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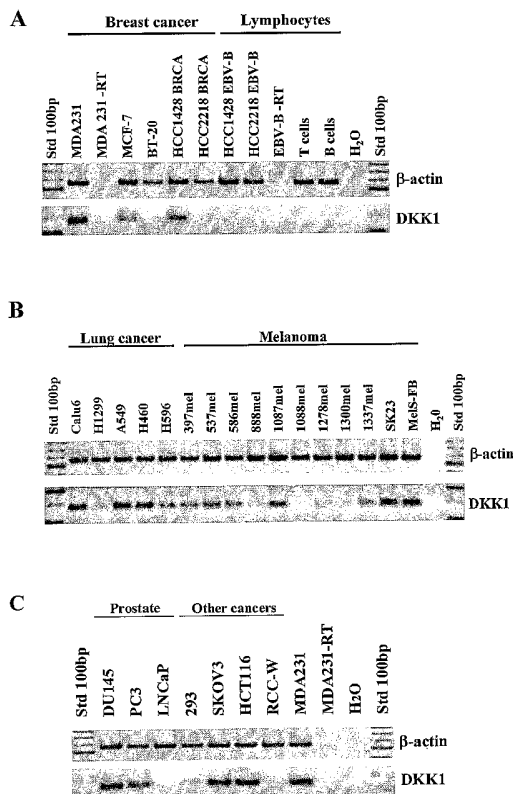
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CODING THE DKK1 PROTEIN OR POLYPEPTIDES, OR LIGANDS THEREOF FOR DETECTING TUMORS; AND FOR
ELICITING IMMUNE RESPONSE AGAINST TUMORS

(57) Abstract: The present invention relates to a use of a purified or recombinant DKK1 protein or of an immunogenic polypeptide comprising at least 8 contiguous amino acids of said DKK1 protein, and to a use of an isolated polynucleotide comprising the coding sequence of a DKK1 protein, or the coding sequence of an immunogenic polypeptide comprising at least 8 contiguous amino acids of the DKK1 protein for activating T cells.



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TITLE OF THE INVENTION

[0001] METHODS OF USE OF A DKK1 PROTEIN, IMMUNOGENIC
POLYPEPTIDES THEREOF, NUCLEIC ACID ENCODING THE DKK1 PROTEIN
OR POLYPEPTIDES, OR LIGANDS THEREOF FOR DETECTING TUMORS;
5 AND FOR ELICITING IMMUNE RESPONSE AGAINST TUMORS

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority on U.S. provisional application no.
60/601,133 filed on August 13, 2004. All documents above are herein incorporated
by reference.

10 **FIELD OF THE INVENTION**

[0003] The present invention relates to methods of use of DKK1 protein,
immunogenic polypeptides thereof, nucleic acid encoding the DKK1 protein or
polypeptides, or ligands thereof for detecting tumors; and for eliciting immune
response against tumors. The present invention also relates to DKK1
15 immunogenic polypeptides and nucleic acids encoding same.

BACKGROUND OF THE INVENTION**Cancer is a major challenge for modern medicine**

[0004] An estimated 69,000 new cases and 30,100 deaths from breast,
lung, kidney and prostate cancers will occur in Canada in 2005¹. For lung cancer,
20 the deaths/cases ratio is estimated to be 0,86 for 2005. An estimated 35,000 new
cases of renal cancers are each year reported in the USA and about 12,000
patients die from this disease each year.

[0005] Despite recent advances in the last 10 years in the field of breast
cancer, the incidence rate is still increasing since 1976¹. Cohorts of patients,

specifically the one with cancer growing independently of hormones, need alternative treatments to improve their chance of long term survival. Also, five to 10% of those cancers occur in a familial context; most of them are associated to a mutation in BRCA1 or BRCA2. Actually, about 60 to 80% of women bearing mutations in these genes will develop the disease in their live.

[0006] A Canadian has a lifetime risk of about 1:80 of developing kidney cancer (www.ncic.cancer.ca). An incomplete understanding of the molecular mechanisms underlying this disease limited up to date the development of successful non-surgical therapies. Surgery therefore remains the mainstay of treatment for kidney cancer. There is currently therefore a mortality rate of 20% within 1 year of kidney cancer diagnosis.

[0007] Although surgery, radiotherapy and chemotherapy can save many lives, cancer is still a major killer. Early detection of cancer helps to improve treatment efficacy. Still alternative strategies need to be elaborated to improve these statistics. One alternative is biological cancer therapy, defined as the use of biological materials or processes to fight tumors. In particular, cancer immunotherapy is based on prompting the immune system to specifically recognize and kill tumors. During the last 12 years, with the improvement of recombinant DNA technologies, several proteins specifically expressed by tumor cells have been identified. These proteins can serve as targets for the immune system, and immunotherapy has been found to be successful in a limited number of patients.

[0008] New diagnostic strategies are required for early detection of cancer, which will increase any therapeutic strategies. The development of an immune vaccine to prevent or delay the apparition of cancers is needed. In particular, major efforts are required to identify more tumor-specific proteins from common cancers, such as breast and kidney cancers, from which a limited number of antigens have been

discovered.

Tumor antigen oriented immunotherapy of cancer

[0009] Tumor antigens (TA) can be defined as proteins expressed in cancer cells/tumors but absent or minimally expressed in normal tissues, such as the heart, lungs and brain. Shared gene expression between tumors and normal cells from important tissues, such as the heart and lung, should be avoided for TA, since immunization against such antigens could result in autoimmunity. However, co-expression in a tissue that is not crucial for survival can be tolerated. For example, immunization against tyrosinase-related protein-1, which is expressed in melanoma and melanocytes, results in tumor rejection and skin depigmentation². The capacity of the immune system to react against TA, referred to as immunogenicity, is an important prerequisite. Although not essential, TAs are also desirably involved in tumor progression.

TA presentation to CD4⁺ and CD8⁺ T lymphocytes

15 [0010] To be recognized by the immune system, antigens are presented at the cell surface as small fragments or peptides (also called epitopes) in association with the major histocompatibility complex (MHC). There are 6 different subtypes of MHC molecules (A, B and C for class I; DP, DQ and DR for class II), and each of them is polymorphic. As illustrated in Figure 3, antigen-derived peptides presented by MHC class I originate from endogenously-expressed antigens trafficking in the cytoplasm and degraded by a protease system, the proteasome. The resulting peptides are then transported in the endoplasmic reticulum, and only peptides with sufficiently high affinity will bind to MHC class I molecules. The MHC/peptide complexes then migrate to the cell surface. Since MHC class I molecules are ubiquitously expressed, such peptide presentation can potentially occur in every tissue. In contrast to MHC class I, MHC class II presentation is mainly achieved by specialized cells of the immune system: the antigen presenting cells (APC). In this case, exogenous antigens are taken up by the APC and reach the

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endosomal/lysosomal compartment where they are degraded to peptides (see Figure 3 right panel). Again, only peptides with high affinity will bind to MHC class II, and the complex will migrate to the cell surface.

5 **[0011]** The overall function of the MHC system is to present antigens to T lymphocytes, MHC class I presentation leading to the cellular response mediated by CD8⁺ T cells, and MHC class II presentation resulting in the humoral response involving CD4⁺ T cells and B lymphocytes. CD4⁺ T cells also strengthen the CD8⁺ T cell-mediated cytotoxic response.

10 **[0012]** Interestingly, immunotherapies in general appear to be safe and well tolerated by patients with minimal side effects. Examples of protocols experienced with success with other TAs are provided below.

Immunizations with HLA-presented peptide epitopes

15 **[0013]** Objective tumor regression was observed in 42% of metastatic melanoma patients immunized with an MHC class I-restricted epitope derived from the melanoma antigen gp100 and high-dose interleukin (IL)-2²⁴.

[0014] Complete clinical responses were noted in 13% of patients immunized with dendritic cells (DC) pulsed with peptides derived from melanoma antigens²⁵.

[0015] Immunization of melanoma patients with a HLA-A1-restricted, MAGE-3-derived peptide resulted in tumor regression in 7 out of 25 treated patients²⁶.

