 for the transduction of pancreatic and colon carcinoma cells: Anticancer Res, v. 30, p. 1931-5.
Thomas, L., 1959, Discussion, *in* H. S. Lawrence, ed., Cellular and Humoral Aspects of the Hypersensitive States: New York, Hoeber-Harper, p. 529-533.

Thompson, I., J. B. Thrasher, G. Aus, A. L. Burnett, E. D. Canby-Hagino, M. S. Cookson, A. V. D'Amico, R. R. Dmochowski, D. T. Eton, J. D. Forman, S. L. Goldenberg, J. Hernandez, C. S. Higano, S. R. Kraus, J. W. Moul, C. M. Tangen, and A. P. C. C. G. U. Panel, 2007, Guideline for the management of clinically localized prostate cancer: 2007 update: J Urol, v. 177, p. 2106-31.

Thompson, J. D., D. Macejak, L. Couture, and D. T. Stinchcomb, 1995, Ribozymes in gene therapy: Nat Med, v. 1, p. 277-8.

Tomihara, K., K. Kato, Y. Masuta, K. Nakamura, H. Uchida, K. Sasaki, T. Tanaka, J. Huang, H. Hiratsuka, and H. Hamada, 2008, Gene transfer of CD40-ligand to dendritic cells stimulates interferon-gamma production to induce growth arrest and apoptosis of tumor cells: Gene Ther, v. 15, p. 203-13.

Torres, M. J., F. Ruiz-Cabello, A. Skoudy, G. Berrozpe, P. Jimenez, A. Serrano, F. X. Real, and F. Garrido, 1996, Loss of an HLA haplotype in pancreas cancer tissue and its corresponding tumor derived cell line: Tissue Antigens, v. 47, p. 372-81.

Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse, 1990, Assembly of MHC class I molecules analyzed in vitro: Cell, v. 62, p. 285-95.

Tripathi, P., and S. Agrawal, 2006, Non-classical HLA-G antigen and its role in the cancer progression: Cancer Invest, v. 24, p. 178-86.

Uyttenhove, C., L. Pilotte, I. Théate, V. Stroobant, D. Colau, N. Parmentier, T. Boon, and B. J. Van den Eynde, 2003, Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase: Nat Med, v. 9, p. 1269-74.

Vacchelli, E., I. Vitale, A. Eggermont, W. H. Fridman, J. Fučíková, I. Cremer, J. Galon, E. Tartour, L. Zitvogel, G. Kroemer, and L. Galluzzi, 2013, Trial watch: Dendritic cell-based interventions for cancer therapy: Oncoimmunology, v. 2, p. e25771.

van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon, 1991, A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma: Science, v. 254, p. 1643-7.

van der Meer, J. W., R. S. Weening, P. T. Schellekens, I. P. van Munster, and F. M. Nagengast, 1993, Colorectal cancer in patients with X-linked agammaglobulinaemia: Lancet, v. 341, p. 1439-40.

Vesely, M. D., M. H. Kershaw, R. D. Schreiber, and M. J. Smyth, 2011, Natural innate and adaptive immunity to cancer: Annu Rev Immunol, v. 29, p. 235-71.

Villablanca, E. J., L. Raccosta, D. Zhou, R. Fontana, D. Maggioni, A. Negro, F. Sanvito, M. Ponzoni, B. Valentinis, M. Bregni, A. Prinetti, K. R. Steffensen, S. Sonnino, J. A. Gustafsson, C. Doglioni, C. Bordignon, C. Traversari, and V. Russo, 2010, Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses: Nat Med, v. 16, p. 98-105.

von Leoprechting, A., P. van der Bruggen, H. L. Pahl, A. Aruffo, and J. C. Simon, 1999, Stimulation of CD40 on immunogenic human malignant melanomas augments their cytotoxic T lymphocyte-mediated lysis and induces apoptosis: Cancer Res, v. 59, p. 1287-94.

Vorburger, S. A., and K. K. Hunt, 2002, Adenoviral gene therapy: Oncologist, v. 7, p. 46-59.

