(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 June 2014 (26.06.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/096367 A1

(51) International Patent Classification:
*G01N 33/574* (2006.01) *C07K 14/74* (2006.01)
*A61K 47/48* (2006.01)

(21) International Application Number:
PCT/EP2013/077702

(22) International Filing Date:
20 December 2013 (20.12.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12382522.6 21 December 2012 (21.12.2012) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published**:

— *with international search report (Art. 21(3))*

WO 2014/096367 A1

(54) **Title**: EXPRESSION OF BETA2-MICROGLOBULIN AS A PROGNOSTIC MARKER FOR TUMOUR IMMUNE ESCAPE AND RESISTANCE TO CANCER IMMUNOTHERAPY AND A DIAGNOSTIC BIOMARKER FOR PATIENT SELECTION FOR SPECIFIC GENE THERAPY

(57) **Abstract**: The present invention refers to a method of predicting or prognosticating the response of a human subject to immunotherapy (from hereinafter method of the invention), wherein the subject is suffering from a cancer disease, and wherein the method comprises using, as an indicator, expression levels of p2-microglobulin from the tumour cells of a biological sample of the subject; wherein the result is indicative of a negative response if the expression levels of β2-microglobulin are under-expressed in comparison to a reference sample and/or a positive control. In a preferred embodiment of the invention, the expression levels are determined after said subject has been treated with immunotherapy.

# Expression of beta2-microglobulin as a prognostic marker for tumour immune escape and resistance to cancer immunotherapy and a diagnostic biomarker for patient selection for specific gene therapy.

## FIELD OF THE INVENTION

The invention relates to the medical field, more specifically to the field of cancer immunotherapy and gene therapy.

## BACKGROUND OF THE INVENTION

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

It is known that the immune system is able to mount a specific T-cell mediated immune response against solid tumours. However, activated tumour-specific T-cells are frequently unable to reject the tumour, leading to cancer progression. This is attributable to the immune escape and expansion of cells with low immunogenicity and high metastatic capacity. One of the central mechanisms of immune evasion is associated with an immune selection that favours the outgrowth of Human Leukocyte Antigen (HLA) class I-negative tumour cells. Cell surface expression of HLA class I molecules in tumour cells is required for the recognition of the class I heavy chain/beta2-microglobulin β2m) /tumour peptide complex by cytotoxic T cells (CTLs). Hence, the elimination of tumour cells by the activated immune system and the success of cancer immunotherapy depends on the proper co-presentation of tumour-specific antigens with HLA class I molecules.

In this sense, HLA class I expression has been reported to be a predictive factor for the response to chemotherapy in early breast cancer patients. Moreover, in patients undergoing immunotherapy, the lack of response and generation of progressing metastases appears to be associated with immune selection of HLA-negative tumour cells variants with irreversible defects.

Consequently, in order to select an appropriate immunotherapy protocol, it is important to know whether the target tumour cells are or will become Human Leukocyte Antigen (HLA) class I-negative tumour cells or not.

In this regard, the present invention provides compelling evidence that β2-microglobulin gene (from hereinafter β2m) deficiencies create immune escape phenotypes in different types of cancer, including melanoma, since these deficiencies result in the generation of Human Leukocyte Antigen (HLA) class I-negative tumour cells.

In this sense, the present invention provides the first experimental evidence of the fate of β2m gene mutations in successive metastatic lesions in a melanoma patient undergoing immunotherapy, demonstrating that the immune escape of HLA class I-negative tumour cells is directly correlated to these genetic events, namely to a mutation of one copy of the β2m gene and the loss of the other copy, i.e., loss of heterozygosity (LOH).

## BRIEF DESCRIPTION OF THE INVENTION

A first aspect of the present invention refers to a method of predicting or prognosticating the response of a human subject to immunotherapy (from hereinafter method of the invention), wherein the subject is suffering from a cancer disease, and wherein the method comprises using, as an indicator, expression levels of β2-microglobulin from the tumour cells of a biological sample of the subject; wherein the result is indicative of a negative response if the expression levels of β2-microglobulin are under-expressed in comparison to a reference sample and/or a positive control. In a preferred embodiment of the invention, the expression levels are determined after said subject has been treated with immunotherapy

In a preferred embodiment of the first aspect of the invention, under-expressed is defined as a level of expression lower than 2/3 of the maximum score achieved in the reference sample and/or positive control.

In another preferred embodiment of the first aspect of the invention, under-expressed is defined as a level of expression lower or equal to ½ of the maximum score achieved in in the reference sample and/or positive control.

In yet another preferred embodiment of the first aspect of the invention, under-expressed is defined as a level of expression lower than 1/10 of the maximum score achieved in in the reference sample and/or positive control.

Additionally, it is particularly preferred that the protein β2-microglobulin is used as an indicator. Furthermore, it is also particularly preferred that the result is obtained by using

immunohistochemistry. Moreover, it is also particularly preferred that the biological sample is fresh tissue or paraffin embedded tissue.

The reference samples used in the above method can be selected from autologous normal cells from the subject.

Furthermore, the cancer disease subjected to prognosis in the above method can be selected from any type of cancer disease, particularly from the list consisting of carcinomas or adeno-carcinomas, more particularly the cancer disease is melanoma or bladder carcinoma.

A second aspect of the invention refers to method for allocating a human subject suffering from cancer in one of two groups, wherein group 1 comprises subjects identifiable by the method of the invention; and wherein group 2 represents the remaining subjects.

A third aspect of the invention refers to a composition comprising a polynucleotide sequence coding for β2-microglobulin protein, for the treatment of a human subject of group 1 as identifiable by the method of the second aspect of the invention.

In a preferred embodiment of the third aspect of the invention, the composition is administered sequentially or simultaneously to a composition suitable for cancer immunotherapy. More preferably, said composition suitable for cancer immunotherapy comprises dendritic cells, obtained from the subject suffering the cancer disease, transfected with mRNA from the tumour cells of the biological sample obtained from the subject. Still more preferable, said composition suitable for cancer immunotherapy comprises Bacillus Calmette-Guerin (BCG), more particularly said treatment comprises the use of the BCG vaccine.

In another preferred embodiment of the third aspect of the invention, the viral vector from the expression system is an adenoviral vector.

A fourth aspect of the invention refers to a composition suitable for cancer immunotherapy for the treatment of a human subject of group 2 as identifiable by the method of the second aspect of the invention. Preferably, said composition suitable for cancer immunotherapy comprises dendritic cells, obtained from the subject suffering the cancer disease, transfected with mRNA from the tumour cells of the biological sample obtained from the subject. More preferably, said composition suitable for cancer immunotherapy comprises dendritic cells, obtained from the subject suffering the cancer disease, transfected with mRNA from the tumour cells of the biological sample obtained from the subject. Still more preferable, said

composition suitable for cancer immunotherapy comprises Bacillus Calmette-Guerin (BCG), more particularly said treatment comprises the use of the BCG vaccine.

In a fifth aspect of the invention, the subject of any of the previous aspects of the invention, suffers from a cancer disease, wherein said cancer disease is melanoma.

In a sixth aspect of the invention, the subject of any of previous aspects one to four suffers from a cancer disease, wherein said cancer disease is bladder carcinoma.

In a preferred embodiment of any of the fifth or sixth aspects of the invention, the treatment of the subject further comprises chemo- or radiotherapy.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1**. Immunohistochemical analysis of HLA class I expression in paraffin-embedded tumour samples with antibodies that recognize free HLA-A, B, C heavy chain (EMR 8-5) and β2m (L-368). Samples 13872, 8755, and 9168 are positive for HLA expression. Tumour 17130 has a heterogeneous (H) pattern of HLA-ABC and β2m expression. The last sample obtained during metastatic progression is positive for HLA-A,B,C and negative for β2m.

**Figure 2.** Tumour infiltration by CD3+, CD8+ T-cells correlates with positive β2m immunostaining; note the very low incidence or absence of CD56+ NK cells in the tumour samples.

**Figure 3.** Negative HLA class I surface expression on DNR-DC-M010 cells analysed by flow cytometry using the anti-β2m antibody L-368 (A, upper FACS image) and the anti-HLAβ2m complex antibody W6/32 (A, lower FACS image). Immunofluorescence was measured either under baseline conditions (black lines) or after 48 h incubation with IFN-gamma (grey lines). Immunocytochemistry of the cells (B) confirmed the loss of both HLA class I complex (W6/32) and β2m (L-368) labeling; however, immunostaining with antibody HC-10 recognizing free HLA heavy chains was positive. (C) β2m, HLA-A,HLA-B, and HLA-C mRNA expression was analyzed by RT-PCR using specific primers.

**Figure 4.** (A) The β2m gene was sequenced in the cell line and in β2m-negative tumour nests and a mutation was found. The sequence from codon 64 to 69 of the β2m gene in exon 2 is shown in the schematic diagram. Asterisk indicates the location of the G-to-T transversion mutation (codon 67) creating a premature stop codon. (B) Sequencing histograms illustrating the G-to-T substitution. Sequencing of the β2m gene from cDNA isolated from autologous patient PBMCs is shown as a control. (C) Heterogeneous pattern of HLA heavy chain (EMR

8-5 and HC-10 antibody) and β2m (L-368 antibody) immunolabeling in tumor sample 17130. Microdissection was performed to isolate β2m -negative nodules.

**Figure 5.** Analysis of the heterozygosity of chromosomes 15 and 6 in melanoma cell line DNR-DC-M010 and in tumour samples. Microsatellite analysis was performed on DNA from DNR-DC-M010 cells and compared to DNA obtained from the patient's autologous PBMC. (A) Scans from the results of PCR analysis of five microsatellite markers on chromosome 15 reveal a pattern of allelic loss. Arrows indicate loss of heterozygosity at five markers.(B) Results obtained from genomic typing of chromosome 6 in DNR-DC-M010 cells compared to autologous PBMC cDNA indicate LOH at chromosome 6.(C) The microsatellite analysis revealed the presence of LOH in 4 out of 5 tumour samples and showed an accumulation of chromosomal loss at specific STRs on chromosome 15 during metastatic progression.

**Figure 6.** (A) Restoration of surface HLA class I expression and function on DNR-DC-M010 cell line 48 hours after infection with AdCMVβ2m virus, as demonstrated by flow cytometry using L-368 (black filled histograms) and W6/32 antibodies (grey histograms). (B) Increase in IFNγ production (ELISPOT) by HLA-A2+ donor T-cells primed with influenza A peptide after co-culture with M010 cells infected with AdCMVβ2m virus. HLA-A2+ melanoma cells with normal HLA class I expression were used as a positive control. (C) The percentage of specific T-cell mediated lysis (calculated by radioactive 51Cr release) of M010 cells transduced with β2m gene was higher than the lysis of luciferase-transfected control cells.

**Figure 7.** Clinical evolution of metastatic melanoma showing the early appearance of β2m mutation (sample 17130). The same mutation was detected in a late metastatic lesion (sample 17790) and in the cell line derived from a lymph node fine needle biopsy. LOH in chromosome 6 is present in all samples; LOH in chromosome 15 was detected in all lesions except for the first tumor specimen. The intensity of HLA class I positive immunolabeling (+++, ++) was determined by using antibodies that recognize HLA-A,B,C heavy chain specificity.

**Figure 8.** Positive expression of NK ligands (MICA/B, ULBP1,2, and 3) (A) and melanoma antigen peptide MelanMart-1 (B) in DNR-DC-M010 cells. C) Analysis of the HLA class I molecule expression on the autologous DCs used for patient vaccination.

**Figure 9**. Positive transduction of human bladder cells with a control AdCMVGFP-vector. Bladder cell lines were incubated with AdCMVGFP for 48 hours and positive GFP expression was confirmed by flow cytometry.

**Figure 10**. Infection with AdCMVβ2m-vector increases HLA class I expression on human bladder carcinoma cells. Upreguation of surface HLA class I expression on bladder cell lines T24, J82 and WIL 48 hours after infection with AdCMVβ2m virus, as demonstrated by flow cytometry using anti-b2m antibody L-368 and anti-HLA class I antibody W6/32. Solid red plots demonstrate upregulation of b2m and HLA class I complex expression after incubation with AdCMVβ2m, while solid grey plots correspond to b2m/HLA expression on cells not infected with the virus. The remaining red-dotted plot and grey-line plot correspond to the measurements done without primary antibodies (negative control for background fluorescence).

**Figure 11**. In vivo experiments on immunodeficient mice by using an intratumoral injection of AdCMV luciferase (control) and AdCMVβ2m adenoviral vectors. Human tumour cells were transplanted into nude/nude mice. Viral vectors were injected into tumours of 5–8 mm in diameters. (A) In vivo imaging of luciferase expression in human xenograft tumours 5 days after injection of adenoviral vector AdCMVluc in a dose of 109 PFU. (B) Time- and dose-dependent intensity of bioluminescence of the luciferase in human tumour xenografts. (C and D) Positive re-expression of b2m protein in the tumours transfected with AdCMVb2m vector demonstrated by immunohistochemistry (C) and western blotting (D) using anti-β2m antibody.

**Figure 12**. Treatment protocol with AdCMVβ2m-vector + BCG in patients with T1G3 urothelial cancer. One month after the resection of recurrent tumor, patients will receive a combined treatment consisting of gene therapy and BCG. Six weekly BCG instillations will be combined with three bi-weekly instillations of adenovirus carrying human b2m gene ( 1011-1012 pfu) diluted in 50ml of PBS, each time five days before BCG administration. Three months later similar, but reduced, treatment cycle will be repeated, including three weekly BCG and two AdCMVb2m administrations (two weeks apart). During the following 10 months patients will be receiving more BCG instillations, but without gene therapy. We expect that this treatment will reduce the incidence of tumor recurrence as compared to patients treated only with BCG.

**Figure 13.** Construction and production of AdCMVβ2m

## DETAILED DESCRIPTION OF THE INVENTION

The authors of the present invention studied the primary tumours and successive metastatic lesions of a melanoma patient who did not responded to treatment with a cancer vaccine. In particular, the patient was treated with an individualized melanoma vaccine based on the

transfection of autologous dendritic cells with autologous tumour-mRNA. Immunohistochemical and mutational analyses of the primary tumours and successive metastatic lesions of the melanoma patient revealed a chronological sequence of β2m gene alterations (Fig. 7). Particularly, β2m-negative tumour nests micro-dissected from an early lesion of the patient with a heterogeneous HLA expression pattern, showed a β2m gene mutation (G to T substitution in exon 2, codon 67, that generated a stop codon) and loss of heterozigosity (LOH) in chromosome 15 (Figures 4 and 5). The same β2m mutation was found in a late metastatic lesion of the disease and in a melanoma cell line established from a fine needle biopsy of a post-vaccination lymph node (Figures 4 and 7). LOH for chromosome 15 was detected in four out of five studied cancer samples suggesting that LOH in chromosome 15 may be an early event in malignant transformation. Based on these results, the authors concluded that the disease was characterized by an early onset of tumour HLA loss due to the reduce expression and ultimate loss of β2m protein due to the above mentioned alterations (Figures 1 and 7), followed by immune selection and outgrowth of β2m-negative melanoma cells.

The above was later ratified by the fact that reconstitution of β2m expression following transduction with an adenoviral vector carrying a wild-type β2m gene was sufficient to restore total HLA class I expression on the studied melanoma cells. This proves that β2m reduced expression and loss was the primary cause of HLA loss in the melanoma cells. Moreover, β2m-transduced melanoma cells became sensitive to lysis by peptide-stimulated HLA-A2-restricted T-cells and recovered the ability to induce peptide-specific IFN-gamma secretion by T-cells in an HLA-restricted manner (Figure 6). In all experiments, the stimulation was induced by both influenza virus peptide (Figure 6) and Melan/Mart-A peptide, for which this cell line is positive, as determined by FACS.