20 **[0016]** Peptides can also be indirectly delivered by vaccination with viral vectors coding for the minimal MHC determinant from a TA. Vaccination of metastatic melanoma patients were performed with recombinant ALVAC virus bearing short MAGE-1 and MAGE-3 sequences coding for antigenic peptides presented by HLA-

A1^{27,28}.

[0017] Peptides from multiple TA pulsed on DCs can be used as a mean of immunizing patients. Eighteen metastatic melanoma patients were immunized with autologous dendritic cells pulsed with peptides derived from melanocyte/melanoma shared antigens (gp100, MART-1 and tyrosinase) and evidence of immunization and clinical benefit were reported²⁹.

[0018] Some strategies seeking to decrease negative T cell regulators such as CTLA-4, have been evaluated in clinical trials to improve the immune response against tumors. Thus, 14 patients with metastatic melanoma were immunized by using serial i.v. administration of a fully human antagonizing anti-CTLA-4 antibody (MDX-010) in conjunction with s.c. vaccination with two modified HLA-A*0201-restricted peptides from the gp100 melanoma-associated antigen, gp100_{209-217(210M)} and gp100_{280-288(288V)}³⁰. Objective cancer regressions have been observed in 21% of treated patients, but several patients suffered grade III/IV side effects, which were resolved by steroid administrations.

Immunization with the whole gene coding for a TA

[0019] Although peptide immunization, directly or presented by efficient dendritic cells (DCs), is achievable, it may be sub-optimal in view of the limited peptide/MHC complex stability at the cell surface. Ideally therefore, the expression of the whole TA in APC enables a stable and continuous production of potentially all relevant epitopes. Thus, the gene, the cDNA, or the mRNA coding for a TA expressed in APC can be used to induce a potent immune reaction and sometimes tumor regressions. Hence, tumor responses have been observed in stage III or IV melanoma patients immunized with full length recombinant MAGE-3 protein. The MAGE-3 recombinant protein was provided by GlaxoSmithKline Biologicals (Rixensart, Belgium).

[0020] Expression of TA can be mediated by transfer in DCs by different means, such as viral vectors³¹, and TA-specific T cells can be expanded using these TA-expressing DCs³¹.

5 [0021] DCs are conveniently categorized as "immature" and "mature" cells and allow for an easy discrimination of two well characterized phenotypes. Immature DCs are CD11c⁺, MHC class II⁺, CD86⁺, CD80^{low}, CD14⁻ and CD83⁻, and fail to secrete IL-12. Following proper stimulation/maturation with a combination of CD40L and lipopolisacharrides, or CD40L and poly I:C for example, CD80 and CD83 increase and they secrete high level of IL-12³³. In order to optimize their
10 capacity of stimulating TA-specific T cells, DCs may need to be properly activated or matured³². Matured-TA-expressing DCs could be a means of expanding TA-specific T lymphocytes dedicated for adoptive transfer³³. It was shown that only DCs matured with a combination of CD40L and LPS were effective in generating IFN- γ secreting MART-1-specific T lymphocytes, demonstrating the importance of
15 delivering strong maturational signals to DCs to get efficient T cell activations.

[0022] TA-expressing DCs have been shown to be effective *in vivo* for immunization in preventive and treatment of a tumor expressing β -galactosidase as a model TA in mouse³⁴. Results demonstrated that only β -galactosidase-expressing DCs or DCs pulsed with a define β -galactosidase peptide were
20 effective in treating pulmonary metastases. This was correlated by the increase in circulating lytic β -galactosidase-specific T lymphocytes.

[0023] Renal cell carcinoma patients were immunized with autologous DCs transfected with RNA coding for TA from RNA prepared from autologous tumors. Both immunological responses and clinical benefits were reported³⁵. Interestingly,
25 an increase of tumor-specific T cells after vaccination with renal tumor RNA-transfected DCs was noted, with specificity to telomerase and G250, two renal cell cancer TA.

[0024] Patients with advanced metastatic colon cancer were treated with DCs transfected with mRNA coding for the carcinoembryonic antigen (CEA). Again, immunizations were well tolerated. Both evidences of immunological responses and clinical benefits were reported³⁶.

- 5 [0025] A phase I/II clinical trial was performed using human autologous DC transfected with cDNA of the human tumor antigen mucin (MUC1) as a vaccine in 10 patients with advanced breast, pancreatic or papillary cancer. Evidences of immunizations were demonstrated and the formulation was well tolerated by patients³⁷.

10 **Adoptive transfer of TA-specific T lymphocytes**

- [0026] Adoptive T cell therapy, involving the *ex vivo* selection and expansion of antigen-specific T cell clones derived from peripheral blood or from T cells infiltrating tumors, provides a mean of augmenting antigen-specific immunity without the *in vivo* constraints that can accompany vaccine-based strategies. It
15 includes the transfer of T lymphocytes expanded *in vitro*, and genetically-modified T cells with a T cell receptor (TCR) specific to a TA.

- [0027] CD8⁺ T cell clones targeting the TAs MART-1/MelanA and gp100, were administered for the treatment of patients with metastatic melanoma³⁸. No toxicity was observed, and adoptively transferred T cell clones persisted *in vivo* in
20 response to low dose IL-2. Importantly, this strategy lead to the regression of multiple metastases.

- [0028] T lymphocytes were expanded from tumor infiltrating lymphocytes (TIL) and adoptively transferred to melanoma patients following the administration of a nonmyeloablative lymphocyte depleting regimen^{39,40}. Of 35 treated patients, 51%
25 demonstrated objective clinical responses using this approach⁴¹. This is by far the most impressive demonstration that bulky tumors can be eradicated by exploiting

the immune system in a treatment regimen. All patients had assessable disease (measurable disease on computed tomography scan or by physical exam) refractory to standard treatments including high-dose IL-2 therapy (except one patient, who did not receive IL-2 before entry into this protocol). Five (14.7%) of the
5 34 patients who received high-dose IL-2 initially responded to IL-2 therapy alone, but then exhibited progressive disease and were enrolled on this protocol.

[0029] T cells recognize peptides derived from TA presented by MHC complexes by a molecule called T cell receptor (TCR) (see Figure 3). TCR is highly polymorphic to respond to a gigantic diversity of pathogens for example. During the
10 development and selection of T lymphocytes, emerge some having TCR with a capacity to react with TA-derived peptides.

[0030] T lymphocytes from an HLA-A*0201 donor were engineered to express a TCR (α and β chains) specific to an HLA-A*0201 epitope from MART-1⁴² or gp100⁴³, both melanoma antigens. Modified T cells were expanded to a very high
15 number and they became reactive against TA-expressing tumors. Clinical assays exploiting these TCR-modified anti-tumor T cells are expected to start in a near future at the Surgery branch of the American National Cancer Institutes. Briefly, RNA isolated from the MART-1-reactive T-cell clone (M1F12) was subjected to RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) and
20 DNA sequence analysis in order to determine TCR α and β chain usage. PCR primers for cloning of the individual chain full-length cDNAs were designed. Briefly, polyA⁺ RNA was isolated from 1×10^7 M1F2 T cells using the Poly (A) Pure mRNA purification kit (Ambion, Austin, TX). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Titan One Tube RT-PCR kit
25 (Roche, Indianapolis, IN) according to the manufacturer's suggestions with the following pairs of oligonucleotide primers: forward primer cccgcggacatgttgcttgaacatttattaataatcttggatgcagc (SEQ ID NO: 1) and reverse primer gttaactagttcagctggaccacagccgcagc (SEQ ID NO: 2) (for the rearranged α