Wadler, S., B. Yu, J. Y. Tan, R. Kaleya, A. Rozenblit, D. Makower, M. Edelman, M. Lane, E. Hyjek, and M. Horwitz, 2003, Persistent replication of the modified chimeric adenovirus ONYX-015 in both tumor and stromal cells from a patient with gall bladder carcinoma implants: Clin Cancer Res, v. 9, p. 33-43.

Wallich, R., N. Bulbuc, G. J. Hämmerling, S. Katzav, S. Segal, and M. Feldman, 1985, Abrogation of metastatic properties of tumour cells by de novo expression of H-2K antigens following H-2 gene transfection: Nature, v. 315, p. 301-5.

Walter, S., T. Weinschenk, C. Reinhardt, and H. Singh-Jasuja, 2013, Single-dose cyclophosphamide synergizes with immune responses to the renal cell cancer vaccine IMA901: Oncoimmunology, v. 2, p. e22246.

Walter, S., T. Weinschenk, A. Stenzl, R. Zdrojowy, A. Pluzanska, C. Szczylik, M. Staehler, W. Brugger, P. Y. Dietrich, R. Mendrzyk, N. Hilf, O. Schoor, J. Fritsche, A. Mahr, D. Maurer, V. Vass, C. Trautwein, P. Lewandrowski, C. Flohr, H. Pohla, J. J. Stanczak, V. Bronte, S. Mandruzzato, T. Biedermann, G. Pawelec, E. Derhovanessian, H. Yamagishi, T. Miki, F. Hongo, N. Takaha, K. Hirakawa, H. Tanaka, S. Stevanovic, J. Frisch, A. Mayer-Mokler, A. Kirner, H. G. Rammensee, C. Reinhardt, and H. Singh-Jasuja, 2012, Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival: Nat Med, v. 18, p. 1254-61.

Wang, E., A. Monaco, V. Monsurró, M. Sabatino, Z. Pos, L. Uccellini, J. Wang, A. Worschech, D. F. Stroncek, and F. M. Marincola, 2009a, Antitumor vaccines, immunotherapy and the immunological constant of rejection: IDrugs, v. 12, p. 297-301.

Wang, L. L., P. Chen, S. Luo, J. Li, K. Liu, H. Z. Hu, and Y. Q. Wei, 2009b, CXC-chemokine-ligand-10 gene therapy efficiently inhibits the growth of cervical carcinoma on the basis of its anti-angiogenic and antiviral activity: Biotechnol Appl Biochem, v. 53, p. 209-16.

Wang, R. F., X. Wang, A. C. Atwood, S. L. Topalian, and S. A. Rosenberg, 1999, Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen: Science, v. 284, p. 1351-4.

Warrington, K. H., and R. W. Herzog, 2006, Treatment of human disease by adeno-associated viral gene transfer: Hum Genet, v. 119, p. 571-603.

Watson, N. F., J. M. Ramage, Z. Madjd, I. Spendlove, I. O. Ellis, J. H. Scholefield, and L. G. Durrant, 2006, Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis: Int J Cancer, v. 118, p. 6-10.

Waye, M. M. Y., and C. W. Sing, 2010, Anti-viral Drugs for Human Adenoviruses: Pharmaceuticals, v. 3.

Wickham, T. J., E. J. Filardo, D. A. Cheresh, and G. R. Nemerow, 1994, Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization: J Cell Biol, v. 127, p. 257-64.

Williams, D. B., 1995, Calnexin leads glycoproteins into the fold: Glycoconj J, v. 12, p. iii-iv.

Wilson, J. M., 2005, Gendicine: the first commercial gene therapy product: Hum Gene Ther, v. 16, p. 1014-5.

Wobus, C. E., B. Hügle-Dörr, A. Girod, G. Petersen, M. Hallek, and J. A. Kleinschmidt, 2000, Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection: J Virol, v. 74, p. 9281-93.

Wold, W. S., and K. Toth, 2013, Adenovirus vectors for gene therapy, vaccination and cancer gene therapy: Curr Gene Ther, v. 13, p. 421-33.