Based on the above facts, the authors of the present invention concluded that early tumour β2m reduced expression and ultimate loss during natural tumour development with no therapy allows malignant cells to evade T-cell recognition. Cells with high class I expression are immune-selected for elimination by T-lymphocyte cells (CTLs), while HLA-negative cells outgrow and disseminate. As a result, immune selective pressure from CTLs leads to the isolation and expansion of tumour cell subpopulations with impaired HLA-class-I-peptide complex expression.

This is an important finding since primary tumours are normally heterogeneous with different molecular mechanisms generating different subsets of cancer cells with distinct metastatic capacity, resulting in distinct clinical courses and variations in the response to therapy. Therefore, the identification of the molecular mechanisms underlying the development of the aggressive phenotypes shall certainly contribute to a better understanding of cancer immune

escape. This better understanding is in turn useful to select the most effective therapy for different tumour cell subsets.

In fact, the above mentioned different subset of cancer cells in the melanoma patient lead to a high degree of infiltration with CD3+ and CD8+ T-cells in HLA-positive tumours and low numbers of these CTLs in HLA-negative nodules. The failure to halt tumour growth and dissemination is, as already stated, due to the inability of HLA-negative tumour cells to present tumour-associated peptides to T-cells. However, loss of tumour HLA class I expression renders malignant cells potentially susceptible to NK cell attack. Nevertheless, surprisingly NK cell accumulation in the studied tumour specimens was found to be practically negative in all of them. This might be the case because NK cells might have eliminated melanoma cells but failed to migrate into the tumour, possibly attributable to suppressive factors in the tumour microenvironment. This highlights the fact that a better understanding of the aggressive phenotypes shall certainly contribute to a better selection of the most effective therapy.

In this sense, it is important to note that immunotherapy induces an additional cycle of immune selection of tumor cells with reduced or negative β2m expression, which are potentially able to escape from T-cell recognition. However, if tumor cells have a reversible HLA alteration (''soft'' lesion), immunotherapy induces the local release of cytokines leading to recovery of normal β2m expression and thus normal HLA class I expression, increased tumor cell immunogenicity, and immune rejection of tumor cells. In contrast, when tumor cells have irreversible β2m expression defects (''hard'' lesions), including LOH and/or gene mutations, immunotherapy will fail to induce sufficient upregulation of tumor β2m expression and thus of HLA class I antigen expression, and the tumor cells will escape from immune recognition and will relapse. Therefore, activation of immune surveillance after immunotherapy leads to the immunoselection and elimination of tumour cells with up-regulated HLA class I expression and to the outgrowth of cancer cells with structural β2m expression alterations.

Consequently, tumour cells with reduced expression of β2m after been treated with immunotherapy result, with time, in HLA-negative tumour cells, wherein HLA class I expression can only be restored with the introduction of wild-type β2m gene. As already stated, adenovirus-mediated transfer of β2m gene restored HLA class I antigen expression and recognition by peptide-specific HLA-A2-restricted cytotoxic T lymphocytes in a melanoma cell line. Thus, reconstitution of a normal HLA class I phenotype is a pivotal strategy for tumour cells to circumvent an effective immune response and is associated with tumour progression in cancer patients. Therefore, the findings of the present invention support the

importance of immunological tumour analysis in order to exclude those patients unlikely to respond to immunotherapy.

Additionally, these results have been further corroborated by the authors of the present invention by the following experimental evidence. First of all by transducing human bladder cancer cells with a control adenoviral vector (AdCMVGFP-vector) carrying green fluorescent protein (GFP) (Fig. 9), the authors of the present invention have shown that adenoviral vectors show a high level tropism for these types of cells. In this sense, human bladder carcinoma cells were incubated with or without AdCMVGFP for 48 hours and the expression of GFP was evaluated by flow cytometry.

Based on the above mentioned results, the authors of the present invention have infected human bladder carcinoma cells having hard lesions in the β2m gene with adenoviral vector AdCMVβ2m. As illustrated in figure 10, this type of infection produced an increased level of HLA class I expression in these types of cells. This experiment was conducted by infecting human bladder carcinoma cells with AdCMVb2m virus for various time periods and up-regulated cell surface expression of β2m and HLA class I complex was evaluated by indirect flow cytometry using anti-b2m and anti-HLA class I antibodies. These results clearly corroborate the fact that HLA class I expression in human bladder carcinoma cells having hard lesions can be restored with the introduction of wild-type β2m gene. Moreover, this has been further corroborated by the *in vivo* experiments illustrated in figure 11, wherein immunodeficient mice with human xenogenic tumors were treated with an intratumoral injection of AdCMV luciferase (control) and adenoviral vector AdCMVβ2m. Infection with adenoviral vector AdCMVβ2m, increased the expression of β2m thus restoring HLA class I expression. Thus, recovery of b2m/HLA class I expression using AdCMVb2m viral vector occurred both *in vitro* and *in vivo*.

Therefore, the authors of the present invention have come to the conclusion that those subjects suffering from a cancer disease, wherein at least part of their tumour cells show hard lesions in the β2m gene, can only be successfully treated with immunotherapy by the introduction of the wild-type β2m gene. In addition, the authors of the present invention have come to a particularly advantageous treatment protocol for those patients suffering from urothelial cancer. In this sense, patients with recurrent bladder carcinoma tumours despite BCG therapy, shall receive several cycles of local AdCMVb2m administration preceding BCG instillations. Each time, virus ($10^{11}$-$10^{12}$ viral particles) diluted in 50ml of PBS will be instilled into the bladder for a period of 60 min. Five days after, the patient shall continue the standard treatment with the local administration of BCG. In this manner, by the time standard treatment with BCG is administered, the HLA-negative tumor escape variants will recover

HLA class I expression leading to increased recognition and elimination of cancer cells by activated cytotoxic T-lymphocytes.

Thus, taken together, these results provide an accumulating body of evidence that immune evasion strategies can develop before the clinical application of immunotherapy, something which should be taken into account at the time of designing an immunological treatment protocol. This is so, since immunological tumour analysis before treatment shall provide a rationale for excluding patients unlikely to respond to immunotherapy.

Thus, a first aspect of the present invention refers to a method of predicting or prognosticating the response of a human subject to immunotherapy (from hereinafter method of the invention), wherein the subject is suffering from a cancer disease, and wherein the method comprises using, as an indicator, expression levels of β2-microglobulin from the tumour cells of a biological sample of the subject; wherein the result is indicative of a negative response if the expression levels of β2-microglobulin are under-expressed in comparison to a reference sample and/or a positive control. In a preferred embodiment of the invention, the expression levels are determined after said subject has been treated with immunotherapy.

Preferably, said reduced expression levels are caused by gene alterations originated by the substitution of a guanine residue for a thymine residue in exon 2, codon 67, of the β2-microglobulin gene sequence and/or the loss of heterozigosity (LOH) in chromosome 15.

The method of the present invention may be applied with samples from individuals of either sex, i.e. men or women, and at any age.

In the invention the method of determining the result, i.e. the expression level of β2-microglobulin, need not be particularly limited, and may be selected from a gene profiling method, such as a microarray, and/or a method comprising PCR, such as real time PCR; and/or Northern Blot or by using immunohistochemistry. Additionally, it is particularly preferred that the protein β2-microglobulin is used as an indicator. Furthermore, it is also particularly preferred that the result is obtained by using immunohistochemistry. Moreover, it is also particularly preferred that the biological sample is fresh tissue or paraffin embedded tissue.

The reference samples used in the above method can be selected from any type of autologous normal cells from the subject.

In the context of the present invention under-expressed is defined in comparison to a normal sample and/or a positive control as a level of expression lower than 2/3 of the maximum score

achieved in the reference sample and/or positive control. Preferably, under-expressed is defined as a level of expression lower or equal to ½ of the maximum score achieved in in the reference sample and/or positive control. More preferably, under-expressed is defined as a level of expression lower to 1/10 of the maximum score achieved in in the reference sample and/or positive control.

In the context of the present invention illustrative non-limiting examples of a biological sample include different types of samples from tissues, as well as from biological fluids, such as blood, serum, plasma, cerebrospinal fluid, peritoneal fluid, faeces. Preferably, said samples are samples from tissues and most preferably, said samples of tissues originate from tumour tissue of the individual the response of which is to be predicted, and may originate from biopsies.

In the context of the present invention "Response" refers to the clinical outcome of the subject, "Response" may be expressed as overall survival or progression-free survival. Survival of cancer patients is generally suitably expressed by Kaplan-Meier curves, named after Edward L. Kaplan and Paul Meier who first described it (Kaplan, Meier: Amer. Statist. Assn. 53:457–481). The Kaplan–Meier estimator is also known as the product limit estimator. It serves for estimating the survival function from life-time data. A plot of the Kaplan–Meier estimate of the survival function is a series of horizontal steps of declining magnitude which, when a large enough sample is taken, approaches the true survival function for that population. The value of the survival function between successive distinct sampled observations is assumed to be constant. With respect to the present invention, the Kaplan-Meier estimator may be used to measure the fraction of patients living for a certain amount of time after beginning of immunotherapy and chemotherapy and/or radiotherapy. The clinical outcome predicted may be the (overall/progression-free) survival in months/years from the time point of taking the sample. It may be survival for a certain period from taking the sample, such as of six months or more, one year or more, two years or more, three years or more, four years or more, five years or more, six years or more. In each case, "survival" may refer to "overall survival" or "progression-free-survival".

Thus, in one embodiment, the response is clinical outcome, which is "overall survival" (OS). "Overall survival" denotes the chances of a patient of staying alive for a group of individuals suffering from a cancer. The decisive question is whether the individual is dead or alive at a given time point.

Additionally, β2-microglobulin might be under-expressed in a great variety of human cancers. Hence, in a still more preferred embodiment of the invention, the cancer disease as is a carcinoma, preferably a carcinoma selected from the list consisting of bladder, ovarian, neck,

colon, stomach, cervix, thyroid gland, lung, uterus, rectum, breast or kidney carcinoma. In a still more preferred embodiment, the cancer disease is melanoma or bladder carcinoma.

Additionally, the present inventors have identified a novel subgroup of patients that will profit from the method of the invention. Hence, a second aspect of the invention refers to method for allocating a human subject suffering from cancer in one of two groups, wherein group 1 comprises subjects identifiable by the method of the invention; and wherein group 2 represents the remaining subjects.

A third aspect of the invention refers to a composition comprising an expression system which in turn comprises a polynucleotide sequence coding for β2-microglobulin protein, for the treatment of a human subject of group 1 as identifiable by the method of the fourth aspect of the invention.

In the context of the present invention, an expression system is a system specifically designed for the production of the β2-microglobulin. Preferably, an expression system consists of the β2-microglobulin gene, normally encoded by DNA or cDNA, and the molecular machinery required to transcribe the DNA into mRNA and translate the mRNA into protein using the reagents provided.

In a preferred embodiment of the third aspect of the invention, the expression system is a viral vector, more preferably the viral vector is an adenoviral vector.

In another preferred embodiment of the third aspect of the invention, the composition is administered sequentially or simultaneously to a composition suitable for cancer immunotherapy. More preferably, said composition suitable for cancer immunotherapy comprises dendritic cells, obtained from the subject suffering the cancer disease, transfected with mRNA from the tumour cells of the biological sample obtained from the subject. Still more preferable, said composition suitable for cancer immunotherapy comprises the use of Bacillus Calmette-Guerin (BCG), more particularly said treatment comprises the use of the BCG vaccine.

Other suitable immunotherapeutic treatments are IL-2, IFN, peptide based therapy or transfer of autologous activated T-cells.

A fourth aspect of the invention refers to a composition suitable for cancer immunotherapy for the treatment of a human subject of group 2 as identifiable by the method of the fourth aspect of the invention. Preferably, said composition suitable for cancer immunotherapy comprises dendritic cells, obtained from the subject suffering the cancer disease, transfected

with mRNA from the tumour cells of the biological sample obtained from the subject. Still more preferable, said composition suitable for cancer immunotherapy comprises the use of Bacillus Calmette-Guerin (BCG), more particularly said treatment comprises the use of the BCG vaccine.

In a preferred embodiment of any of the third or fourth aspects of the invention, the treatment of the subject further comprises chemo- or radiotherapy.

In a fifth aspect of the invention the subject of any of the previous aspects of the invention, suffers from a cancer disease, wherein said cancer disease is a melanoma.

In a sixth aspect of the invention the subject of any of previous aspects one to four, suffers from a cancer disease, wherein said cancer disease is bladder carcinoma.

The following examples serve to illustrate the present invention; these examples are in no way intended to limit the scope of the invention.

EXAMPLES

**Example 1. Material and methods**

1.1. Tumour tissue samples and cells

Tumour samples and the melanoma cell line DNR-DC-M010, which was established from a lymph node fine needle biopsy, were obtained from a 72 years old patient with metastatic melanoma (received as part of European collaborative project ENACT). Autologous PBMCs were also received from the same patient. This patient was treated in Norwegian Radium Hospital using dendritic cells transfected with autologous tumour-mRNA. Table 1 below depicts a schematic summary of the patient's clinical time course from October 2001 to February 2003. In October 2001, a primary tumour was surgically removed, and a second lesion was removed two months later. In June 2002, several metastases from lymph nodes at both axillae and a cutaneous lesion were surgically removed. In October 2002, a metastasis in the axilla sinister was used to isolate the tumour mRNA and to develop a dendritic cell vaccine that was administered in December 2002. In January 2003, the DNR-DC-M010 cell line was established from a fine needle biopsy of the right inguinal lymph node. The patient died in February 2003. After detection of a β2m mutation in the cell line, we retrospectively analysed 20 tumour samples from six paraffin-embedded biopsies obtained from this patient at different time points during the disease course. The cell line was grown in RPMI medium (Biochrom KG, Berlin, Germany) supplemented with 10% FCS (Gibco BRL, Life

Technologies,Karlsruhe, Germany), 2% glutamine (Biochrom KG), and 1% penicillin/streptomycin (Biochrom KG) at 37°C in a humidified atmosphere with 5% CO2. Autologous PBMCs (A*0205, 3201; B*5001, 5101; Cw*0602, 1502) were used for LOH analysis and genome sequencing.

Human bladder carcinoma cell lines T24, J82 and WIL were obtained from ATTC and used in viral gene transfer experiments.

1.2. Monoclonal antibodies

HLA class I expression in the melanoma cell line was studied by flow cytometry using monoclonal antibody (mAb) W6⁄32, which recognizes a cell surface complex of β2-microglobulin with HLA heavy chain. This antibody and an anti-human β2m mAb L-368 were a kind gift from Dr. Bodmer (Tissue Antigen Laboratory, Imperial Cancer Research Fund Laboratories, London, UK). HC-10 mAb against free HLA heavy chain (Stam et al. 1986: J Immunol 137:2299–2306) was also used to detect free HLA heavy chain in melanoma cells. To study HLA expression in paraffin-embedded tumour sections, we used anti-β2m antibody L-368 and anti-pan-HLA class I mAb EMR8-5, which recognizes a common HLA-A,B,C heavy chain sequence (MBL, Naka-ku Nagoya, Japan). Antibodies against various components of antigen processing machinery (APM) (TAP-1, TAP-2, Tapasin, LMP-2, LMP-7, calnexin, calreticulin and ERP-57) were purchased from Abcam (UK) and used for flow cytometry and immunocytochemistry. The mAbs against human NK ligands, including anti-ULBP-1-Fluorescein monoclonal antibody (for intracellular staining), anti-human ULBP-2-Phycoerythrin antibody, and the human ULBP-3 antibody were purchased from R&D systems (Minneapolis, MN, USA), and anti-MICA-MICB (clone 2C10) from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to CD3, CD4, CD8, and CD56 markers for immunohistochemical detection of tumour infiltrating lymphocytes (TILs) were purchased from Master Diagnostica (Granada, Spain). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma, St Louis, MO, USA) was used as a secondary antibody for flow cytometry.