chain), forward primer cccatgggcacaaaggtgtcttctatgtggc (SEQ ID NO: 3) and reverse primer cgggttaactagttcagaaatcctttctcttgaccatggc (SEQ ID NO: 4) (for the rearranged β chain). The amplified products were gel purified and cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and subsequently confirmed by sequencing. The retroviral vector backbone used in this study, pMSGV1, is a derivative of the vector pMSGV (MSCV-based splice-gag vector) that utilizes a murine stem cell virus (MSCV) long terminal repeat (LTR; Hawley et al., 1994), and contains the extended gag region and env splice site from vector SFGtcLuc⁺ITE4⁻. A modification incorporates a naturally occurring Kozak sequence to enhance translational efficiency. Production of biologically active virus by clones with the highest relative physical titer was tested by retroviral vector transduction of SupT1 cells and analysis by fluorescenceactivated cell sorter (FACS) staining for CD3 or anti-V β 12. Southern blot analysis was used to confirm vector integration and copy number in the PG13 clone AIB 18. Its genomic DNA was extracted using Easy-DNA Kit (Invitrogen, Carlsbad, CA). PBL were obtained by leukapheresis from two HLAA2-positive melanoma patients. Lymphocytes were purified by centrifugation on a Ficoll/Hypaque cushion, washed in Hanks' balanced saline solution (HBSS) and resuspended in AIM-V medium supplemented with 50 ng/ml of OKT3, 300 IU/ml IL-2 and 5% human AB serum at a concentration of 1×10^6 cells per milliliter. The lymphocytes were cultured in 24-well plates (Costar, Cambridge, MA) for 48 hr prior to transduction. Nontissue culture-treated six-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) were treated with 25 μ g/ml of recombinant fibronectin fragment as directed by the manufacturer (RetroNectin, Takara, Otsu, Japan). Retroviral vector supernatant (4–6 ml) was added and the plates were incubated at 32°C for 2–4 hr after storage at 4°C overnight. Plates were warmed to room temperature, supernatant was removed and 10^6 stimulated PBL per milliliter were added to each well with 3–5 ml per well. These plates were incubated overnight and the transduction process was repeated the following day. After transduction, the cells were grown in the above media without OKT3 and the cultures split to maintain a density between $0.5\text{--}3 \times 10^6$ cells per milliliter.

Specificity of TCR-transduced PBMC was determined by co-culture with T2 cells pulsed with either HLA-A2–restricted influenza peptide (GILGFVFTL), MART-1₂₇₋₃₅ peptide or gp100_{209-217(210M)} in AIM-V and 5% human serum. For all of these assays, the responder cells and the stimulators (T2 pulsed) were co-cultured in a ratio of 1:1 with 100,000 cells each in 96-well U-bottom plate (Costar, Corning, NY) with a total volume of 0,2 ml for 24 hr except TNF- α , which was harvested at 6 hr. Cytokine secretion (IFN- γ or GM-CSF) was measured via enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, MA).

Need for new TA for common cancers such as breast cancer

10 **[0031]** Some breast cancer TA, such as HER-2/neu, carcinoembryonic antigen (CEA)³, MUC-1⁴, mammaglobin⁵, NY-ESO-1⁶ and MAGE-1⁷, have been identified previously. However, concerns have been raised about several of these TA. For example, CEA is also expressed in epithelial-derived normal tissues, such as the colon and stomach³, and in the context of immunotherapy, raising an immune response against this antigen could also result in a response against normal tissues from important organs. Also, some of them, such as NY-ESO-1 or HER-2/neu, are expressed in less than 25% of breast carcinomas⁸, limiting the applicability of these TA. Finally, the generation of tumor-specific T cells, using some of these antigens in patient immunization, has been inconsistent⁹. Additional TA are definitely required to include a large number of patients, and to improve the efficacy in patient TA-directed immunization strategies¹⁰.

25 **[0032]** Although some of these antigens could credibly be of clinical relevance, the identification of additional breast cancer TA might help to optimize clinical applicability¹¹. Recently, several interesting breast cancer TA candidates were identified by different means. For example, serological analysis of recombinant cDNA expression libraries¹² (SEREX) has certainly resulted in the identification of several TA, by finding antibodies from the blood of cancer patients reacting against certain TA. One recent example is metalloproteinase-1¹³, but the expression of

this particular protein was detected in several normal tissues, such as the heart, liver, muscle, pancreas and colon, limiting its applicability. Also, a family of potential breast cancer TA, known as SART was recently identified¹⁴⁻¹⁶. Although some MHC class I epitopes of this protein have been identified, mRNA expression
5 of members of the SART family was detected in several normal tissues, including the heart, spleen, pancreas and PBMC^{17,18}. Surprisingly, no SART proteins were found from the same normal tissues where mRNA was detected, and this discrepancy remains unexplained. Consequently, the clinical applicability of the SART family antigens remains questionable until it is clear that there is no
10 expression in normal tissues.

[0033] This is certainly not a complete list of all potential breast cancer TA from the scientific literature, and perhaps there is an apparent abundance of these TA. However, more TA derived from ductal or lobular breast carcinomas are required to develop immunotherapeutic strategies for a large proportion of patients.
15 Also, several TA are required to develop a vaccine strategy directed against multiple antigens, which could help to prevent loss of antigen expression in tumors, defined as immuno-selection¹⁹. The facts that available TA are frequently expressed in a low proportion of tumors, and that some candidate TA are also expressed in important tissues, such as the heart or digestive tract, are limiting the
20 applicability of immunotherapy. It is indeed critical to demonstrate immunogenicity to further translate TA identification to clinical use.

[0034] Wirths et al. (50) have described human DKK1, an antagonist of wingless/WNT signaling (involved in fetal development), as a marker for Hepatoblastomas and Wilms' tumors. Other cell types (normal and tumor cells)
25 have been tested and, for most, were DKK1-negative. Breast carcinoma samples, particularly, tested negatively, leaving DKK1 as a putative marker for a very limited number of cancer types. There is no teaching in this reference that DKK1 is a marker for largely spread cancers like breast, prostate, lung, colon, kidney and

skin cancers. There is no further teaching that DKK1 is a TA usable in an antitumoral vaccine. DKK1 binds LRP5/6 and a protein called Kremen (the product of Krm gene). This association inhibits the interaction of LRP5/6 to Fz (Frizzled), which is a key interaction for beta-cathenin pathway engagement.

- 5 **[0035]** Therefore, new TAs, alone or combined to other TAs for a given cancer type, would be extremely useful, especially for the cancers non-responding to anti-hormonal therapy.

[0036] The present invention seeks to meet these needs and other needs.

- 10 **[0037]** The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

- 15 **[0038]** The present invention concerns the use of DKK1 as a marker of tumors. It further concerns the use of DKK1 and of immunogenic polypeptides derived therefrom as a useful TA. The tumors shown to be expressing DKK1 are melanomas, prostate carcinomas, breast carcinomas, lung carcinomas renal carcinomas, ovarian and colon cancer.

- 20 **[0039]** The present invention encompasses immunization of cancer patients with various means including administering to patients DKK1 protein or immunogenic polypeptides or cells expressing same, polynucleotides encoding DKK1 protein or immunogenic polypeptides thereof.

[0040] When the immunization of cancer patients is performed with administration of a polynucleotide encoding a DKK1 protein or an immunogenic polypeptide thereof, such polynucleotide encoding a DKK1 protein can be included in a plasmid

and linked to a promoter sequence/expression control sequence, such as that from the cytomegalovirus (CMV). DKK1 under the control of a promoter can then be transferred in DCs by chemical (lipofectamine or other) means, or using non-viral or viral vectors (adenoviral or other). DKK1 expressing DCs can then administered so that reaches the lymphatic circulation, under the skin or directly in the lymphatic circulation. Vectors encoding DKK1 or an immunogenic polypeptide thereof can also be administered directly, alone or with other genes having immunogenicity enhancing capacity.

[0041] It is also concerned with the adoptive transfer in the patients of T cells activated with DKK1 protein, immunogenic polypeptides thereof, polynucleotides encoding DKK1 protein or immunogenic polypeptides thereof or APCs expressing DKK1 protein or immunogenic polypeptides thereof. MHC class I or class II restricted immunogenic polypeptides or a combination of such polypeptides can be administered s.c., or intradermal (i.d.) from 100 μ g to 1 mg several times at different intervals. Peptides can be administered as is, or in combination with adjuvants. Polypeptides can be administered after loading on matured antigen presenting cells such as DCs. In a particular embodiment, adoptively transferred T cells can be T lymphocytes from an donor engineered to express a TCR (α and β chains) specific to an epitope from DKK1.