Wong, S. F., 2005, Cetuximab: an epidermal growth factor receptor monoclonal antibody for the treatment of colorectal cancer: Clin Ther, v. 27, p. 684-94.

Wu, J., W. Zhao, L. Zhong, Z. Han, B. Li, W. Ma, K. A. Weigel-Kelley, K. H. Warrington, and A. Srivastava, 2007, Self-complementary recombinant adeno-associated viral vectors: packaging capacity and the role of rep proteins in vector purity: Hum Gene Ther, v. 18, p. 171-82.

Wu, Q. J., C. Y. Gong, S. T. Luo, D. M. Zhang, S. Zhang, H. S. Shi, L. Lu, H. X. Yan, S. S. He, D. D. Li, L. Yang, X. Zhao, and Y. Q. Wei, 2012, AAV-mediated human PEDF inhibits tumor growth and metastasis in murine colorectal peritoneal carcinomatosis model: BMC Cancer, v. 12, p. 129.

Wölfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wölfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K. H. Meyer zum Büschenfelde, and D. Beach, 1995, A p16INK4a-insensitive

CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma: Science, v. 269, p. 1281-4.

Xiao, X., J. Li, and R. J. Samulski, 1998, Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus: J Virol, v. 72, p. 2224-32.

Xie, Q., W. Bu, S. Bhatia, J. Hare, T. Somasundaram, A. Azzi, and M. S. Chapman, 2002, The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy: Proc Natl Acad Sci U S A, v. 99, p. 10405-10.

Yang, J., and Q. Yi, 2010, Killing tumor cells through their surface beta(2)-microglobulin or major histocompatibility complex class I molecules: Cancer, v. 116, p. 1638-45.

Yang, Y., 2003, Generation of major histocompatibility complex class I antigens: Microbes Infect, v. 5, p. 39-47.

Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson, 1995, Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses: J Virol, v. 69, p. 2004-15.

Yang, Y., and J. M. Wilson, 1995, Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo: J Immunol, v. 155, p. 2564-70.

Yiang, G. T., P. L. Chou, H. F. Tsai, L. A. Chen, W. J. Chang, Y. L. Yu, and C. W. Wei, 2012, Immunotherapy for SV40 T/t antigen-induced breast cancer by recombinant adeno-associated virus serotype 2 carrying interleukin-15 in mice: Int J Mol Med, v. 29, p. 809-14.

Yiang, G. T., H. J. Harn, Y. L. Yu, S. C. Hu, Y. T. Hung, C. J. Hsieh, S. Z. Lin, and C. W. Wei, 2009, Immunotherapy: rAAV2 expressing interleukin-15 inhibits HeLa cell tumor growth in mice: J Biomed Sci, v. 16, p. 47.

Ylä-Herttuala, S., 2012, Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union: Mol Ther, v. 20, p. 1831-2.

Yoon, S. J., J. O. Kang, J. S. Park, N. K. Kim, and D. S. Heo, 2000, Reduced expression of MHC class I antigen in human cancer cell lines with defective LMP-7: Anticancer Res, v. 20, p. 949-53.

Zhang, H., J. Melamed, P. Wei, K. Cox, W. Frankel, R. R. Bahnson, N. Robinson, R. Pyka, Y. Liu, and P. Zheng, 2003, Concordant down-regulation of proto-oncogene PML and major histocompatibility antigen HLA class I expression in high-grade prostate cancer: Cancer Immun, v. 3, p. 2.

Zhang, H., Y. Wang, C. Liu, L. Zhang, Q. Xia, Y. Zhang, J. Wu, C. Jiang, Y. Chen, Y. Wu, X. Zha, X. Yu, and W. Kong, 2012, DNA and adenovirus tumor vaccine expressing truncated survivin generates specific immune responses and anti-tumor effects in a murine melanoma model: Cancer Immunol Immunother, v. 61, p. 1857-67.

Zhang, Y. X., L. Wang, P. Y. Ji, G. G. Zhao, G. P. Zhong, and Z. P. Wang, 2013, Correlation of serum β2-microglobulin levels with prostate-specific antigen, Gleason score, clinical stage, tumor metastasis and therapy efficacy in prostate cancer: Arch Med Res, v. 44, p. 259-65.