1.3. Immunostaining of melanoma cells and tumor specimens

Immunocytochemical analysis of the DNR-DC-M010 cell line was performed on cytospin glass slides as previously described. Immunohistochemical analysis of the tumour biopsies was performed by cutting consecutive 4–6 μm thick sections from formalin-fixed paraffin-embedded tissue blocks using a SLEE MAINZ CUT 5062 microtome and mounting them on coated glass slides. After deparafinization and antigen retrieval using heat, EDTA, or citrate buffer treatment, depending on the antibody used, slides were rehydrated and used for antibody staining with the biotin–streptavidin system (supersensitive Multilink-HRP/ DAB kit, BioGenex). HLA class I expression was studied with the monoclonal antibodies L-368,

EMR8-5, and HC-10. TILs were analysed with antibodies against CD3, CD4, CD8 and CD56. The primary antibody was replaced with PBS in negative controls, in which no labelling was detected.

1.4. RNA isolation, reverse transcription, PCR, and quantitative real time-PCR

Total RNA was extracted from cultured melanoma cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and utilized for reverse transcriptase-PCR analysis with the iSriptTMcDNA Synthesis Kit (Bio-Rad). Primers used for PCR amplification of □2-microglobulin cDNA were: forward 5′- GGGCATTCCTGAAGCTGACA-3′, and backward: 5′- GGTTGCTCCACAGGTAGCTCTA-3′ with 618 bp of predicted PCR product size. The primers used for HLA-A, HLA-B and HLA-C heavy chain amplification have been described by Ruano et al. (Ruano et al, 2010), and their predicted PCR product sizes are 197bp, 140 bp, and 151 bp, respectively. Primers used for Melan-Mart-1 analysis were: Fw 5′-CACGGCCACTCTTACACCAC-3′ and Bw: 5′- GGAGCATTGGGAACCACAGG-3 with a predicted PCR product size of 254bp. Reverse transcription products were analyzed by quantitative real-time PCR for the expression of various target genes (HLA class I heavy chain, β2m, TAP-1, TAP-2, LMP-2, LMP-7 and tapasin). To control for variations in amounts of mRNA, the glucose-6-phosphate dehydrogenase (G6PDH) and beta-actin genes were also amplified. All PCR reactions were performed in a Light Cycler instrument using the LC-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Manheim, Germany). The primer sequences, amplicon size, and annealing temperatures used are described in (Romero et al 2005), with the exception of G6PDH and beta-actin amplification, for which Housekeeping Gene Set Kits were used (Roche Diagnostics and Search LC, GmbH Heidelberg). The primers used for β2m gene amplification were: forward 5′-CAGGAAATTTGACTTTCCATTC3′ and backward: 5′- TTCTGGCCTGGAGGCTATC-3′.The final expression levels of target genes were given relative to G6PDH expression levels.

1.5. Genomic DNA Extraction

DNA extraction from the cultured melanoma cells was performed with the blood and cell culture DNA Midi Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Immunolabeled paraffin sections were micro-dissected using a laser micromanipulator (PALM Microlaser Systems, Olympus). Micro-dissected fragments were collected in PALM Adhesive Caps. These fragments were used for DNA extraction using the QIAamp®DNR FFPE Tissue Kit (QIAGEN, Westburg, Leusden, The Netherlands) following manufacturer's recommendations with few modifications.

1.6. Sequencing of β2-microglobulin gene

The β2m cDNA from the cell line was sequenced using the same primers as in the PCR analysis. The amplification of β2m gene from the micro-dissected tumour nests of paraffin-

embedded samples was performed on genomic DNA with Illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare Life Sciences) using the following forward primers: 5′-CGATATTCCTCAGGTACTCC-3′ and 5′- GGTGAATTCAGTGTAGTACAAG-3′, and with one backward primer: 5′-ACACAACTTTCAGCAGCTTAC-3′. The predicted PCR product sizes are 311 bp and 114 bp, respectively. Sequencing was performed by using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK) and the same primers as in the amplification reaction. The sequencing reaction was performed in Centri-Sep Columns (Applied Byosistems) following the manufacturer's recommendations. ABI 3130x/ Genetic Analyzer and SequencingAnalysis v5.2 software (Applied Biosystems) were used for the analyses.

1.7. Microsatellite analysis

To determine the possible loss of heterozygocity (LOH) in chromosome 6, DNA obtained from the cell line, from micro-dissected tumor and from the patient`s PBMCs was studied with seven short tandem repeats (STRs) (D6S291, D6S273, C.1.2.C, C.1.2.5, D6S265, D6S105, and D6S276) mapping the HLA region and an additional STR, D6S311, in the 6q region. The β2-microglobulin studies used five STR markers that flanked the gene in chromosome 15 (DS15209, DS15126, DS15146, DS151028 and DS15153). The 5'end of one primer of each primer set was tagged with a fluorescent label (Applied Biosystems). Details of the PCR reactions, electrophoresis, and data analysis were previously described (Maleno et al. 2006: Immunogenetics 58(7):503–510; Koene et al. 2004 Tissue Antigens 64(4):452–461). Microsatellite instability was determined by using the ABI 3130x/ Genetic Analyzer with 16 capillaries and GeneMapper v4.0 software (Applied Biosystems). LOH was assigned when the signal of one allele in the tumor sample was reduced by more than 25% in comparison to the PBMCs. LOH was determined by the following calculation: (intensity of tumor allele one/intensity of tumor allele two)/ (intensity of normal allele one/intensity of normal allele two) (referred to as the percent LOH index).

1.8. Flow cytometry

FACS analysis using a panel of specific antibodies directed against HLA class I antigens and APM components was performed on a BD FACS Canto apparatus (Becton Dickinson, Franklin Lakes, NJ) in baseline conditions and after 48 h incubation with IFN-gamma (800 U/ml) as previously described . Secondary FITC labeled antibodies were used. Results were presented as representative fluorescence plots and expressed as relative mean fluorescence intensity (MFI).

Restoration of tumour HLA class I expression by adenoviral-mediated β2m gene transfer

The purified recombinant adenovirus carrying human β2m (AdCMVβ2m) under the control of cytomegalovirus (CMV) promoter was added to the cells in a 2% FCS supplemented

medium at MOI 2,5 and the medium was replaced 24 hours later. The recovery of β2m expression and restoration of normal surface HLA class I expression was confirmed by FACS, immunocytochemistry, and in ELISPOT and IFN-gamma production functional assays. In some experiments, cells were infected with a similar adenoviral vector carrying GFP or Luciferase gene (AdCMVGFP and AdCMVLuc, respectively).

1.9. IFN-gamma production and chromium release assays for CTL analysis

Donor HLA-A2 PBMCs were primed with HLA-A2-restricted influenza A virus matrix 58–66 peptide (GILGFVFTL, flu peptide) a kind gift from Dr.Esther Larrea (CIMA, Pamplona, Spain (Larrea et al. 2009 J Virology)). PBMCs (105/well) were co-cultured in 96-well round-bottom plates with untreated DNR-DC-M010 melanoma cells (5x104cells/well) or with cells manipulated in one of the following ways: pulsed with flu peptide, transduced with AdCMVβ2m, peptide-pulsed and transduced with AdCMVβ2m, transduced with a control AdCMVLuc vector, or peptide-pulsed and transduced with AdCMVLuc. After 48 h of co-culture, T cells producing IFN-gamma were counted using an ELISPOT kit (BD-Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.

Briefly, plates (Multiscreen HTS; Millipore, Bedford, MA, USA) were coated with purified anti-IFN-gamma NA/LETM antibody. After overnight incubation, plates were washed with phosphate-buffered saline (PBS) and blocked for 3 h with RPMI containing 10% fetal calf serum. After that, (105/well) peptide-pulsed PBMCs were co-cultured in 96-well round-bottom plates with HLA-A2 positive control melanoma cells (E-….) and untreated DNR-DC-M010 melanoma cells (5x104cells/well) or with cells manipulated in one of the following ways: pulsed with flu peptide, transduced with AdCMVb2m, peptide-pulsed and transduced with AdCMVb2m, transduced with a control AdCMVLuc vector, or peptide-pulsed and transduced with AdCMVLuc.. Next day plates were washed with PBS and incubated with biotinlylated anti-IFN-g antibody for 4 h. Then plates were washed and incubated with a 1/500 dilution of streptavidin-peroxidase. One hour later plates were washed and developed with AEC Substrate Reagent. The reaction was stopped with distilled water and spots were counted using an automated ELISPOT reader (CTL; Aalen, Germany).

Tumour cell lysis was measured by incubating the effector T-cells with 3000 Na251CrO4-labeled target cells at different tumor-effector cell ratios. Supernatants were harvested 4 h later and the percentage of specific lysis was calculated according to the formula: [(cpm experimental-cpm spontaneous) / (cpm maximum-cpm spontaneous)]x100, where spontaneous lysis corresponds to target cells incubated in the absence of effector cells, and maximum lysis is obtained by incubating target cells with 5% Triton X-100.

1.10. Statistical analysis

Fisher's exact test was used to analyze the data. Statistical significance was established at $p<0.05$.

The statistical analysis was performed using SPSS 10.0 software (SPSS, Chicago, IL).

**Example 2. Immunohistochemical analysis of HLA class I expression in tumour specimens.**

To investigate whether the disease progression was correlated with the progressive evolution of alterations in HLA antigens, we analysed HLA class I expression in primary tumours and five metastases (all paraffin-embedded) that have been surgically removed from the patient at different time points during disease progression before the administration of the DC vaccine (Table 1).

**Table 1.** Sampling time points with clinical and pathological characteristics of tumor specimens and a cell line obtained from patient with metastatic melanoma,

| DATE | EVENT | BIOPSY NUMBER | LOCATION | PATHOLOGY REPORT |
|---|---|---|---|---|
| **Sample 1** October 2001 | Lesion surgically removed | 13872 (1 block) | Cutis (dorsum) | Primary tumor, likely origin of later metastases |
| **Sample 2** December 2001 | Lesion surgically removed | 17130 (6 blocks ) | Cutis (dorsum) | Second primary tumor or metastasis |
| **Sample 3** June 2002 | Lesion surgically removed | 8755 (4 blocks) | Lymph node (axilla dexter) | Melanoma in 3/5 lymph nodes. Metastasis |
| **Sample 4** June 2002 | Lesion surgically removed | 9168 (blocks # 1-5) | Lymph node (axilla sinister) | Melanoma in 4/9 lymph nodes. Metastasis |
| **Sample 4** June 2002 | Lesion surgically removed | 9168 (blocks # 6-8) | Cutis (dorsum) | Metastasis |
| **Sample 5** October 2002 | Lesion surgically removed | 17790 (1 block) | Lymph node (axilla sinister) | Metastasis |
| December 2002 | DC vaccine administration | | | |
| **Cell line** January 2003 | Fine needle biopsy | | Right inguinal lymph node | |

| | | | | |
|---|---|---|---|---|
| February 2003 | Death of patient | | | |

The mRNA of lesion 17790 was used to prepare the DC vaccine. Positive labelling for HLA-A, B, C heavy chain (EMR 8-5 antibody) and β2m (L-368 antibody) was detected in the primary tumor (sample 13872) and metastatic lesions 8755 (lymph node metastasis), 9168-1 (lymph node lesions), and 9168-2 (cutaneous lesions) (Fig. 1). Sample 17790 (lesion used for immunotherapy) showed a heterogeneous pattern of HLA-A, B, C labeling with β2m-negative areas. Monoclonal anti-pan HLA class I antibody EMR 8-5 detects a common domain of HLA-A, -B, and -C heavy chain in formalin-fixed paraffin embedded tissues and does not recognize a heavy chain-β2m complex, which is recognized by antibody W6/32 (Tsukahara T et al, 2006). Unfortunately, there is no antibody available for HLA class I complex detection in paraffin-embedded tissue. The second metastatic nodule (17130) was heterogeneous for both HLA class I complex and β2m (Fig.1). In this sample, both positive and negative tumor nests were detected by antibodies directed against HLA heavy chain (HC-10 and EMR8-5) and by anti-β2m antibody (L-368) (Fig. 4).

**Example 3. Immunohistochemical detection of tumour infiltrating lymphocytes.**

The presence of TILs was analysed in all studied paraffin-embedded samples and, as shown in Figure 2, a larger number of CD3+ and CD8+ lymphocytes were found in β2m-positive samples (samples 8755 and 9168) than in β2m-negative tumour nests (biopsies 17130 and 17790), where they were only observed on the periphery of the tumour node. These results demonstrate that cytotoxic T cells have a better ability to penetrate HLA-positive tumours. We also analysed NK cell accumulation in the studied tumour specimens and found that tumour infiltration with CD56+ NK cells was practically negative in all the studied samples. Notably, expressions of activating NK ligands MICA/B and ULBP-1,-2, and -3 were positive on DNR-DC-M010 cells as analysed by FACS.

**Example 4. Total loss of HLA-class I expression on DNR-DC-M010 cell line.**

Melanoma cell line DNR-DC-M010 (M010) was established from a fine needle biopsy of a lymph node metastasis obtained after immunotherapy with dendritic cells transfected with autologous tumor mRNA. FACS analysis of cells stained with W6/32 and L-368 antibodies showed total absence of cell surface expression of HLA class I complex and β2m, both under baseline conditions and after incubation with IFN-gamma (Figure 3A). HLA class I complex and free β2m were also not detectable in immunocytochemical analysis (Fig.3B). In contrast, intracellular labeling of free HLA class I heavy chain (HC-10 antibody) was positive (Fig 3B). In addition, intracellular immunostaining of APM components, including LMP2/7, TAP1/2, tapasin, calreticulin, ERp57, and calnexin, was also positive (data not shown). These results and the lack of HLA upregulation by IFN-gamma suggest that the loss of HLA

expression in this cell line is caused by the loss of β2m. We further confirmed this hypothesis by adenoviral-mediated β2m gene transfer and by molecular analysis of the β2m gene.

**Example 5. Molecular analysis of β2m gene alterations**

We analysed the transcription of the β2m gene and of HLA heavy chain A, B, and C genes by PCR amplification of cDNA obtained after reverse transcription of mRNA isolated from melanoma cells. A cell line with normal HLA class I expression was used as a control. Figure 3C demonstrates that β2m and HLA-A, -B, and -C genes were transcribed correctly, with a predicted amplicon size similar to that of the control cell line. To investigate whether a structural defect in β2m gene was responsible for the total loss of HLA class I expression, cDNA from M010 cells was amplified and nucleotide sequencing of the open reading frame of the gene was performed. The cDNA isolated from the patients` peripheral blood mononuclear cells (PBMC) was used as a sequencing control. We detected a point mutation at codon 67 in exon 2, where guanine was substituted for thymine (G to T substitution), leading to generation of an early stop codon (Fig. 4A,B) with a 66-amino-acid-long predicted size of the truncated β2m protein . This short protein cannot form a functional complex with HLA heavy chain due to the absence of the key amino acids necessary for binding with the heavy chain. We also analysed HLA class I-negative tumour samples for β2m mutation. We micro-dissected β2m-negative tumour nests from samples 17130 and 17790 Figure 4C and detected the same β2m mutation (G-T substitution at codon 67 in exon 2). This tumour was used to isolate mRNA for DC transfection to generate a cancer vaccine. Therefore, the β2m gene mutation first detected in the second consecutive lesion, which had a heterogeneous HLA phenotype, re-appeared again in another heterogeneous lesion used for the vaccine preparation. Finally, the same mutation causing total HLA class I loss was detected in a cell line derived from a post-vaccination lesion.