20 DEFINITIONS

[0042] As used herein the terminology "DKK1 immunogenic polypeptide" refers to peptides from 8 to 25 residues derived from DKK1 protein sequence that can be presented by MHC molecules to stimulate either CD4⁺ or CD8⁺ T lymphocytes or both. This includes modified peptides in which a substitution of one or more amino acids were made to allow a better binding to MHC molecules, or to improve interaction with TCR. Without being so limited, the residues at position 2, 6 and 9 (Figure 9) which being the putative anchor residues of the HLA-A*0201 restricted DKK1 immunogenic polypeptides can be substituted by a leucine or

methionine residue, a valine residue, or a valine or a leucine residues, respectively. This also includes such peptides synthetically prepared with post-translational modifications (such as glycosilation, phosphorylation etc.) necessary for proper TCR or MHC molecule interaction. The sequences of polypeptides more likely to promote these interactions can be routinely predicted exploiting tools such as the SYFPEITHI⁴⁸ (<http://www.syfpeithi.de/>) tool, or Dr. Kenneth Parker's algorithms⁴⁹ (http://bimas.dcrf.nih.gov/molbio/hla_bind/). Valid epitopes after proteasome cleavage can also be predicted with the PAPROCTM tool (<http://www.paproc.de/>)^{46,47}. Although DKK1 immunogenic polypeptides are exemplified herein with HLA-A*0201 restricted DKK1 polypeptides, DKK1-derived peptides from other HLA alleles can be predicted. For example, HLA-A1 or HLA-A3 restricted DKK1 immunogenic polypeptides can be routinely identified with bioinformatics tools such as those described above.

[0043] As used herein the terminology "immune response" refers to any reaction of the immune system against a foreign biological material (i.e. antigen). As used herein the terminology "immune system" refers to the collection of organs and tissues and cells involved in the adaptive defense of a body against foreign biological material. It may be broken down into the adaptive immune system, composed of four lymphoid organs (thymus, lymph nodes, spleen and submucosal lymphoid nodules) and the group motile cells that are involved in the body's defense against foreign bodies. Without being so limited, immune response include *in vivo* or *ex vivo* "T lymphocytes activation" in an antigen-specific manner by triggering of the TCR, as illustrated by T cell proliferation, or secretion of an array of cytokines such as but not limited to GM-CSF, TNF- α , IFN- γ , IL-2, IL-4 and IL-10, or evidence of cytolytic activity such as but not limited to secretion of perforin, granzyme family members, or migration of CD107a (LAMP-1) to the cell surface or any functional assay demonstrating lysis of a relevant target. Upregulation of some surface or intracellular molecules can also serve as T cell activation markers, such as but not limited to CTLA-4, CD25 (high affinity IL-2 receptor) KI-67, or MHC

class II molecules.

[0044] Upon stimulation through the T cell receptor, T lymphocytes frequently proliferate and secrete an array of cytokines, which can be diverse, depending on cell polarization. Helper CD4⁺ T cells (Th) and cytotoxic CD8⁺ T cells (Tc) progress from naive to effector, and memory T cells to Th1, Th2, Tc1 or Tc2 profiles. Typically, Th1/Tc1-type cells and associated cytokines, such as interferon (IFN)- γ and IL-2, are secreted by lymphocytes committed to cellular responses. Conversely, Th2/Tc2-type cells and cytokines such as IL-4, IL-5 and IL-10, are secreted by lymphocytes committed to an allergic reaction and to humoral responses. Nevertheless, functions and responses to antigens diverge, depending on T cell polarization and the anatomical site. Of course, this is a simplistic view of T cell diversity, but cytokines such as IFN- γ and IL-4 help to define T cell polarization and activation. Interestingly, granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine secreted by a wide array of T cells, such as Tc1 and Tc2, and helps to identify activated cells. Qualitative evaluation of cytokine secretion by T cells can be done by ELISA. In such assays, T cells are co-cultured with their target from which specificity is assumed. When T cells are cultured with their specific target, IFN- γ is secreted and evaluated by ELISA. This approach is useful to assess T cell specificity in a cell population with a relatively high proportion of cytokine secreting antigen-specific T cells. Quantitative evaluation of cytokine secretion can also be performed by ELISPOT assays. In this assay, T cells are co-cultured with relevant targets such as DKK1 exposing cells such as tumor cells and DKK1 expressing DCs in a 96-well plate pre-coated with an antibody specific to a cytokine of interest. These cells are then removed after cytokine secretion, and cytokines captured by coated antibodies are revealed in a sandwich assay similar to an ELISA reaction. This results in spots in individual wells, which reflects the number of reactive T cells originally dispersed in the well. The spots are enumerated by an automated ELISPOT counter, so discrimination criteria are applied equally throughout all the plate. Identification of specific T cells

can be achieved using peptide/MHC class I tetramers. Soluble MHC molecules are first synthesized and complexed with a peptide from the antigen of interest. The soluble MHC/peptide molecule is then labeled and combined in a multimer structure with biotin-streptavidin reagents. TA-specific T cells are then revealed by
5 flow cytometric analyses.

[0045] In a method for monitoring the progressing of a cancer in a patient encompassed by the present invention, the subsequent point in time after having detecting for the first time DKK1 in the patient may be any convenient time after a previous detection of DKK1 in the patient. It may depend on the type of cancer and
10 could be adapted to coincide with other recommended periodical tests for that cancer. Without being so limited, it could be any time after the previous detection of DKK1 in the patient from about 1 week to about 7 years. Without being so limited, when the cancer is a breast cancer, a convenient subsequent point in time could be one year or 15 months after the previous test. For lung cancer, a
15 convenient subsequent point in time after could be about a month or two months after the previous test.

[0046] The present invention encompasses the use of a DKK1 polynucleotide sequences as disclosed in Figure 1 along with DKK1 polynucleotide sequences substantially identical to those. Namely any DKK1 polynucleotide
20 sequence that has at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence such as
25 those disclosed in Figure 1 using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid

similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 70%, more preferably at least 80%, 90%, and most preferably at least 95%.

5 [0047] Another indication that two polynucleotide sequences are substantially identical is they hybridize to each other under stringent conditions (see below). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are
10 substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

15 [0048] The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces
20 minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

[0049] "Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as
25 Southern and Northern hybridization are sequence dependent, and are different under different environmental parameters. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a

perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, 1984; $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, $\%GC$ is the percentage of guanosine and cytosine nucleotides in the DNA, $\% \text{ form}$ is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $>90\%$ identity are sought, the T_m can be decreased 10°C . Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point T_m ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point T_m ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point T_m . Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific sequence at a defined ionic strength and pH.

[0050] An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC

wash at 65°C for 15 minutes. Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1X SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6X SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C and at least about 60°C for long robes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2X (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

[0051] The term "substantial identity" in the context of a DKK1 polypeptide indicates that a polypeptide comprises a sequence with at least 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, or even more preferably, 95%, 96%, 97%, 98% or 99%, sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a polypeptide is substantially identical to a second peptide, for example, where the two polypeptides differ only by a conservative substitution e.g.

not substantially affecting the folding, the charge, the molecular weight, the lipophilicity or the final composition (glycosylation for exemple) of a polypeptide.

[0052] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0053] The invention encompasses isolated or substantially purified nucleic acid or protein compositions. In the context of the present invention, an "isolated" or "substantially purified" DNA molecule or an "isolated" or "substantially purified" polypeptide is a DNA molecule or polypeptide that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated DNA molecule or polypeptide may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell. An isolated or purified DNA or polypeptide may be synthesized chemically, may be produced using recombinant DNA techniques and then isolated or purified or may be isolated or purified from its natural host. An "isolated" or "substantially purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques and, in some circumstances, further purified, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5kb,

4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A DKK1 protein, or an immunogenic polypeptide thereof, that is purified or substantially free of cellular material includes preparations of protein or polypeptide having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the DKK1 protein, or immunogenic polypeptide thereof, is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein of interest chemicals.