Zhong, L., B. Li, C. S. Mah, L. Govindasamy, M. Agbandje-McKenna, M. Cooper, R. W. Herzog, I. Zolotukhin, K. H. Warrington, K. A. Weigel-Van Aken, J. A. Hobbs, S. Zolotukhin, N. Muzyczka, and A. Srivastava, 2008, Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses: Proc Natl Acad Sci U S A, v. 105, p. 7827-32.

Ziegler, R. J., S. M. Lonning, D. Armentano, C. Li, D. W. Souza, M. Cherry, C. Ford, C. M. Barbon, R. J. Desnick, G. Gao, J. M. Wilson, R. Peluso, S. Godwin, B. J. Carter, R. J. Gregory, S. C. Wadsworth, and S. H. Cheng, 2004, AAV2 vector harboring a liver-restricted promoter facilitates sustained expression of therapeutic levels of alpha-galactosidase A and the induction of immune tolerance in Fabry mice: Mol Ther, v. 9, p. 231-40.

Ziello, J. E., Y. Huang, and I. S. Jovin, 2010, Cellular endocytosis and gene delivery: Mol Med, v. 16, p. 222-9.

Zitvogel, L., A. Tesniere, and G. Kroemer, 2006, Cancer despite immunosurveillance: immunoselection and immunosubversion: Nat Rev Immunol, v. 6, p. 715-27.

Zolotukhin, S., B. J. Byrne, E. Mason, I. Zolotukhin, M. Potter, K. Chesnut, C. Summerford, R. J. Samulski, and N. Muzyczka, 1999, Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield: Gene Ther, v. 6, p. 973-85.

Zou, W., S. Chen, X. Liu, P. Yue, M. B. Sporn, F. R. Khuri, and S. Y. Sun, 2007, c-FLIP downregulation contributes to apoptosis induction by the novel synthetic triterpenoid methyl-2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate (CDDO-Me) in human lung cancer cells: Cancer Biol Ther, v. 6, p. 1614-20.

# APENDIX

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## LIST OF ABREVIATURES

AAV: Adeno-associated vector

Ad: Adenovirus

APC: Antigen Presenting Cell

APM: Antigen Presentation Machinery

β2m: Beta-2-microglobulin

BCG: Bacilus Calmette-Guerin

BH: Benign Prostate Hyperplasia

CAR: Coxsackievirus Adenovirus Receptor

CMV: Cytomegalovirus

CMV: Cytomegalovirus

CRAdV: Conditionally Replicating Vectors

CRPC: Castration Resistant Prostate Cancer

CTL: Citotoxic T lymphocyte

DC: Dendritic Cell

EBV: Epstein Barr virus

ER: Endoplasmic Reticulum

FACS: Fluorescence-Activated Cell Sorting

FDA: Food and Drug administration

GFP: Green Fluorescent Protein

GM-CSF: Granulocyte macrophage colony-stimulating factor

HC: Heavy chain

HLA: Human leukocyte antigens

HLA-I: Human leukocyte Antigen class I

HLA-II: Human leukocyte Antigen class II

HSPG: Heparan Sulfate Proteoglycan

IFN: Interferon

IL: Interleukin

ITR: Inverted Terminal Repeat

LHRH: Luteinizing Hormone-Releasing Hormones

LOH: Loss of heterocygocity

mAb: Monoclonal Antibody

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

MOI: Multiplicity Of Infection

NK: Natural killer cell

NLRC5: NOD-, LRR- and CARD-containing 5 factor

PBMC: Peripheral Blood Mononuclear Cells

PC: Prostate cancer

pfu: Plaque-forming units

Ph: Phenotype

PLC: Peptide Loading Complex

PNI: Perineural invasion

PSA: Prostate Specific Antigen

scAAV: Self-Complementary AAV

SCID: Severe Combined Immune Deficiency

TAA: Tumor associated antigens

TCR: T-cell receptor

TNF: Tumor necrosis factor

Treg: Regulatory T cells

TSA: Tumor specific antigens

vg: Viral genomes