**Example 6. Loss of heterozygosity (LOH) in chromosomes 15 and 6.**

LOH was defined by microsatellite analysis of the M010 cell line using five polymorphic markers spanning chromosome 15q, where the β2m gene has been mapped. Genomic DNA extracted from DNR-DC-M010 patient's PBMC served as control. As shown in Figure 5A, LOH was detected at chromosome 15 for the five markers, showing a single allele at STR sites D15S1028 and D15S146, which flank β2m gene, and also in STR D15S153, D15S126 and D15S209, whereas PBMC control showed retention of heterozygosity with two bands at the studied sites. We also checked the status of heterozygosity on chromosome 6. HLA-typing was performed using a low-resolution genomic sequence-specific oligonucleotide analysis, which indicated LOH in chromosome 6 (HLA typing results for melanoma cell line DNR-DC-M010 were: A*0205, B*5001, and Cw*0602) (Figure 5B); this finding was verified by microsatellite analysis of this chromosome, which detected LOH in 6 out of 8 STR (the other two were non-informative), whereas the autologous PBMC retained heterozygosity (data not

shown). We also analysed micro-dissected tumour specimens for LOH at chromosomes 15 and 6. LOH at chromosome 15 was detected for all five specific markers in two tumour samples: 17790 (used for tumour mRNA extraction for the vaccine) and 9168 (Figure 5C). The 8755 sample exhibited LOH for three markers, two of which (D15S126 and D15S209) flank the β2m region. Tumour sample 17130 exhibited LOH for three markers, one of which (D15S1269) was located close to the β2m region, whereas the other two of the five markers retained heterozygosity. Finally, the primary tumour (13872) presented LOH for two markers, one of them flanking the β2m region. Figure 7 summarizes the evolution of β2m gene alterations in recurrent metastases during the melanoma progression, showing a growth and dissemination advantage for HLA class I-negative cells.

**Example 7. Reconstitution of normal HLA class I expression and its functional activity after transfer of wild-type β2m gene**

A non-replicating adenovirus (AdCMVβ2m) expressing the wild-type human β2m gene under CMV promoter was constructed using the Cre-Lox recombination system as previously described (Efficient recovery of HLA class I expression in human tumor cells after beta2-microglobulin gene transfer using adenoviral vector: implications for cancer immunotherapy.del Campo AB, Aptsiauri N, Méndez R, Zinchenko S, Vales A, Paschen A, Ward S, Ruiz-Cabello F, González-Aseguinolaza G, Garrido F.Scand J Immunol. 2009 Aug;70(2):125-35)

In this sense, recombinant adenovirus carrying human β2m (AdCMVb2m) under the control of cytomegalovirus (CMV) promoter were construed by homologous recombination between W5 (as a donor virus to supply the viral backbone) and pAdlox β2m (a shuttle plasmid with a single loxP site carrying the b2m gene) using the Cre-lox recombination system.

Total cellular RNA from peripheral blood mononuclear cells from a healthy donor was isolated with Ultraspec (Biotecx Laboratories,Houston, TX, USA) and amplified by reverse transcription– polymerase chain reaction (RT-PCR) using specific primers for human β2m (forward primer: 5¢-AAGCTTGCCACCATGTCTCGCTCC- 3¢; reverse primer: 5¢-GGATCCTGCGGCATCTTCAAACCTCCATG-3¢) including the sites for restriction enzymes HindIII and BamHI. The 600-bp fragment corresponding to β2m cDNA was cloned into pCR4-TOPO plasmid generating TOPOb2m (TOPO TA cloning Kit; Invitrogen, Carlsbad, CA, USA). The HindIII / BamHI β2m fragment excised from TOPO-b2m was ligated into HindIII/BamHI-digested alkaline phosphatase-treated pAdlox.

AdCMVb2m construction. The new pAdlox β2m, linearized with SfiI, was co-transfected along with W5 DNA (W5 is an E1–E3 deleted version of Ad5-containing loxP sites flanking

the packaging site) into 116 cells using jet PeiTM, (Redox Lab. S.L., Malaga, Spain) After development of complete cytopathic effect (7 or 8 days), the cell lysate was passaged three times in 116 cells to eliminate the W5 virus contamination. An AdCMVb2m single clone was expanded to produce a large-scale concentrated stock in HEK293 cells, purified on CsCl gradient, desalted using PD-10 columns (Amersham Biosciences, Uppsala, Sweden) and stored in Tris–HCl 0.1 M and 10% glycerol at 80°C. 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., Temecula, CA, USA) and the titre was expressed as plaque-forming units. Concentrated stocks typically had titres in the range of $10^8$– $10^{10}$ PFU / ml. Adenoviral vectors containing green fluorescent protein (GFP) and Luciferase reporter genes under the control of CMV promoter (AdCMVGFP and AdCMVLuc) were produced similarly.

Melanoma cells transduced with this adenovirus recovered normal HLA class I expression, as demonstrated by FACS analysis using anti-β2m (L-368) and anti-HLA-class-I/β2m complex (W6/32) antibodies (Fig. 6A). Immunocytochemical analysis also confirmed the recovery of HLA class I expression on the surface of M010 cells (data not shown). The functional restoration of tumour HLA class I complex was confirmed by analysing the ability of virally transduced tumour cells to stimulate IFN-gamma production by autologous and HLA-matched T-cells and to induce anti-tumor cytotoxic activity in an HLA-A2-restricted manner. IFN-gamma secretion by HLA-matched T-cells was measured by ELISPOT after 48 hours of co-culture with the original β2m-negative melanoma cells or AdCMVβ2m-infected melanoma cells pulsed with HLA-A2-restricted virus matrix 58–66 peptide (GILGFVFTL). As shown in Figure 6B, the number of IFNγ–positive spots (per 100.000 PBMC) observed for T cell responses against non-transduced melanoma cells were as low as those in HLA-A2+ control melanoma cells (ESTDAB-064) not stimulated with peptide. In contrast, significant elevation of ELISPOT reactivity to the peptide was observed in melanoma cells infected with AdCMVβ2m virus. Recovery of normal HLA class I expression also restored the sensitivity of melanoma cell to HLA-restricted peptide-dependent T-cell lysis as demonstrated in the 51Cr release assay. The percentage of T-cell lysis of peptide-pulsed melanoma cells transduced with AdCMVβ2m virus was higher than that of melanoma cells transduced with the control vector carrying luciferase gene (AdCMVLuc) (Fig. 6C).

## CLAIMS

1. A method of predicting or prognosticating the response of a human subject to immunotherapy, wherein the subject is suffering from a cancer disease, and wherein the method comprises using, as an indicator, expression levels of β2-microglobulin from the tumour cells of a biological sample of the subject after said subject has been treated with immunotherapy; wherein the result is indicative of a negative response if the expression levels of β2-microglobulin are under-expressed in comparison to a reference sample and/or a positive control.

2. The method of claim 1 wherein protein β2-microglobulin is used as an indicator.

3. The method of claim 1 or claim 2, wherein said result is obtainable by an immunohistochemistry method.

4. The method of any one of the preceding claims, wherein under-expressed is defined as a level of expression lower than 2/3 of the maximum score achieved in the reference sample and/or positive control.

5. The method of any one of claims 1-3, wherein under-expressed is defined as a level of expression lower or equal to ½ of the maximum score achieved in in the reference sample and/or positive control.

6. The method of any one of claims 1-3, wherein under-expressed is defined as a level of expression lower to 1/10 of the maximum score achieved in in the reference sample and/or positive control.

7. The method of any one of claims 1 to 6, wherein the reference sample are autologous normal cells from the subject.

8. The method of any one of claims 1 to 7, wherein the cancer disease is selected from the list consisting of carcinomas or adenocarcinomas.

9. The method of any one of the preceding claims, wherein the response refers to the overall survival rate.

10. The method of any one of claims 8 or 9, wherein the cancer or carcinoma is selected from the list consisting of melanoma or bladder carcinoma.

11. The method of any one of the precedent claims, wherein the biological sample is fresh tissue or paraffin embedded tissue.

12. A method for allocating a human subject suffering from cancer in one of two groups, wherein group 1 comprises subjects identifiable by the method according to claims 1 to 11; and wherein group 2 represents the remaining subjects.

13. A composition comprising a polynucleotide sequence coding for β2-microglobulin protein, for the treatment of a human subject of group 1 as identifiable by the method of claim 12.

14. A composition suitable for cancer immunotherapy for its sequential or simultaneous administration to an expression system comprising a viral vector which in turn comprises a polynucleotide sequence coding for β2-microglobulin protein, for the treatment of a human subject of group 1 as identifiable by the method of claim 12.

15. The composition of claim 14, wherein said composition comprises dendritic cells from the subject transfected with mRNA from the tumour cells of the biological sample of the subject and wherein the viral vector from the expression system is an adenoviral vector.

16. The composition of claim 14, wherein said composition comprises the BCG vaccine and wherein the viral vector from the expression system is an adenoviral vector.

17. A composition suitable for cancer immunotherapy for the treatment of a human subject of group 2 as identifiable by the method of claim 12.

18. The composition of claim 17, wherein said composition comprises dendritic cells from the subject transfected with mRNA from the tumour cells of the biological sample of the subject.

19. The treatment of any one of claims 15 to 18 which further comprises chemo- or radiotherapy.

20. The method of claim 12 or the composition of any of claims 13 to 18, wherein the cancer is melanoma of bladder carcinoma.

21. A composition suitable for cancer immunotherapy for its sequential or simultaneous administration to an expression system comprising a viral vector which in turn comprises a polynucleotide sequence coding for β2-microglobulin protein, for the treatment of a human subject of group 1 as identifiable by the method of claim 12, wherein the composition suitable for cancer immunotherapy is the BCG vaccine and wherein the subject suffers from bladder cancer.