10 **[0054]** Recombinant DKK1 proteins or immunogenic polypeptides thereof encompassed by the present invention also include fusion protein comprising not only DKK1 proteins or immunogenic polypeptides thereof but also heterologous domains for enhancing immunogenicity, including adjuvants and amino acid sequences enabling epitope cross-presentation. The term cross-presentation
15 denotes the ability to take up, process and present exogenous antigens with MHC class I molecules to CD8⁺ T cells (= cytotoxic T cells). Without being so limited, a helper epitope from an other protein such a protein D from *H. influenzae* as described in Example 7 is useful for enhancing immunogenicity. Recombinant DKK1 proteins or polypeptides encompassed by the present invention may further
20 include domains for facilitating purification such as a histidine tag. The DKK1 proteins useful in the present invention encompass mature a DKK1 protein i.e. without its signal peptide (see Figures 1). The mature protein of the DKK1 presented in figure 2 consists of residues 32 to 266 of this protein.

[0055] Useful vectors for transfecting cells to be used as APCs include
25 plasmids where DKK1 polynucleotide expression would be controlled by a promoter sequence, such as that from the cytomegalovirus (CMV). Viral vectors can also be exploited to transduce APCs, such as but not limited to adenoviruses, lentiviruses or classical retroviruses.

[0056] Useful vectors for direct injections (i.e. without APCs) of DKK1 or immunogenic polypeptides thereof include the ALVAC virus, and non-viral plasmids including those from VICAL technologies (See <http://www.vical.com/company/dnatech.htm>).

- 5 **[0057]** The term "ligand" when used herein in reference to a DKK1 protein or immunogenic polypeptide refers to antibodies, or a natural DKK1-binding domain from natural receptors including LPR5/6 and the Kremen protein⁵¹, agonists, antagonists and to any molecule found to bind DKK1 protein or immunogenic polypeptide from an assay on a combinatorial library namely, from a
10 phage display library.

[0058] The term "ligand" when used herein in reference to a DKK1 polynucleotide encoding a DKK1 protein or immunogenic polypeptide refers to a complementary strand to one of the strands of a DNA, a cDNA, or an RNA, the complementary strand being used as a probe or as an amplification primer.

- 15 **[0059]** Useful APCs for the present invention include any cells with the ability to engulf DKK1 protein or immunogenic polypeptide thereof and present them to the cells of the immune system in a form that can be recognized by those immunocompetent cells. Without being so limited it includes DCs expressing DKK1 protein or immunogenic polypeptide thereof and B lymphocytes. In both
20 instances (ligand to polypeptide or to polynucleotide), the ligands may be labeled by any direct or indirect means for proper detection.

- [0060]** As used herein the term "expression control sequence" refers to a sequence that promotes expression of a DKK1 protein or an immunogenic polypeptide thereof in a host cell including a tumor cell and a natural or
25 recombinant APCs. Without being so limited, such sequence includes the CMV expression control sequence.

Pharmaceutical Compositions

[0061] The present invention also provides a pharmaceutical composition comprising a immunogenically effective amount of DKK1 or immunogenic polypeptide thereof, and one or more pharmaceutically or physiologically acceptable carriers, adjuvants and diluents. Examples of adjuvants include incomplete Freund's adjuvant (IFA, also called Montanide, commercialized as ISA-51, Seppic Company, Paris, France), CpG sequences such as but not limited to CpG 7909 (TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 5); Coley Pharmaceutical Group), different cytokines with T cells stimulatory capacity such as IL-2, IL-7, IL-12, IL-15, or others, cytokines promoting activation of antigen presenting cells such as GM-CSF, CD40L, FLT3L, or others. Adjuvants can also include products derived from pathogens such as detoxified lipopolysaccharides (Monophosphoryl lipid A or MPL, Corixa Corporation) or mycobacterial walls (Bioniche). Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or weekly, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. The active compound may be administered in a convenient manner such as by the oral, intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (e.g., using slow release molecules or devices). The DKK1 or immunogenic polypeptide thereof, may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0062] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders of the extemporaneous dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action

of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, buffered saline such as PBS, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0063] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

[0064] More particularly, the present invention thus relates to a use of a purified or recombinant DKK1 protein or of an immunogenic polypeptide comprising at least 8 contiguous amino acids of said DKK1 protein, for activating T cells. In a specific embodiment, the DKK1 protein comprises an amino acid sequence as set forth in any one of SEQ ID NO: 8 and residues 32 to 266 of SEQ

ID NO: 8. In an other specific embodiment, the DKK1 immunogenic polypeptide consists of a maximum of 25 amino acid residues and comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15, 34 and 38-41. In an other specific embodiment, the DKK1 immunogenic polypeptide consists of a maximum
5 of 25 amino acid residues and comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15. In an other specific embodiment, the purified or recombinant DKK1 protein or the immunogenic polypeptide is expressed on an antigen presenting cell (APC). In an other specific embodiment, the APC is a dendritic cell transformed to express the purified or recombinant DKK1 protein or
10 immunogenic polypeptide comprising at least 8 contiguous amino acids of the DKK1 protein.

[0065] According to an other aspect of the invention, there is provided a use of an isolated polynucleotide comprising the coding sequence of a DKK1 protein, or the coding sequence of an immunogenic polypeptide comprising at least 8
15 contiguous amino acids of the DKK1 protein, for activating T cells. In a specific embodiment, said isolated polynucleotide is comprised in an expression vector. In an other specific embodiment, the vector further comprises a polynucleotide encoding an immunogenicity enhancing polypeptide. In an other specific embodiment, the coding sequence is as set forth in any one of SEQ ID NO: 7 and
20 nucleotides 248 to 952 of SEQ ID NO: 6. In an other specific embodiment, the coding sequence encodes an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15, 34 and 38-41. In an other specific embodiment, the coding sequence encodes an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15.

25 **[0066]** According to an other aspect of the invention, there is provided a use of a purified or recombinant DKK1 protein or of an immunogenic polypeptide comprising at least 8 contiguous amino acids of said DKK1 protein, in the making of a medicament for activating T cells. There is also provided a use of an isolated

polynucleotide comprising the coding sequence of a DKK1 protein, or the coding sequence of an immunogenic polypeptide comprising at least 8 contiguous amino acids of the DKK1 protein, in the making of a medicament for activating T cells.

[0067] According to a further aspect of the invention, there is provided a method for monitoring the progression of breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in a patient, with the proviso that the kidney cancer is not Wilms' tumor, comprising the steps of: (a) obtaining from the patient a biological sample susceptible of containing tumor cells; (b) contacting the biological sample with a ligand to a DKK1 protein or an immunogenic polypeptide thereof, or to a polynucleotide encoding said protein or said polypeptide to form a complex; (c) measuring the amount of the complex if any, d) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time, (e) comparing the amount of the complex measured in step (c) with that in step (d) thereby monitoring the progression of breast, lung, kidney, colon, melanoma , prostate, ovarian or colon cancer in the patient.

[0068] According to a further aspect of the invention, there is provided a method for detecting the presence of breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in a patient, with the proviso that the kidney cancer is not Wilms' tumor, comprising the steps of: (a) obtaining from the patient a biological sample susceptible of containing tumor cells; (b) contacting the biological sample with a ligand to a DKK1 protein or an immunogenic polypeptide thereof, or to a polynucleotide encoding said protein or said polypeptide to form a complex; (c) determining the presence or absence of the complex, wherein the presence of the complex is an indication of the presence of a breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in the patient.

[0069] According to a further aspect of the invention, there is provided a method for monitoring the progression of breast, lung, kidney, colon, melanoma,

prostate, ovarian or colon cancer in a patient, with the proviso that the kidney cancer is not Wilms' tumor, comprising the steps of: (a) contacting a biological sample from the patient, susceptible of containing tumor cells, with at least two oligonucleotide primers under conditions wherein said oligonucleotide primers are effective for specifically amplifying a polynucleotide sequence of DKK1 in a reverse transcription polymerase chain reaction; (b) detecting in the sample an amount of polynucleotide amplified in step (a); (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polynucleotide detected in step (c) to the amount detected in step (b) thereby monitoring the progression of breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in the patient.