FIGURES

**Fig. 1**

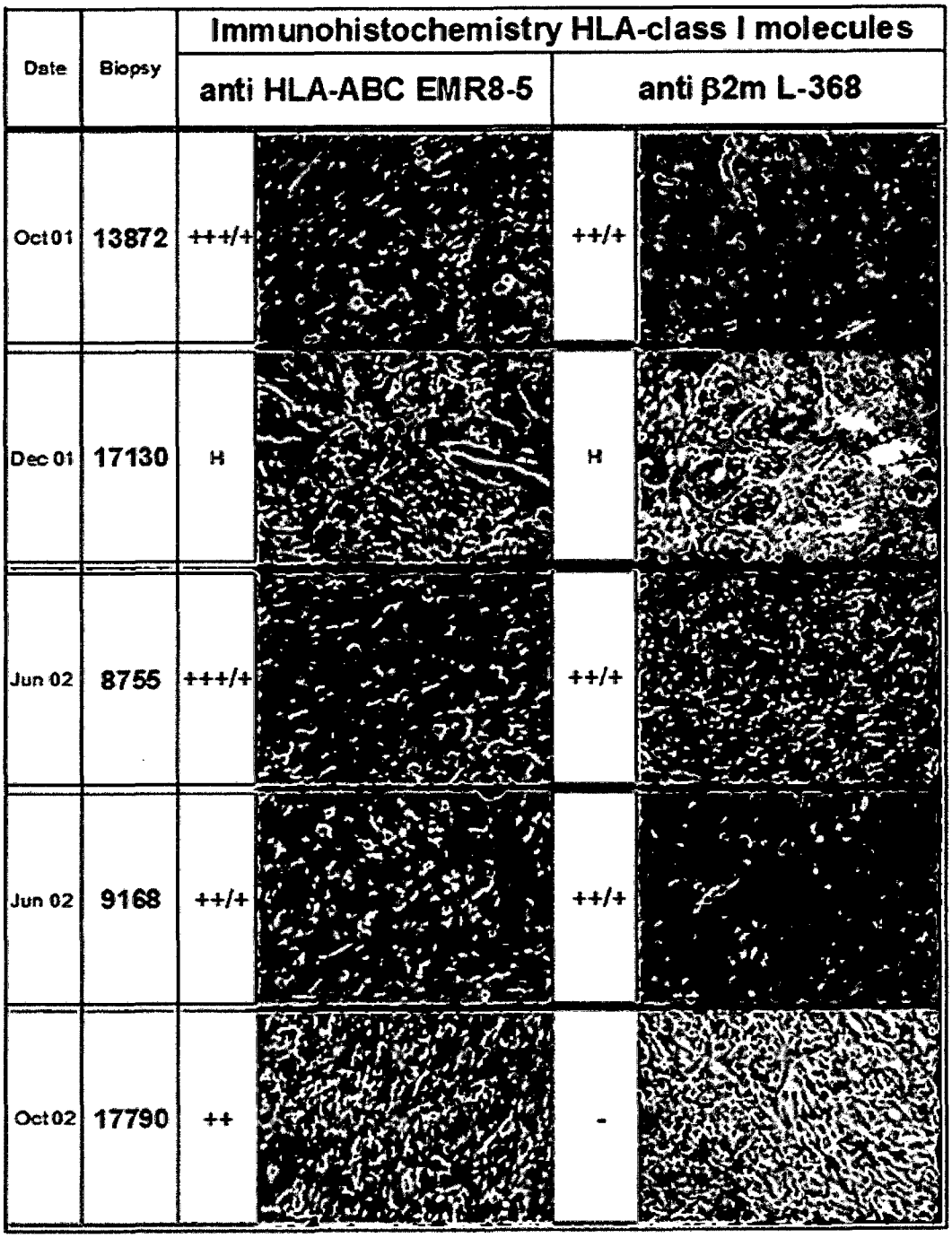


Fig. 2

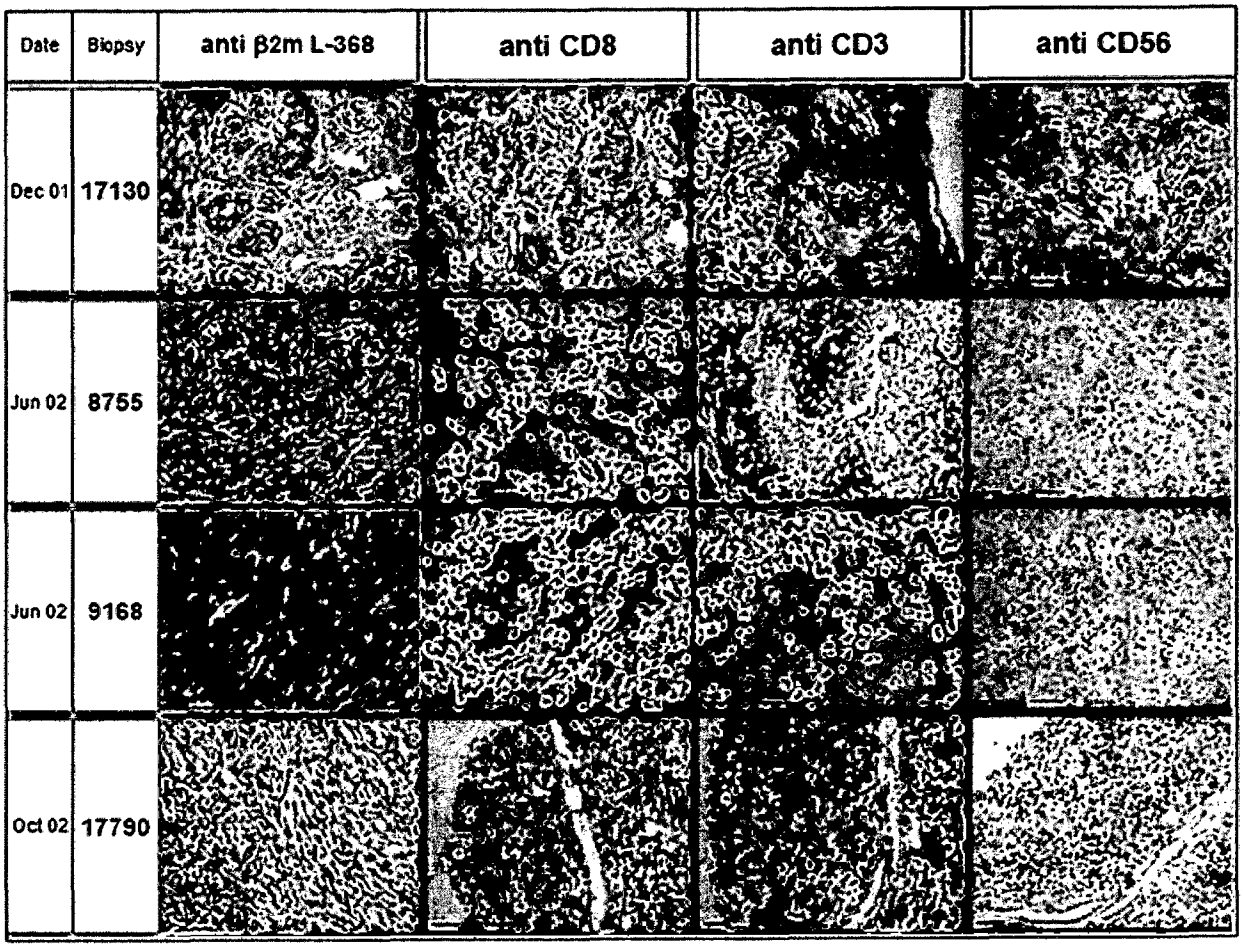
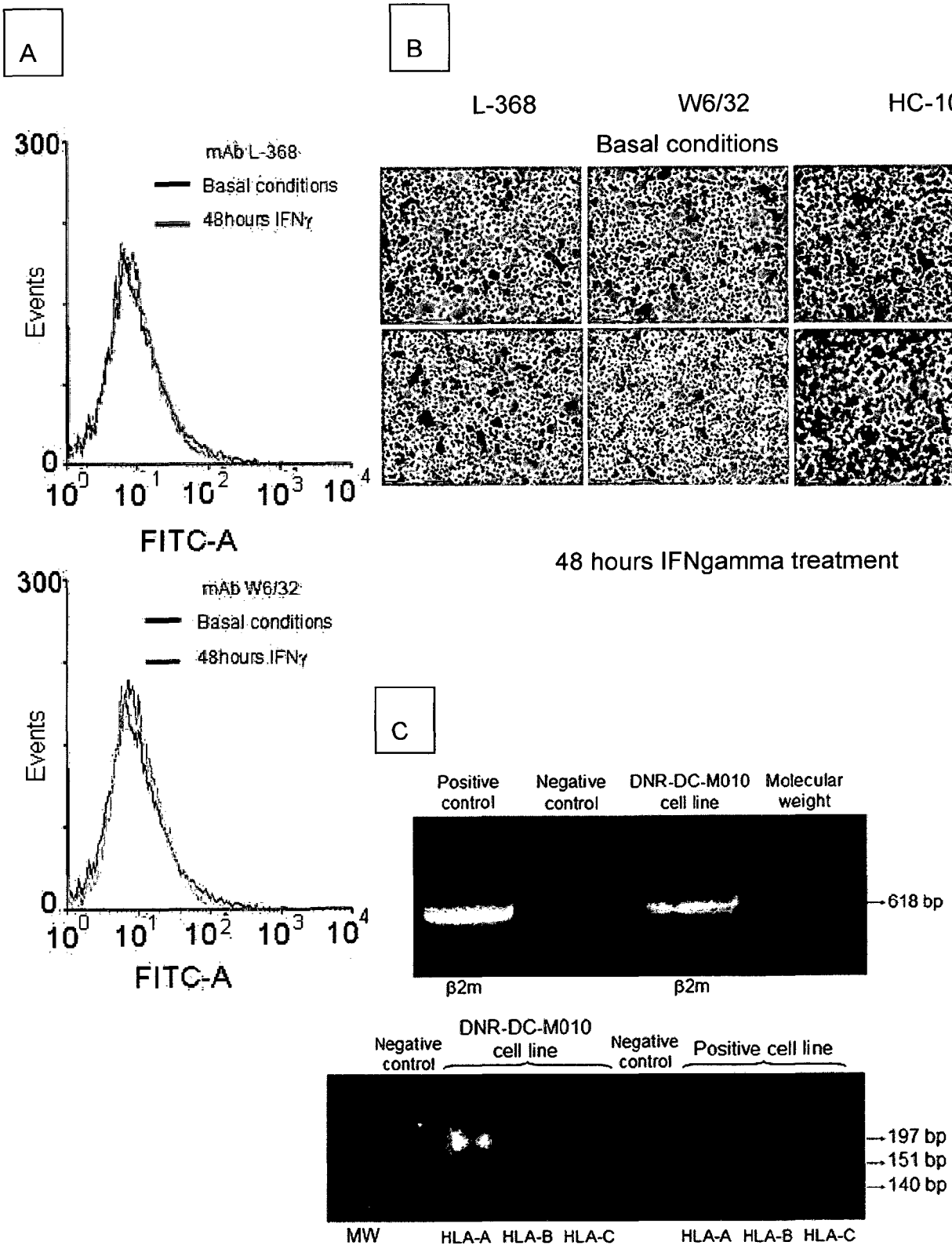

Date
Biopsy
anti β2m L-368
anti CD8
anti CD3
anti CD56
Dec 01
17130
Jun 02
8755
Jun 02
9168
Oct 02
17790

Fig. 3


A
300
mAb L-368
Basal conditions
48hours IFNγ
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FITC-A
300
mAb W6/32
Basal conditions
48hours IFNγ
Events
0
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FITC-A
B
L-368
W6/32
HC-10
Basal conditions
48 hours IFNgamma treatment
C
Positive control
Negative control
DNR-DC-M010 cell line
Molecular weight
618 bp
β2m
β2m
Negative control
DNR-DC-M010 cell line
Negative control
Positive cell line
197 bp
151 bp
140 bp
MW
HLA-A HLA-B HLA-C
HLA-A HLA-B HLA-C

Fig. 4

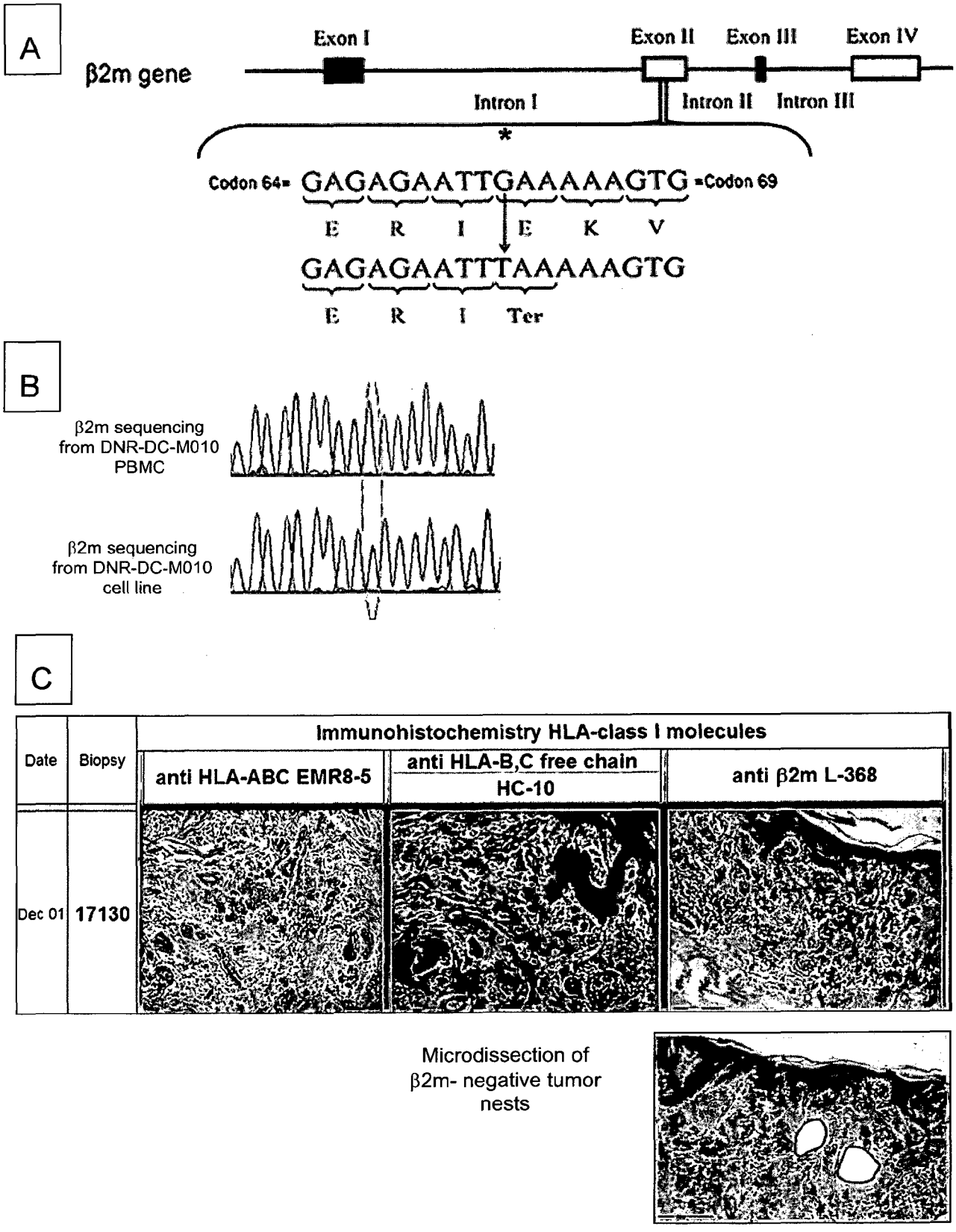
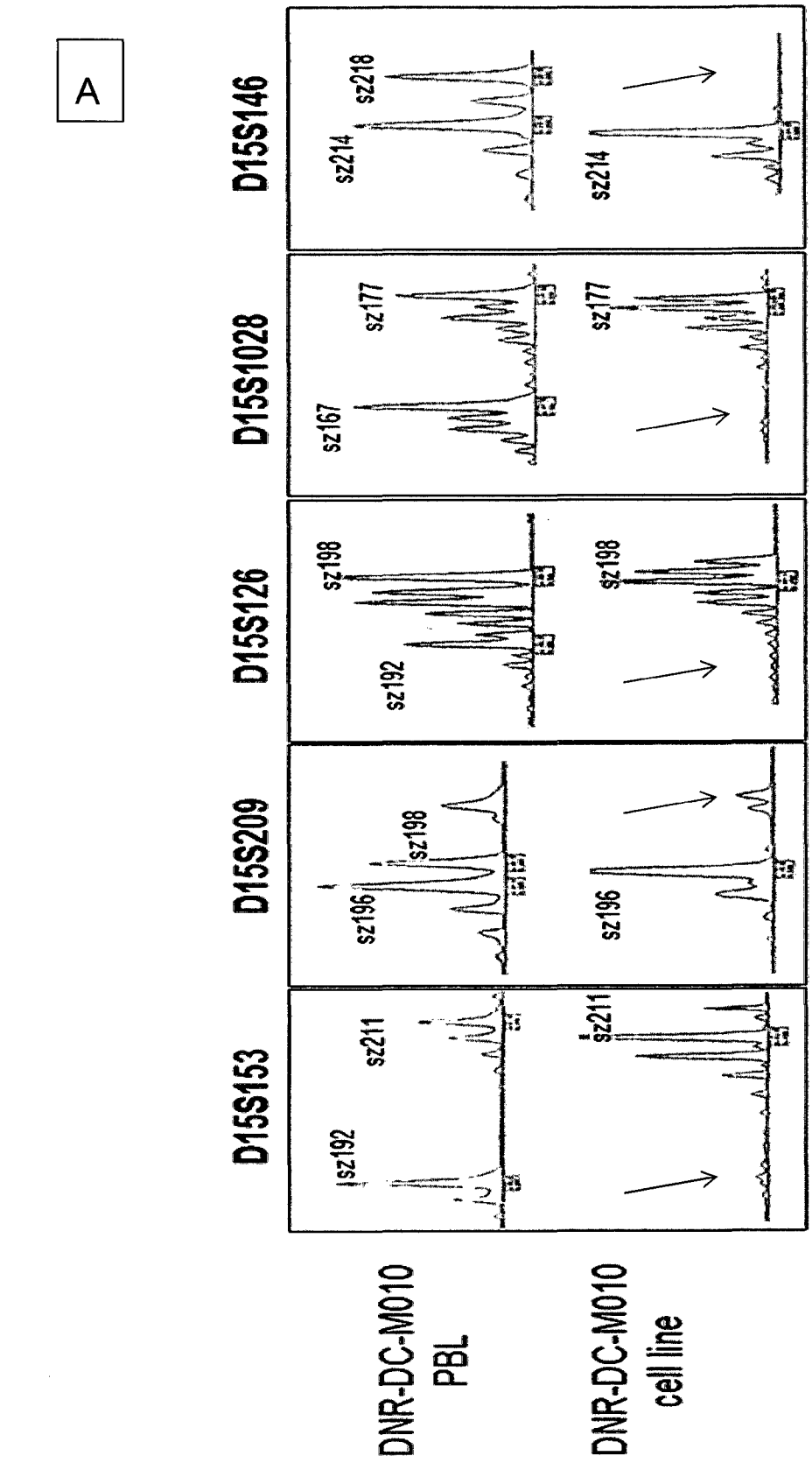

A
β2m gene
Exon I
Exon II
Exon III
Exon IV
Intron I
Intron II
Intron III
*
Codon 64= GAGAGAATTGAAAAAGTG =Codon 69
E R I E K V
GAGAGAATTTAAAAAGTG
E R I Ter
B
β2m sequencing from DNR-DC-M010 PBMC
β2m sequencing from DNR-DC-M010 cell line
C
Date
Biopsy
Immunohistochemistry HLA-class I molecules
anti HLA-ABC EMR8-5
anti HLA-B,C free chain
HC-10
anti β2m L-368
Dec 01
17130
Microdissection of β2m- negative tumor nests

Fig.5


A
D15S153
D15S209
D15S126
D15S1028
D15S146
sz192
sz211
sz196
sz198
sz192
sz198
sz167
sz177
sz214
sz218
DNR-DC-M010 PBL
DNR-DC-M010 cell line

**Continuation Fig. 5.**

B

| | | | |
|---|---|---|---|
| DNR-DC-M010-PBL | A*0205, 3201 | B*5001, 5101 | Cw* 0602, 1502 |
| DNR-DC-M010 | A*0205 | B*5001 | Cw* 0602 |

C

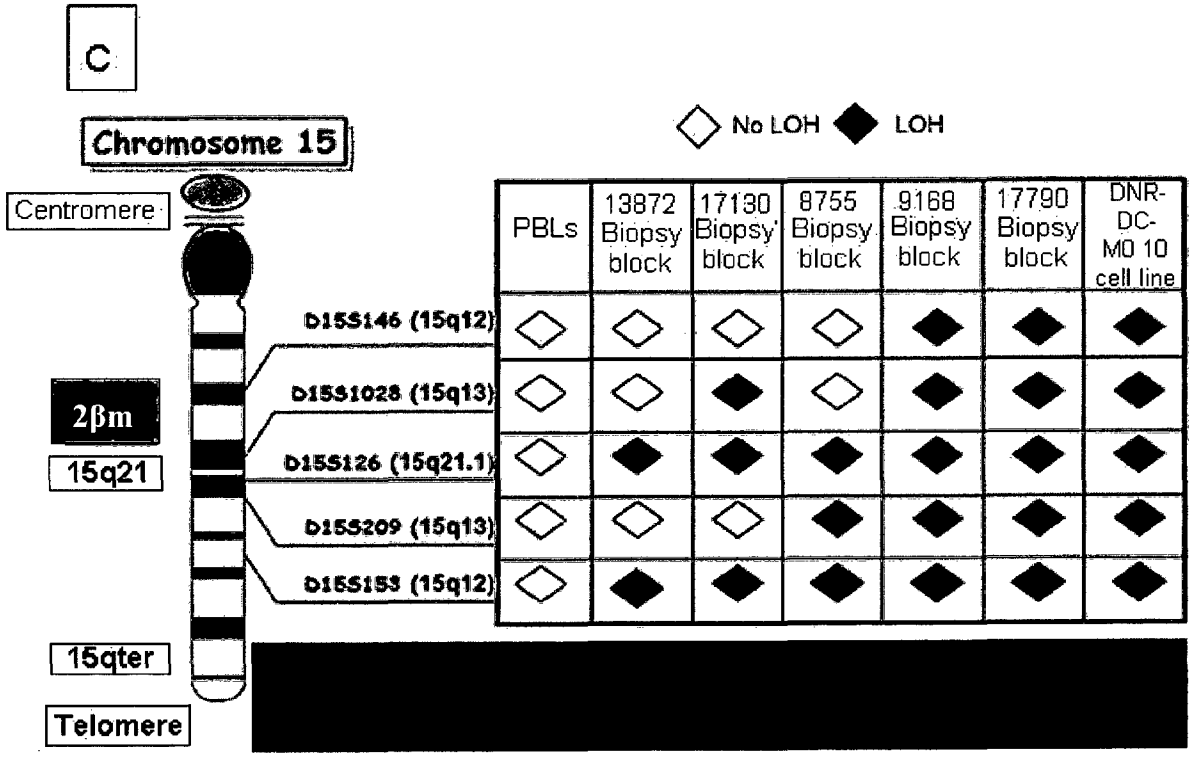
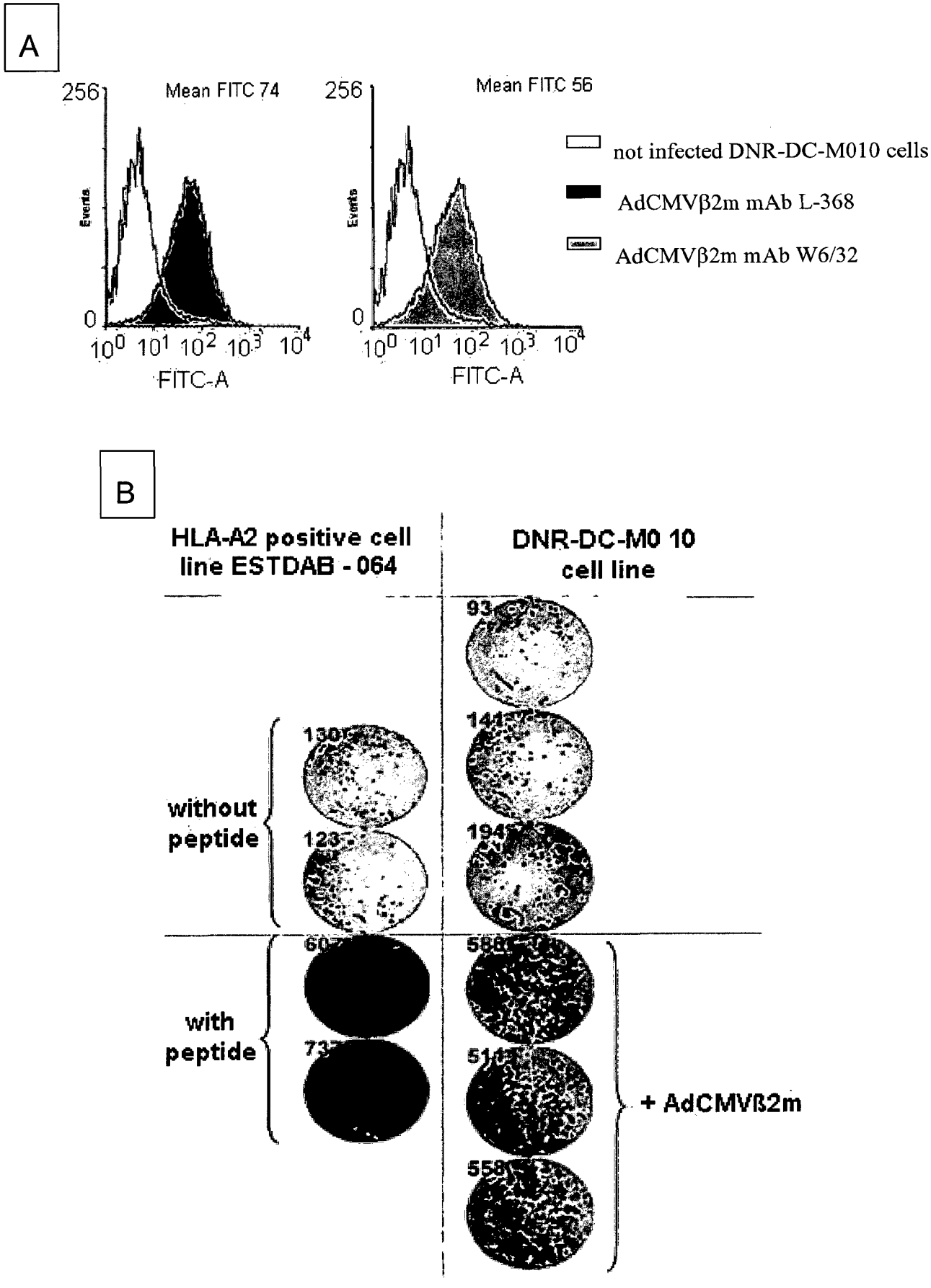


Fig. 6


A
256
Mean FITC 74
Events
0
$10^0$ $10^1$ $10^2$ $10^3$ $10^4$
FITC-A
256
Mean FITC 56
Events
0
$10^0$ $10^1$ $10^2$ $10^3$ $10^4$
FITC-A
not infected DNR-DC-M010 cells
AdCMVβ2m mAb L-368
AdCMVβ2m mAb W6/32
B
HLA-A2 positive cell line ESTDAB - 064
DNR-DC-M0 10 cell line
93
141
130
123
194
without peptide
60
588
73
511
558
with peptide
+ AdCMVß2m

**Continuation Fig. 6.**

C

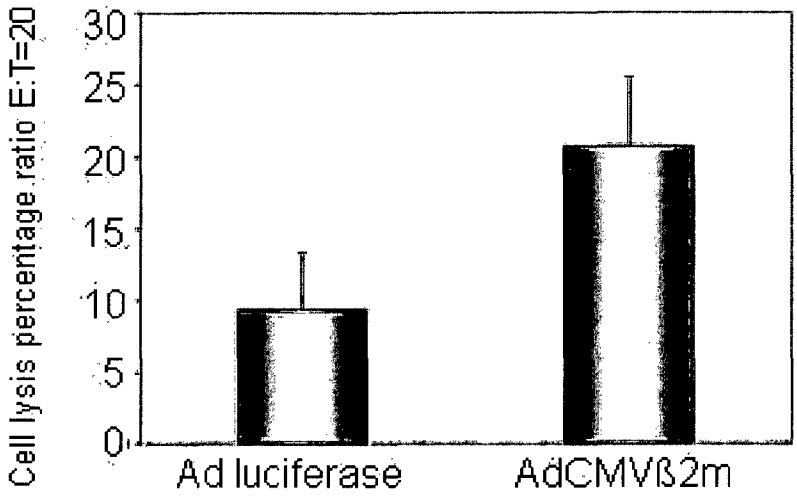


Fig. 7

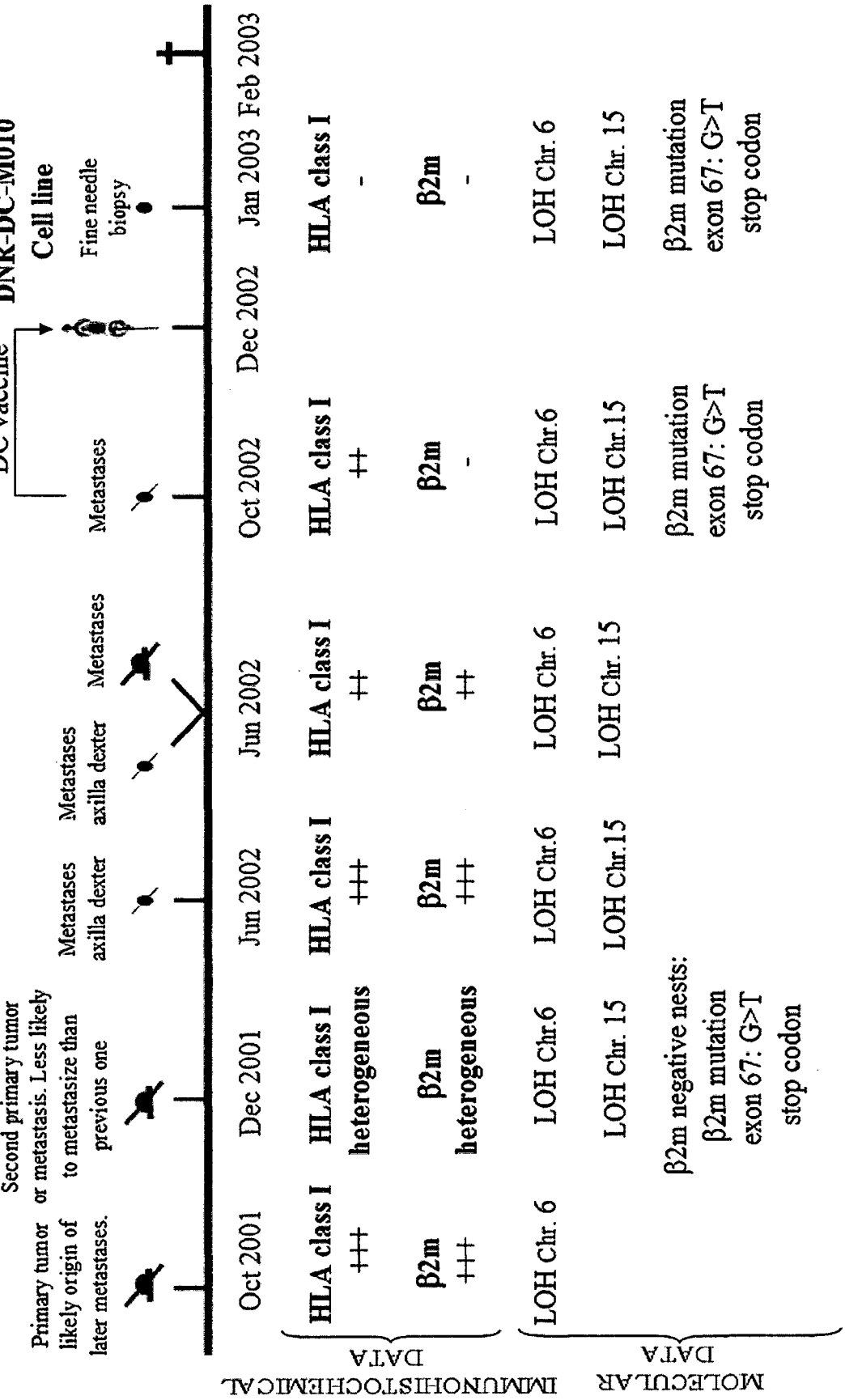

Primary tumor likely origin of later metastases.
Second primary tumor or metastasis. Less likely to metastasize than previous one
Metastases axilla dexter
Metastases axilla dexter
Metastases
Metastases
DC vaccine
DNR-DC-M010
Cell line
Fine needle biopsy
Oct 2001
Dec 2001
Jun 2002
Jun 2002
Oct 2002
Dec 2002
Jan 2003 Feb 2003
HLA class I +++
β2m +++
HLA class I heterogeneous
β2m heterogeneous
HLA class I +++
β2m +++
HLA class I ++
β2m -
HLA class I -
β2m -
LOH Chr. 6
LOH Chr.6
LOH Chr.6
LOH Chr. 6
LOH Chr.6
LOH Chr. 6
LOH Chr. 15
LOH Chr.15
LOH Chr. 15
LOH Chr.15
LOH Chr. 15
β2m negative nests: β2m mutation exon 67: G>T stop codon
β2m mutation exon 67: G>T stop codon
β2m mutation exon 67: G>T stop codon
IMMUNOHISTOCHEMICAL DATA
MOLECULAR DATA

**Fig. 8**

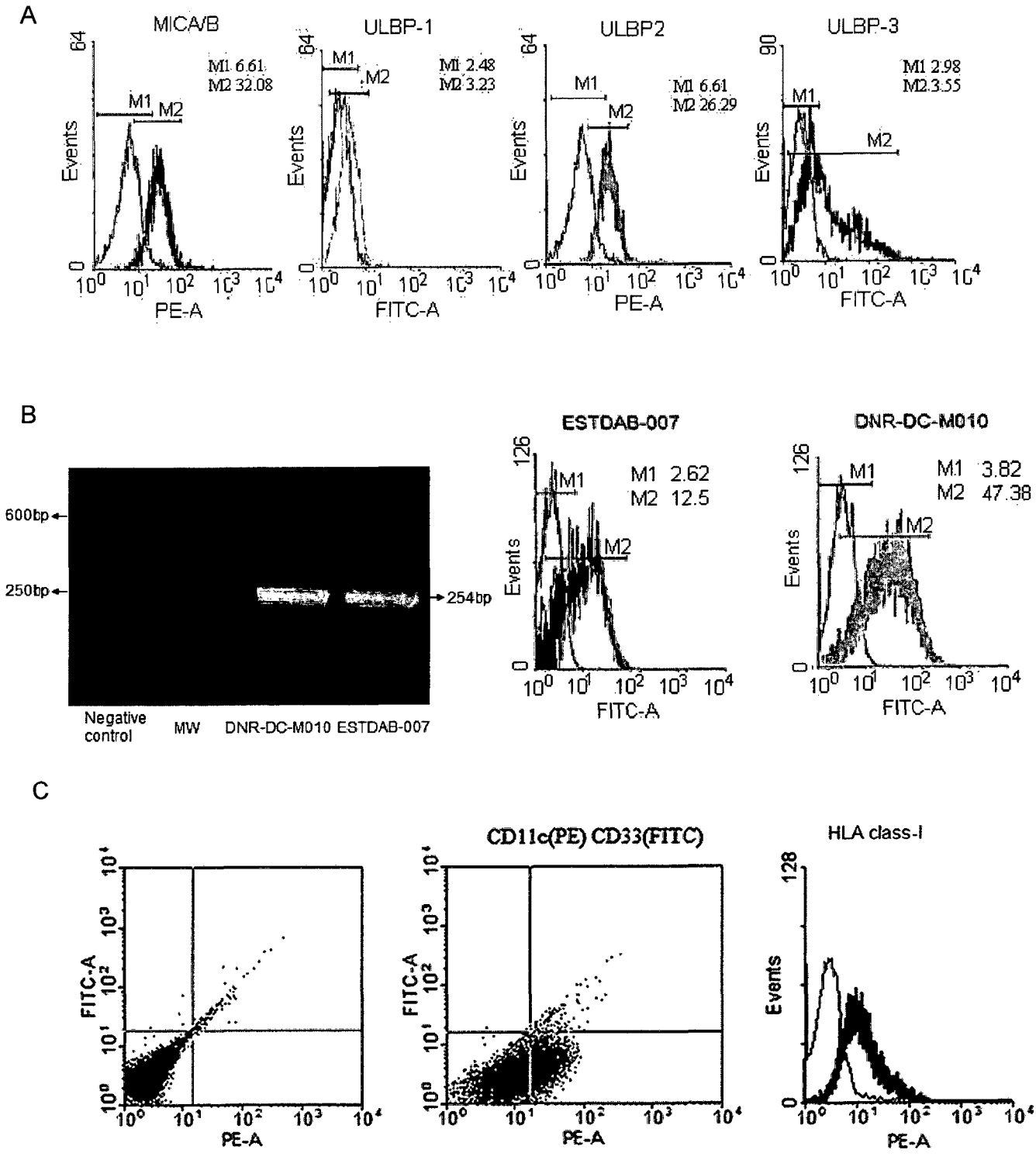

A
MICA/B
M1 6.61
M2 32.08
ULBP-1
M1 2.48
M2 3.23
ULBP2
M1 6.61
M2 26.29
ULBP-3
M1 2.98
M2 3.55
Events
PE-A
FITC-A
B
600bp
250bp
254bp
Negative control
MW
DNR-DC-M010
ESTDAB-007
ESTDAB-007
M1 2.62
M2 12.5
DNR-DC-M010
M1 3.82
M2 47.38
C
CD11c(PE) CD33(FITC)
HLA class-I