[0070] According to a further aspect of the invention, there is provided a method for activating T cells specific for a DKK1 protein, or an immunogenic polypeptide thereof, comprising incubating the T cells with at least one component selected from the group consisting of: (i) a purified or recombinant DKK1 protein or an immunogenic polypeptide thereof; (ii) a polynucleotide comprising the coding sequence of a DKK1 protein or an immunogenic polypeptide thereof; and (iii) antigen presenting cells transformed to express a recombinant DKK1 protein or an immunogenic polypeptide thereof, whereby T cells are activated.

[0071] According to a further aspect of the invention, there is provided a method for activating T cells specific for a DKK1 protein, or an immunogenic polypeptide thereof in a patient, comprising administering to the patient at least one component selected from the group consisting of: (i) a purified or recombinant DKK1 protein or an immunogenic polypeptide thereof; (ii) a polynucleotide comprising the coding sequence of a DKK1 protein or an immunogenic polypeptide thereof; and (iii) antigen presenting cells transformed to express a recombinant DKK1 protein or an immunogenic polypeptide thereof, whereby T cells are activated in the patient.

[0072] According to a further aspect of the invention, there is provided an expression vector comprising a polynucleotide encoding a DKK1 protein operably associated with an expression control sequence, and a polynucleotide encoding an immunogenicity enhancing polypeptide. In an other aspect of the invention, the expression vector comprises an immunogenic polypeptide derived from a DKK1 protein operably associated with an expression control sequence. In a specific embodiment, the expression vector further comprises a polynucleotide encoding an immunogenicity enhancing polypeptide. According to a further aspect of the invention, there is provided a host cell expressing the vector of the present invention. In a specific embodiment, the cell is an antigen-presenting cell (APC).

[0073] According to a further aspect of the invention, there is provided a fusion protein comprising a DKK1 protein or an immunogenic polypeptide thereof and an immunogenicity enhancing polypeptide.

[0074] According to a further aspect of the invention, there is provided a composition comprising a physiologically acceptable carrier, and a second component selected from the group consisting of: (a) a purified or recombinant DKK1 protein or an immunogenic polypeptide of DKK1; (b) a polynucleotide comprising the coding sequence of a DKK1 protein or an immunogenic polypeptide of DKK1; (c) an antibody specific to a DKK1 protein or to an immunogenic polypeptide thereof; (d) a fusion protein of the present invention; (e) a T cell population activated by cells expressing a DKK1 protein or expressing an immunogenic polypeptide thereof; and (f) APC cells of the present invention. In a specific embodiment, the method comprises contacting the T cells with a composition of the present invention.

[0075] According to a further aspect of the invention, there is provided an immunogenic polypeptide derived from a DKK1 protein. In a specific embodiment, the polypeptide consists of up to 25 amino acid residues and comprises an amino

acid sequence as set forth in any one of SEQ ID NOs: 9-15, 34 and 38-41. According to a further aspect of the invention, there is provided an isolated polynucleotide encoding a polypeptide of the present invention.

5 **[0076]** Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

10 **[0077]** Figure 1 presents the DKK1 mRNA sequence (SEQ ID NO: 6), wherein residues 155 to 952 (SEQ ID NO: 7) represent the coding sequence, and the DKK1 polypeptide sequence (SEQ ID NO: 8) obtained from Genbank™ accession number NM_012242 and SwissProt™ accession number: O94907, respectively;

15 **[0078]** Figure 2 presents the polypeptide sequence of DKK1 alone (SEQ ID NO: 8);

[0079] Figure 3 illustrates the presentation of endogenous antigens by MHC class I to CD8⁺ T cells (left panel) and of exogenous antigens by MHC class II to CD4⁺ T cells (right panel) (From S. A. Rosenberg, The Cancer Journal from Scientific American, July/August 1995);

20 **[0080]** Figure 4 shows the expression profile of DKK1 in tumor cell lines and PBMC. A, B and C - mRNA was prepared from the indicated cell lines, and RT-PCR analyses were performed with the indicated specific primers. Normal primary cell lines were prepared by stimulation of PBMC with anti-CD3 and IL-2 (T cells), or with soluble CD40L and IL-4, which stimulate B lymphocytes to proliferate

(B cells). Reverse transcriptase was omitted in the MDA231-RT group and HCC2218 EBVB cells (EBV-B -RT). HCC2218 EBV-B and HCC1428 EBV-B are EBV-immortalized B lymphocytes prepared from breast cancer patients HCC2218 and HCC1428 respectively. Amplification was detected by ethidium bromide staining after electrophoresis migration in agarose gel. Legend : Mel: melanoma;

[0081] Figure 5 shows the expression profile of genes selected by the bio-informatic approach in tumor cell lines and PBMC. mRNA was prepared from the indicated cell lines, and RT-PCR analyses were performed with the indicated specific primers. Normal primary cell lines were prepared by stimulation of PBMC with anti-CD3 and IL-2 (T cells #1 and #2), or with soluble CD40L and IL-4, which stimulate B lymphocytes to proliferate (CD40B #1 and #2), and fresh PBMC (PBMC #1 and #2). Reverse transcriptase was omitted in the MCF-7 -RT group. HCC2218EBV and HCC1428EBV are EBV-immortalized B lymphocytes prepared from breast cancer patients HCC2218 and HCC1428 respectively. Amplification was detected by ethidium bromide staining after electrophoresis migration in agarose gel;

[0082] Figure 6 shows the expression profile of DKK1 in normal tissues. A and B - mRNA was prepared from the indicated normal tissues (acquired from Origene Technologies in A, and from BD-Clontech in B), and controls. RT-PCR analyses were performed with DKK1 and β -actin specific primers. B - Expression was determined by quantitative real time RT-PCR (LightCycler™, Roche);

[0083] Figure 7 graphically shows the results of quantitative real-time, two-step RT-PCR analysis of DKK1 and β -actin from mRNA-prepared clinical samples. cDNA from mRNA primed with oligo-dt, were prepared from the indicated controls and clinical samples from breast (A), lung (B) or renal cell cancers (C). Amplification was undertaken by real-time quantitative PCR and revealed by SYBr green staining. Standard curves for each gene were established to quantify the

number of copies for each sample, and expression was considered only for the Ct of samples within the limit of each standard. Amplification of the relevant amplicon was further confirmed by separation on agarose gel revealed by ethidium bromide staining. Samples from breast cancer patients (A) are clustered by ER and PR status, as evaluated by the clinical pathology service (score: -: negative, +: positive);

[0084] Figure 8 graphically shows a cluster analysis for breast cancer specimens. Samples were clustered according to A the expression of hormonal receptors; B – the reported family history or C – the histological grade. Legend:
10 ER: estrogen receptor; PR: progesterone receptor; +: positive; -: negative; and

[0085] Figure 9 shows the determination of putative anchor residues for DKK1 immunogenic polypeptides (i.e. polypeptide 20 (SEQ ID NO: 9); polypeptide 40 (SEQ ID NO: 10); polypeptide 32 (SEQ ID NO: 11); polypeptide 37 (SEQ ID NO: 12); polypeptide 61 (SEQ ID NO: 13); polypeptide 68 (SEQ ID NO: 14);
15 polypeptide 182 (SEQ ID NO: 15)) from an alignment of these polypeptides. Residues two, six and nine (shaded) within a nine or 10-mer polypeptide sequence have been defined as anchor residues for HLA-A*0201 allele⁴⁴.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0086] This invention is further described in details, referring to specific
20 embodiments, examples and appended figures, the purpose of which is to illustrate the invention rather than to limit its scope.