Fig. 9

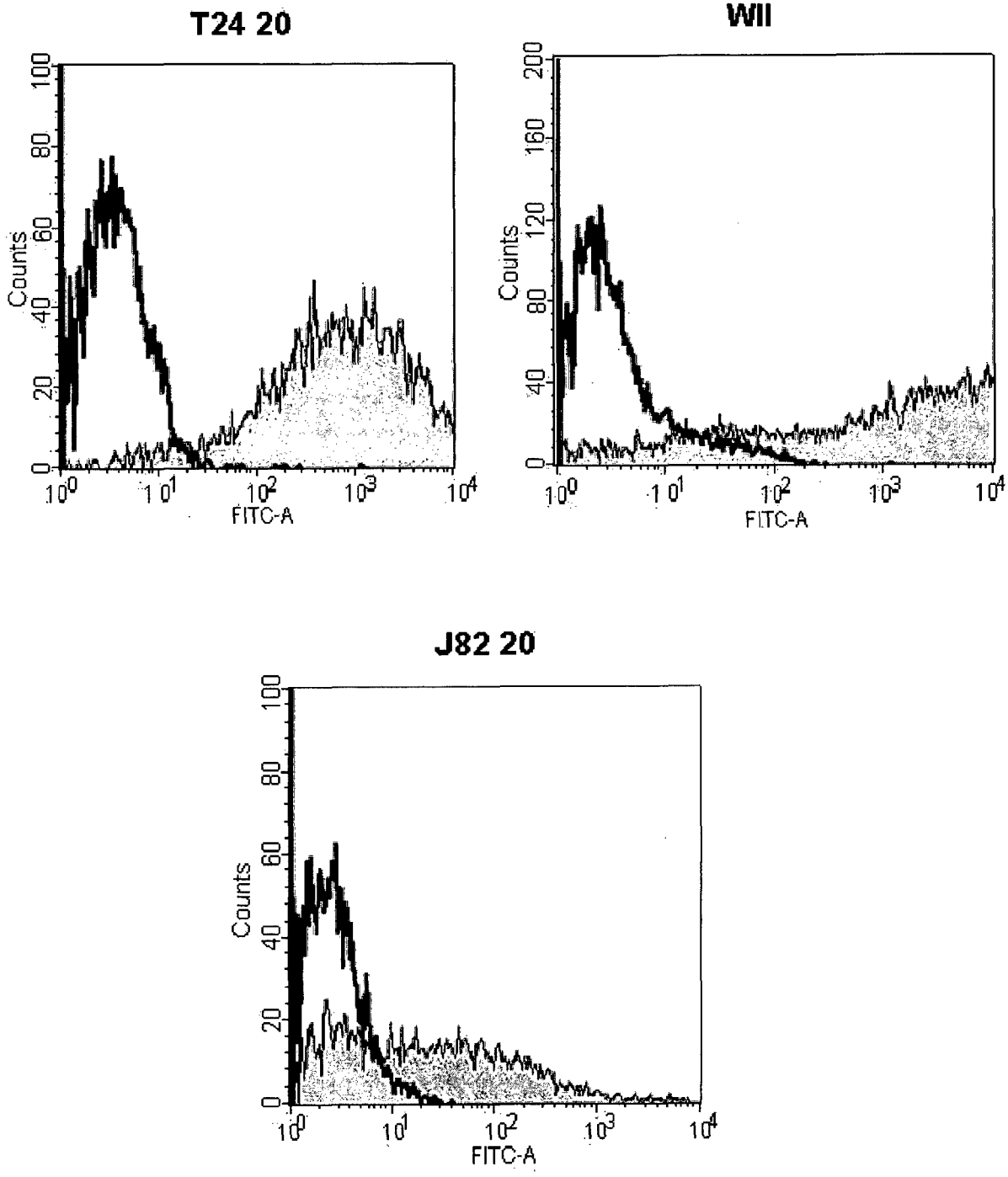
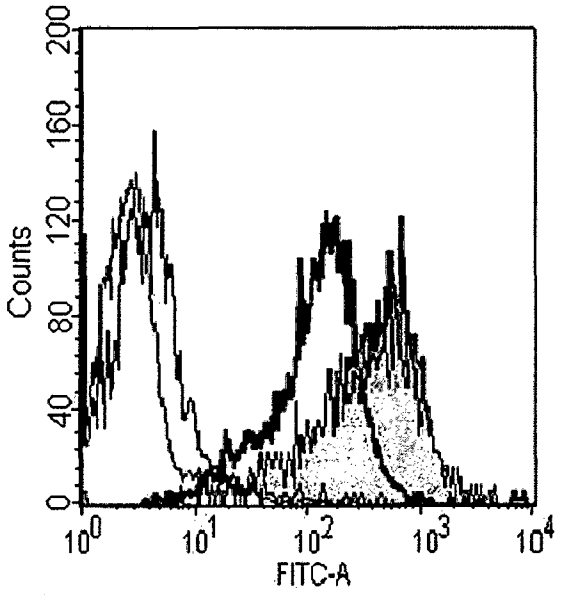


Fig. 10

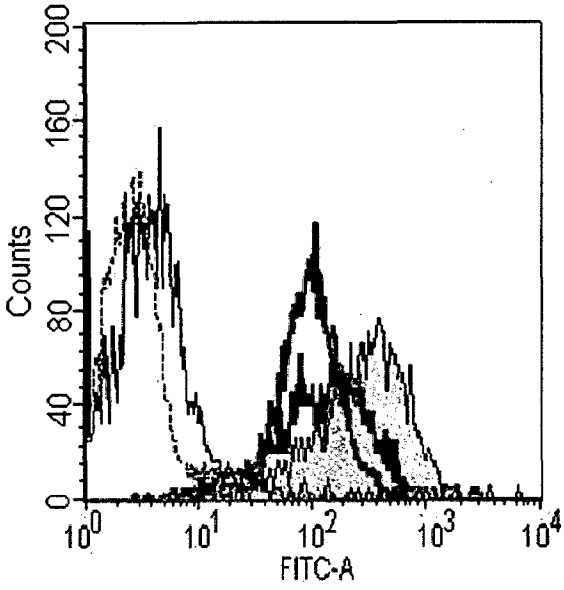
J82

Anti-β2m Ab (L-368)



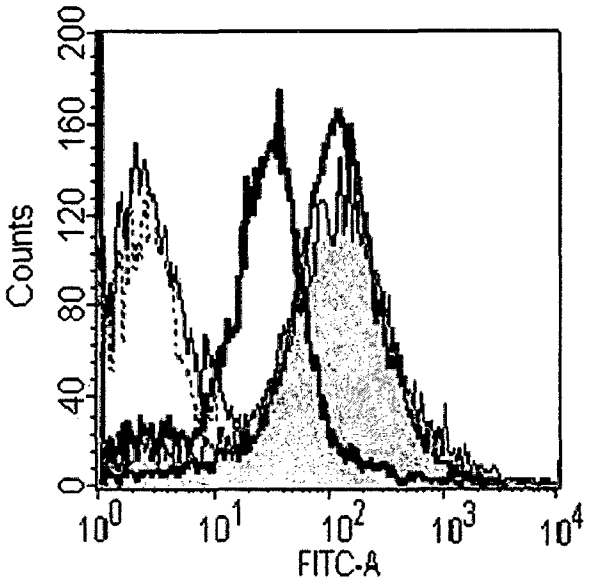

Anti-β2m Ab

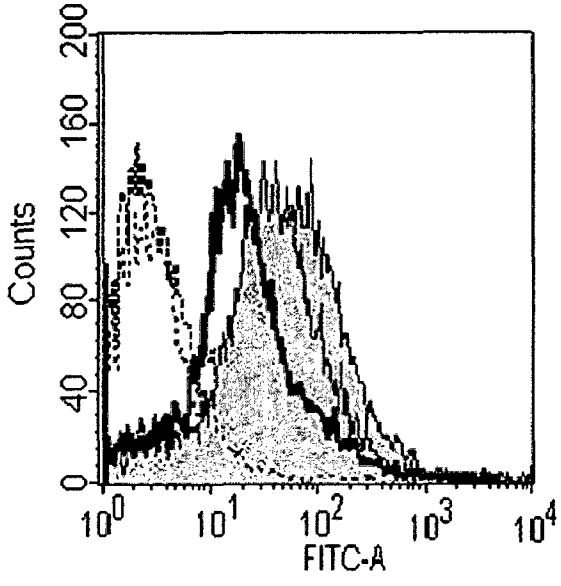
Anti-HLA complex Ab (W6/32)




Anti-HLA Ab

T24, has LOH-15



**Continuation Fig. 10.**

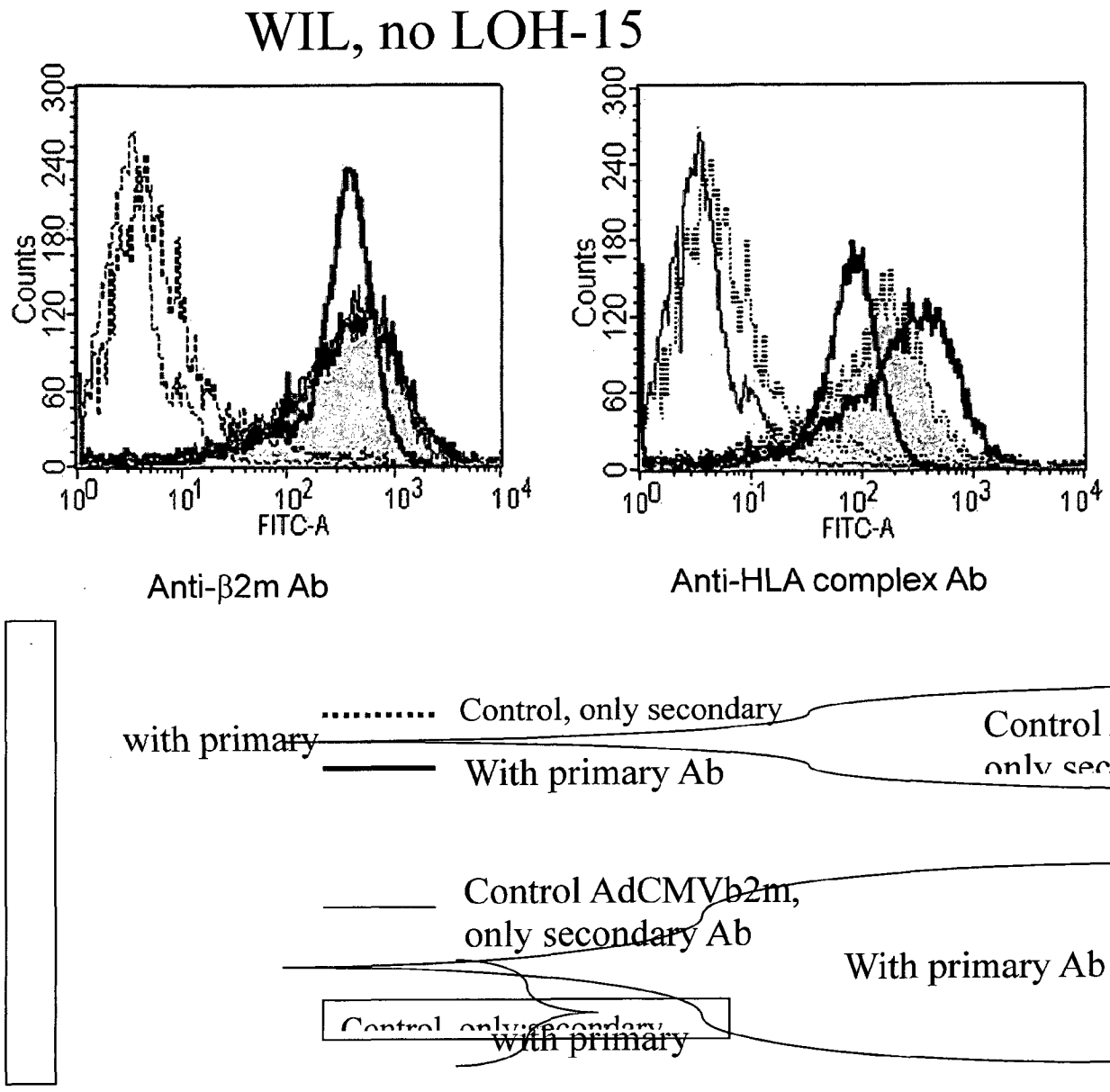
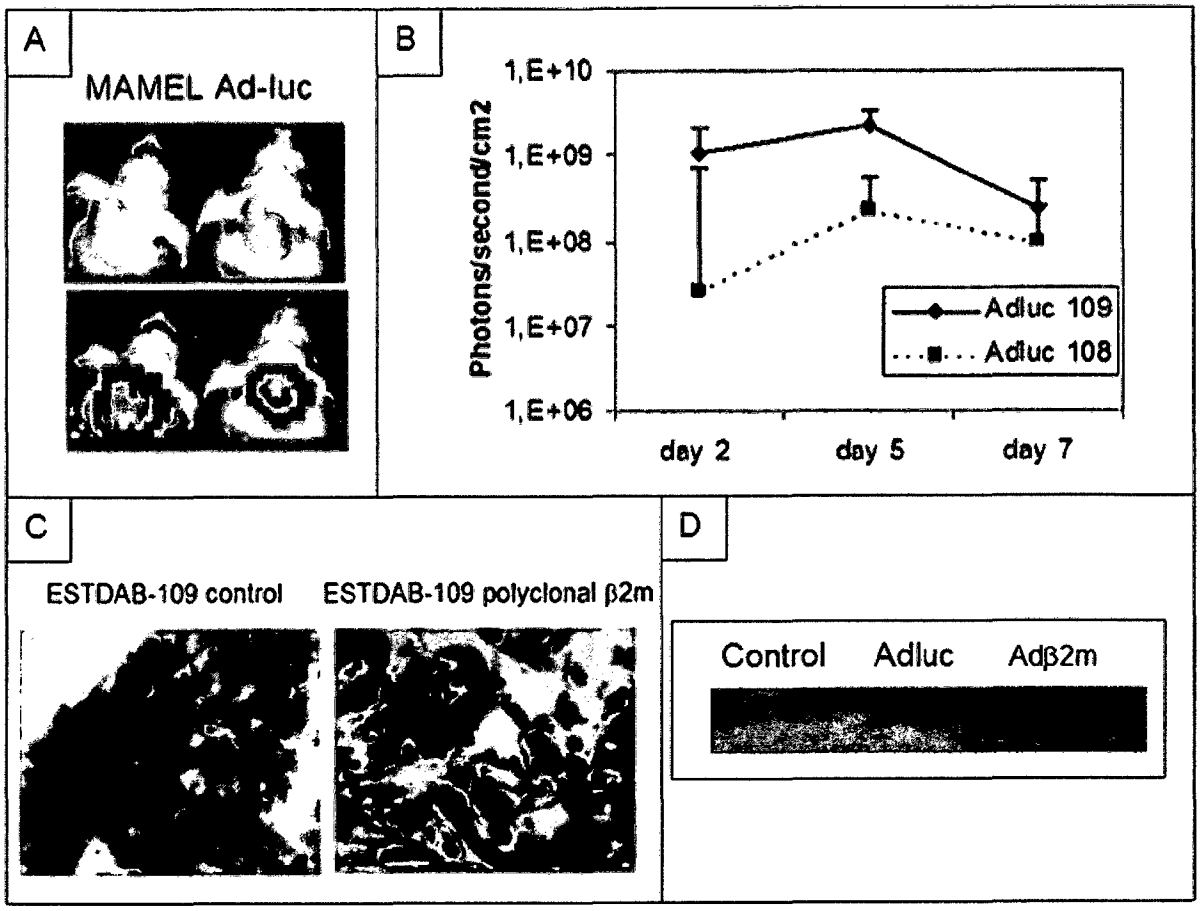


Fig. 11


A
MAMEL Ad-luc
B
Photons/second/cm2
1,E+10
1,E+09
1,E+08
1,E+07
1,E+06
day 2
day 5
day 7
Adluc 109
Adluc 108
C
ESTDAB-109 control
ESTDAB-109 polyclonal β2m
D
Control
Adluc
Adβ2m

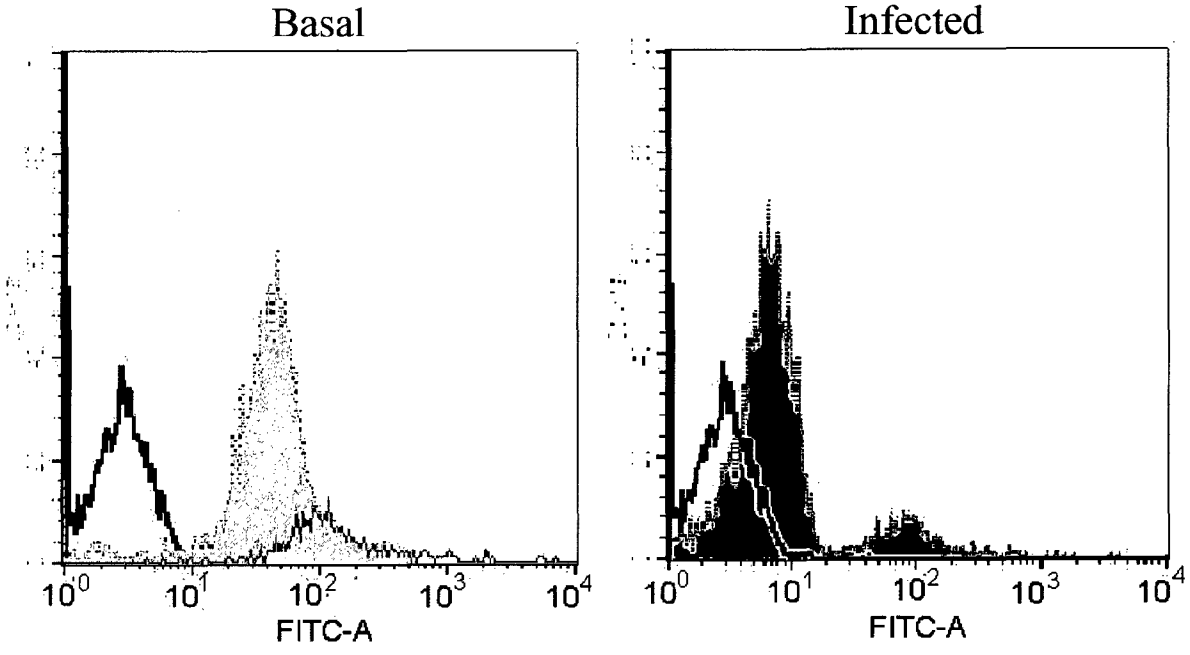

Basal
Infected
FITC-A
FITC-A

Fig.12

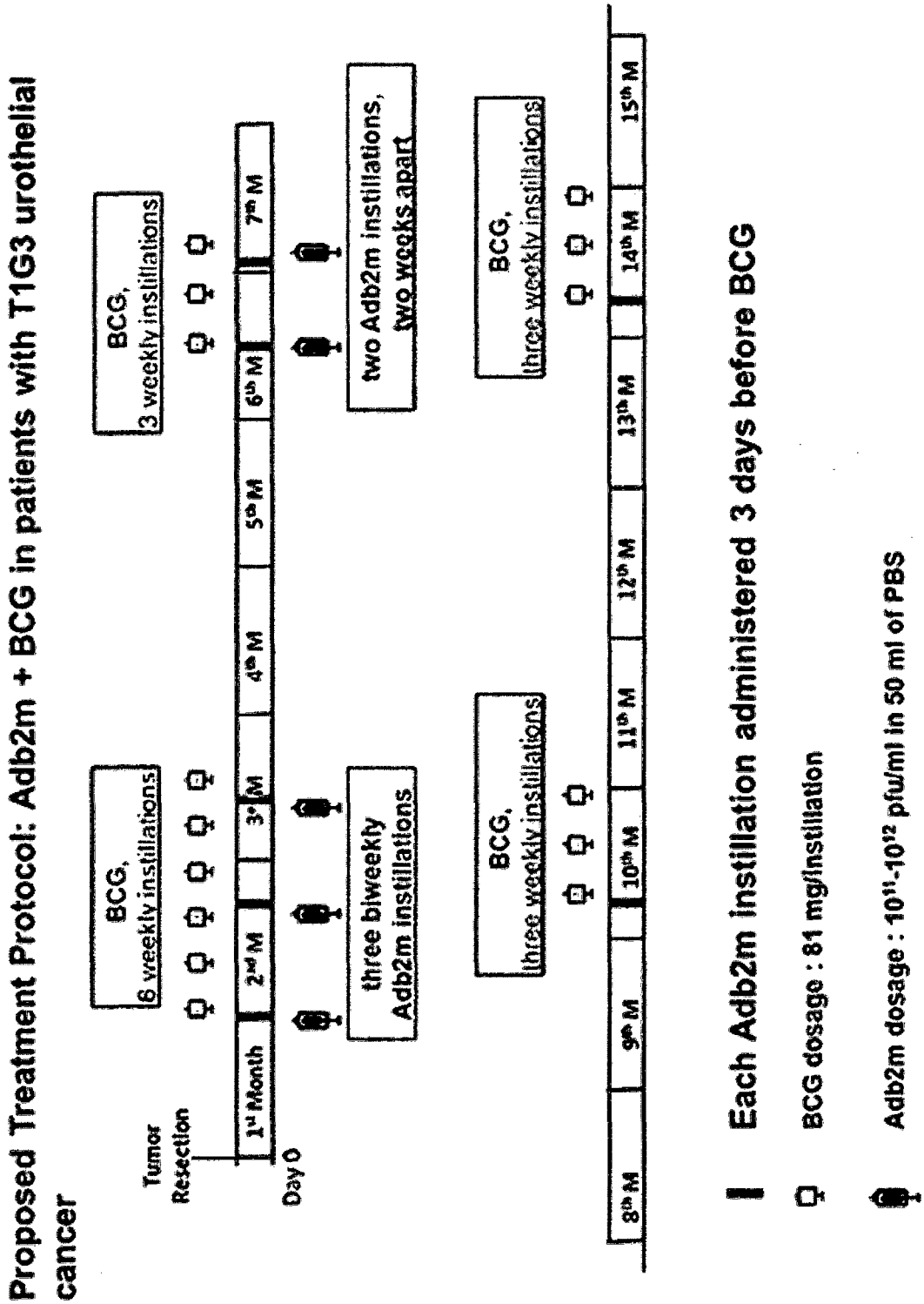
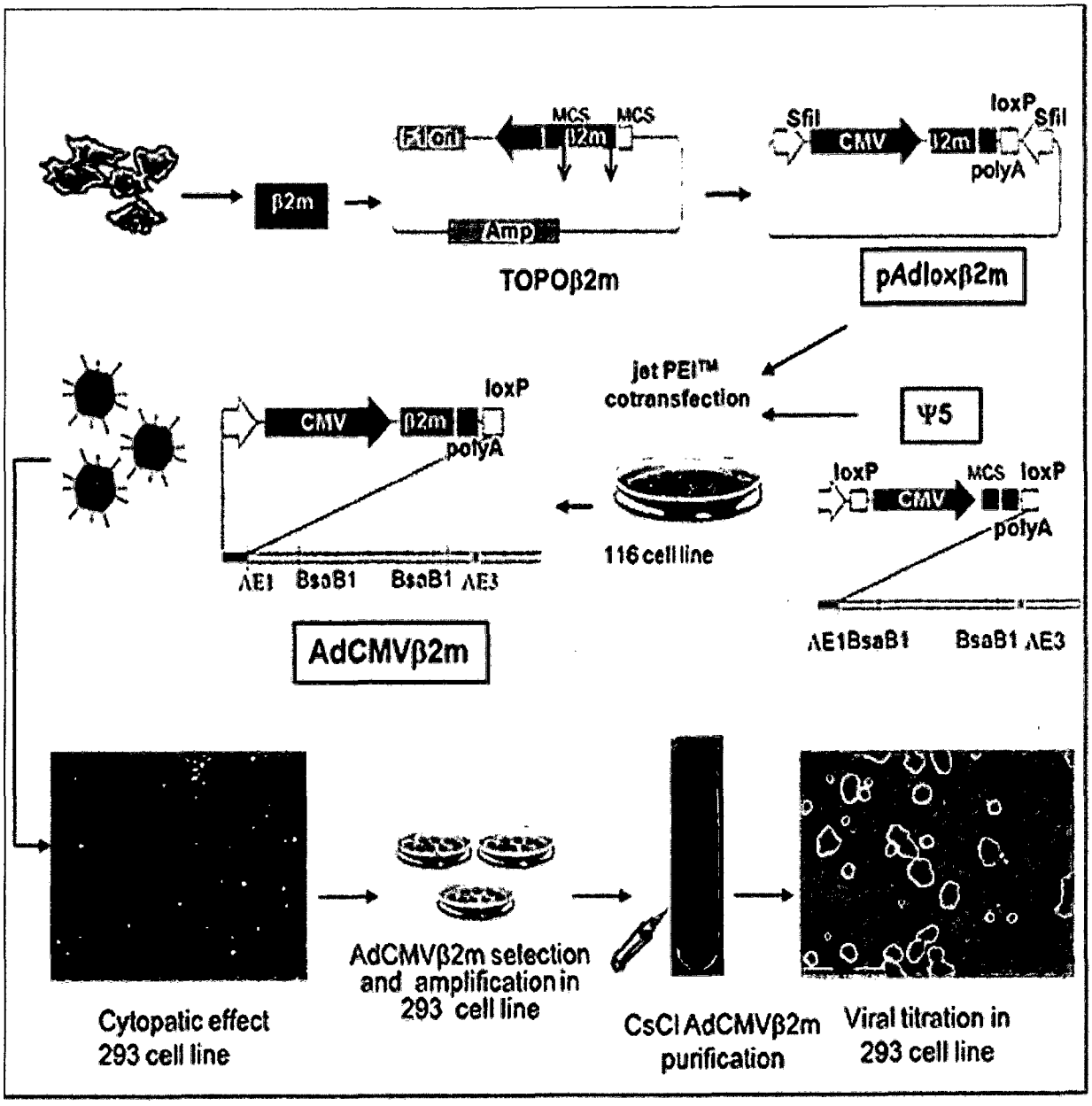

Proposed Treatment Protocol: Adb2m + BCG in patients with T1G3 urothelial cancer
Tumor Resection
Day 0
1st Month
2nd M
3rd M
4th M
5th M
6th M
7th M
8th M
9th M
10th M
11th M
12th M
13th M
14th M
15th M
BCG, 6 weekly instillations
BCG, 3 weekly instillations
three biweekly Adb2m instillations
two Adb2m instillations, two weeks apart
BCG, three weekly instillations
BCG, three weekly instillations
Each Adb2m instillation administered 3 days before BCG
BCG dosage : 81 mg/instillation
Adb2m dosage : 10^11-10^12 pfu/ml in 50 ml of PBS

Fig. 13


β2m
MCS
MCS
F1 ori
β2m
Amp
TOPOβ2m
SfiI
CMV
β2m
loxP
SfiI
polyA
pAdloxβ2m
jet PEI™
cotransfection
Ψ5
116 cell line
loxP
CMV
β2m
polyA
ΔE1
BsaB1
BsaB1
ΔE3
AdCMVβ2m
loxP
MCS
loxP
CMV
polyA
ΔE1BsaB1
BsaB1 ΔE3
Cytopatic effect
293 cell line
AdCMVβ2m selection
and amplification in
293 cell line
CsCl AdCMVβ2m
purification
Viral titration in
293 cell line

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/077702

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 A61K47/48 C07K14/74
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X | WO 2011/020839 A1 (UNIV DUBLIN CITY [IE]; RAJPAL RAJESH [IE]; DOWLING PAUL [IE]; CLYNES M) 24 February 2011 (2011-02-24) | 1-12,17 |
| Y | the whole document<br>claims 1-26<br>page 1, last paragraph - page 9, paragraph first<br>----- | 13-16,<br>18-21 |
| X | WO 2011/153514 A2 (PHARMACYCLICS INC [US]; BUGGY JOSEPH J [US]; ELIAS LAURENCE [US]; FYFE) 8 December 2011 (2011-12-08) | 1-12,17 |
| Y | the whole document<br>paragraphs [0331] - [0335], [0339]<br>paragraphs [0004], [0269] - [0272], [0330] - [0341], [0345], [0350] - [0361]; claims 1,4,18-20,44<br>claims 1-129<br>----- | 13-16,<br>18-21 |
| | -/-- | |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 27 March 2014 | 08/04/2014 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Boiangiu, Clara |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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| Y | WO 2010/037397 A1 (DAKO DENMARK AS [DK]; BRIX LISELOTTE [DK]; SCHOELLER JOERGEN [DK]; PED) 8 April 2010 (2010-04-08)<br>the whole document<br>pages 6-8; claims 1291-1293,1446-1448,<br>pages 90-100; claims 1558-1565<br>claims 803-806,1093,1422 | 1-21 |
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| Y | US 2012/046185 A1 (CHAN DANIEL W [US] ET AL) 23 February 2012 (2012-02-23)<br>paragraphs [0008] - [0027], [0132] - [0135], [0168] - [0173]; claims 1-20 | 1-21 |
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| Y | WO 2010/120897 A1 (UNIV NORTHEASTERN OHIO [US]; CEL SCI CORP [US]; ROSENTHAL KENNETH S [U) 21 October 2010 (2010-10-21)<br>the whole document | 1-21 |
| Y | WO 2011/050344 A2 (MANNKIND CORP [US]; BOT ADRIAN ION [US]; QIU ZHIYONG [US]; DIAMOND DAV) 28 April 2011 (2011-04-28)<br>the whole document<br>paragraphs [0014] - [0036], [0114] - [0118]; claims 1-62 | 1-21 |
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3

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International application No
PCT/EP2013/077702

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| | | CA 2611524 A1 | 04-01-2007 |
| | | EP 1904852 A2 | 02-04-2008 |
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| | | WO 2007002527 A2 | 04-01-2007 |
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| | | EP 2490713 A2 | 29-08-2012 |
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| | | WO 2011050344 A2 | 28-04-2011 |
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Information on patent family members

International application No
PCT/EP2013/077702

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | WO 2010110910 A1 | 30-09-2010 |

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