EXAMPLE 1

Identification of candidate TAs with bio-informatic tools

[0087] A bio-informatic tool from the CGAP server probing two different cDNA

expression libraries, the expressed sequence tags (EST) and the serial analysis of gene expression (SAGE) (i.e. the Digital Gene Expression Displayer™ (DGED)) was used to find candidate genes. The bio-informatic tool allows the analysis of expression profiles from the EST and SAGE databases by the clustering of libraries by origin, such as from normal or tumoral tissues. Candidate genes were selected on the basis of high expression levels in available libraries prepared from human breast cancer, and absent or low expression levels in normal human tissues derived from important organs. A list of the most relevant candidates appears in Table I. β -actin and ubiquitin C genes were included as positive controls, and, as expected, the reported expression levels are relatively equivalent in the normal and tumor tissues selected. PDEF and CLSP were overexpressed in breast carcinomas according to both the EST and SAGE libraries. Interestingly, some genes, such as DCD and DKK1, were detected in a higher proportion of breast cancer tissue samples based on the SAGE only, which was not correlated by expression assessment in the EST database. Also, some genes were found to be potentially overexpressed in breast cancer according to the EST database, which is in contrast to the SAGE approach.

Table I. List of overexpressed candidate genes in breast cancer compared with normal tissues, identified by cDNA and the SAGE Digital Gene Expression Displayer (DGED) from the CGAP server. β -actin and ubiquitin C are referenced as ubiquitously-expressed genes.

Identification ^a	SAGE ^b		EST ^b	
	Normal	Tumor ^c	Normal	Tumor ^c
Hs.288061 β -Actin	1 104	713	1 423	2 985
Hs.183704 Ubiquitin C	501	366	356	241
Hs.180142 calmodulin-like skin protein (CLSP)	11	<u>126</u>	0	<u>144</u>
Hs.79414 prostate Ets transcription factor (PDEF)	45	<u>545</u>	219	<u>4 205</u>
Hs.112408 S100 calcium-binding protein A7	17	<u>1 088</u>	0	0
Hs.130239	0	<u>24</u>	0	48
Hs.144479	0	<u>33</u>	0	0
Hs.350570 dermcidin (DCD)	0	<u>565</u>	0	0
EST 602281305F1 (SAGE tag TTCGGTTGGT) ^d	+	++++	NA	NA
Hs.199713	0	<u>268</u>	0	32
Hs.40499 dickkopf (Xenopus laevis) homolog 1 (dkk-1)	17	<u>304</u>	137	0
Hs.46452 mammaglobin 1	39	33	0	<u>273</u>
Hs.215937	0	0	55	<u>369</u>
Hs.170482 myosin light polypeptide 5	11	0	82	<u>2 006</u>
Hs.1852	39	29	0	<u>1 332</u>

Legend: ^a - Unigene ID and names when available; ^b - Number of positive sequences from libraries prepared from breast tumors or selected normal tissues, among all available tags or sequences. Values are reported as number of positives/1 000 000 sequences; NA: not available;

5 ^c - Numbers are bolded and underlined when $p < 0,05$ AND five times the value from normal cells; ^d - No assigned Unigene ID (EST prepared from osteocarcinoma). The expression level is subjectively reported from 0 to +++++.

10

EXAMPLE 2

Expression Profile of DKK1 Gene in Breast Cancer Cells

Determination of DKK1 Gene Expression in Breast Tumor Cell Lines as Compared to that in Normal Cell Lines

15 [0088] Although the tools used for evaluating global gene expression are powerful, the discrepancies observed between the two methods show the need to further

validate the results. To maximize the identification of relevant genes, the expression profile of the genes was further thus evaluated in tumor cell lines.

[0089] The expression profile of selected genes identified in the SAGE and EST databases was thus assessed by reverse-transcription polymerase chain reaction (RT-PCR) analysis. RNA was prepared using the RNeasy™ Mini or micro Kit (QIAGEN GmbH; Hilden, Germany) according to manufacturer's instructions. Breast cancer cell lines MCF-7, MDA231, BT20, HCC1428 BRCA, and HCC2218 BRCA, the lung cancer cell lines Calu6, H1299, A549, H460 and H596, were all obtained from the American Type Culture Collection (ATCC, Manassas, VA). Immortalized EBV-B cells from HCC1428 and HCC2218 patients were purchased from the ATCC. The melanoma cell lines 397mel, 537mel, 586mel, 888mel, 1087mel, 1088mel, 1278mel, 1300mel, 1337mel and Mels-FB, the kidney cancer line RCC-W were established at the Surgery Branch (NCI/NIH), and the melanoma cell line SK23 was acquired from the ATCC. The ovarian cancer line SKOV3 was also kindly provided by the Surgery Branch. Most of cell lines were cultured in RPMI 1640 (Invitrogen; Carlsbad, CA; and Wisent; St-Bruno, Québec, Canada) supplemented with 10% heat-inactivated FBS (Invitrogen and Wisent), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin (Invitrogen) and 10 µg/ml gentamicin (Invitrogen). For the HCC breast cancer cell lines and corresponding EBV-B lines, 10 mM HEPES Solution (Invitrogen) and 1 mM Sodium Pyruvate (Invitrogen) were added to the culture medium. Negative controls included cells prepared from normal peripheral blood mononuclear cells (PBMC) as well as cultured activated lymphocytes to eliminate genes expressed in normal proliferating cells. PBMC were collected from healthy donors recruited by the McGill University Health Center (MUHC, Royal-Victoria hospital, Montréal, Québec, Canada). PBMC were prepared from aphaeresis samples on lymphocyte separation medium (Cellgro, Herndon, VA, USA). To generate CD40-activated B cell cultures, B cells from bulk PBMC were cultured with 500 ng/ml of a soluble trimeric CD40L (CD40Ls, Immunex Corporation, Seattle, WA) in complete media complemented with 200

U/ml of recombinant human IL-4 (Peprotech, Rocky Hill, NJ). Fresh complete media was added again on day three with IL-4 and 500 ng/ml CD40Ls. After the first round of proliferation (d5-8), cells were either frozen for future use or re-stimulated every two-three days when the culture reached a density of $1.5-2 \times 10^6$ cells/ml. They were re-plated at about $3-5 \times 10^5$ cells/ml of medium containing 500 ng/ml of CD40Ls and 200 U/ml IL-4. For T lymphocyte cultures, PBMC were cultured in complete medium consisted of AIM-V medium (Invitrogen) supplemented with 5% human AB serum (heat-inactivated; Gemini Bio-Products; Calabasas, CA), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10 μ g/ml gentamicin (all from Invitrogen), and supplemented with 300 IU/ml recombinant human IL-2 (Chiron; Emeryville, CA) and 30 ng/ml of an agonistic anti-CD3 (OKT3, eBiosciences, San Diego, CA), or 5 μ g/ml of phytohemagglutinin (PHA, Sigma). Intron-spanning PCR primers were designed from eight of the candidate genes presented in Table I above. To perform the RT-PCR analyses, cDNA was first synthesized from mRNA (0.2 to 1 μ g) with oligo-dt (Invitrogen) using the Omniscript Reverse Transcriptase Kit (QIAGEN) and then amplified using the HotStartTaq DNA Polymerase (QIAGEN). The cycling conditions were 15 min at 95°C, 24 (β -actin) or 32 (DKK1 and other candidate TA) cycles of 45 sec at 94°C, 45 sec at 55°C, 1 min at 72°C, with a final extension of 10 min at 72°C. Primer sequences for β -actin : 5': GGAAGGCTGGAAGAGTGCC (SEQ ID NO: 16); 3': GTGATGGTGGGCATGGGTC (SEQ ID NO: 17), 700 bp amplicon. Amplification was detected by ethidium bromide staining after electrophoresis migration in agarose (2%) gel (all apparatus from Bio-Rad, Hercules, CA). CLSP (5' primer: GTGAGCTGACTCCTGAGGAG (SEQ ID NO: 18); 3' primer: CTCGCGAACTCCTCGTAGTTC (SEQ ID NO: 19), amplicon 410 bp), MYL5 (5' primer: ACCAAGCAGGAGCTTAAGATG (SEQ ID NO: 20); 3' primer: AGAATGGTCTCCTCGGCGTC (SEQ ID NO: 21), amplicon 390 bp) (Figure 5), Hs.199713 and Hs.1582 (data not shown) were expressed in several breast cancer lines but also in PBMC and activated lymphocytes (i.e. T cells #1, T cells #2, CD40-B#1, CD40-B#2) and, consequently, they were rejected as potential TA. The

expression of PDEF (5' primer: TGACATGCTGTACCCTGAGGA (SEQ ID NO: 22); 3' primer: GCTCTGGAAGGTCAGAGCAGCA (SEQ ID NO: 23), amplicon 940 bp) and S100A7 (5' primer: AAGATGAGCAACACTCAAGCTG (SEQ ID NO: 24); 3' primer: GTCTCCCAGCAAGGACAGAAAC (SEQ ID NO: 25), amplicon 247 bp) was confirmed in breast cancer lines but not in normal PBMC or activated lymphocytes (Figure 5). S100A7, also known as psoriasin, has been described previously as being expressed preferentially in invasive breast cancer²⁰. PDEF has also been reported to be expressed with high frequency in breast cancer with no expression in important tissues²¹.

10 [0090] DKK1 (5' primer: ATTCCAACGCTATCAAGAACC (SEQ ID NO: 26); 3' primer: CCAAGGTGCTATGATCATTACC (SEQ ID NO: 27), amplicon 383 bp) and DCD (5' primer: AGCATGAGGTTTCATGACTCTC (SEQ ID NO: 28); 3' primer: CACGCTTTCTAGATCTTCGAC (SEQ ID NO: 29), amplicon 284 bp)²² were also expressed in breast cancer lines, but absent from normal cells tested (Figure 4-A and Figure 5).

[0091] The expression of the DKK1, DCD, PDEF and S100A7 genes previously selected with the bio-informatics approach was thus confirmed in breast cancer lines. Furthermore, the expression of DKK1 was also confirmed in ovarian, colon, prostate, lung cancers as well as melanoma was also identified (Figure 4-B and -

20 C).

Determination of DKK1 gene expression in normal tissues

[0092] Expression of DKK1 in normal tissues was evaluated to further confirm DKK1 specificity of expression to tumoral cells.

[0093] DKK1 is known to be expressed in fetal development, and its expression in mature humans has been detected in normal placenta and prostate, which are not targeted tissues in breast cancer immunotherapy²³. DKK1's relative expression in

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normal cells was further determined here.

[0094] Classical and quantitative real-time RT-PCR approaches were next taken to evaluate the expression of selected genes in panels of normal tissues. Using the cDNA panel from Origen (Origen Technologies; Rockville, MD), and amplified as described earlier (PCR kits from Qiagen), S100A7 and DCD were detected in multiple normal tissues such as the colon and brain (Figure 6-A), and, consequently, these genes were rejected as candidate TA. Interestingly, PDEF appears to be weakly expressed in salivary glands (Figure 6-A). DKK1 is expressed mainly in the placenta, with a weak expression in the prostate, as reported previously (Figure 6-A)²³. Restricted expression of DKK1 to the placenta and prostate was further confirmed in a second panel of normal tissues mRNA, and cDNA was prepared as described above (BD Biosciences Clontech; Palo Alto, CA) (Figure 6-B) using quantitative real-time RT-PCR. For quantitative real-time RT-PCR, amplification was realized with the LightCycler™ system (LightCycler™, Roche; Mannheim, Germany). The amplification was revealed with a SYBR Green™ kit (Quantitect™ SYBR Green PCR, QIAGEN). Standard curves for each gene were established to quantify the number of copies for each sample, and expression was considered only when the sample Ct was within the limit of each standard curves. The cycling conditions were 15 min at 95°C, 40 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C and 5 sec at 82°C (β-actin) or 84°C (DKK1). The primer sequences used for the real-time PCR were the following: β-actin forward: AAGGCCAACCGCGAG (SEQ ID NO: 30) ; reverse: TAATGTCACGCACGATTCCCG (SEQ ID NO: 31); DKK1 5': CTCGGTTCTCAATTCCAACG (SEQ ID NO: 32); 3': GCACTCCTCGTCCTCTG (SEQ ID NO: 33). Finally, amplification of the relevant amplicon was further confirmed by separation on agarose (2%) gel revealed as mentioned earlier. The increased sensitivity of this assay revealed a very low expression in few other tissues (Figure 6-B). DKK1 expression in the placenta and prostate was thus confirmed with two distinct commercially-available mRNAs prepared from normal

tissues (Origene kit and BD-Clontech) along with its absence in tissues from essential normal organs, such as the heart or lungs. Very low DKK1 expression was revealed by real-time quantitative RT-PCR in the prostate, trachea, uterus, and testis, which are not essential organs and targeting DKK1 as a TA in immunotherapy are not expected to be detrimental against these organs. Finally, low detection from the brain is not problematic considering that this is an immune-privileged site, meaning that the systemic immune system is not expected to reach this organ. Historically, auto-immune responses as a consequence of immunotherapy strategies are extremely rare even if some targeted TA were expressed in several normal organs. Auto-immune responses in this context are usually manageable by administration of steroids. Actually, expression levels are higher in tumors from clinical samples (as demonstrated in Figure 7 and discussed in the next section).

Expression Profile of DKK1 Gene in Clinical Samples of Breast Cancer

[0095] Although TA are frequently expressed in both fresh tumor samples and tumor cell lines, confirmation of expression in fresh breast cancer specimens was desirable. The DKK1 expression profiles was thus assessed in clinical samples from different cancer specimens.

[0096] Using the resources from the FRSQ cancer network – breast tissue library from CHUM Notre-Dame hospital, DKK1 expression was evaluated by quantitative real time RT-PCR. RNA was prepared from freshly resected breast cancer and the pieces were preserved in RNA stabilization solution within 15 minutes following surgery (RNAlater™, Sigma, Oakville, Canada). RNA was prepared as described above and the sample was homogenized with a Medimachine™ (DakoCytomation Denmark) according to manufacturer's instructions. cDNA was synthesized as described above. Real time quantitative RT-PCR was performed as described above. DKK1 was detected in 18 out of 70 specimens evaluated (Table II). Expression levels of representative specimens is also presented in Figure 7-A.

Interestingly, eight of the 18 positive tumors originated from patients who reported familial cases of breast cancer (Figure 8), which is in net contrast with the total population included in the cohort. Clearly, DKK1 seems to be preferentially expressed in tumors of women with familial history of breast cancer.

5 Table II Expression profile of DKK1 by cancer types

Breast		
	Cell lines	3/5
	Clinical specimens	18/70
Lung		
	Cell lines	5/5
	Clinical specimens	5/8
Kidney		
	Cell line	0/1
	Clinical specimens	6/20
Prostate cell lines		2/3
Melanoma cell lines		7/11
Ovary cell line		1/1
Colon cell line		1/1

10 [0097] DKK1⁺ and DKK1⁻ specimens were clustered according to different clinical parameters and to evaluate the discriminating potential for relevant clinical information. Interestingly, on the 70 specimens analyzed, only three were positive for HER-2/neu, however, the 18 DKK⁺ tumors turned out to be negative for HER-2/neu. This is of critical relevance considering the efficiency of Herceptin™, a monoclonal antibody specific to HER-2/neu, which will be ineffective to DKK1⁺

patients. This is relevant in so far that patients that will respond to TA vaccine from DKK1 would not have been responsive to Herceptin™. Hence, at least a fraction of patients that were untreatable by Herceptin™, namely that which expresses DKK1, could now be treated.

5 [0098] As presented in Figure 8-A, the majority of DKK1⁺ expressing tumors originated from patients with a bad prognostic based on the absence of estrogen and progesterone hormone receptors (ER-/PR- according to the pathology report). Actually, 61% of DKK1⁺ tumors were ER-/PR-, as opposed to 23% for DKK1-
10 tumors. Finally, the DKK1 expression seems to be higher in tumors with a high histological grade. This is of particular importance considering that the 5-year survival for grade 1 tumors is about 95%, 65% for grade 2 and 50% for grade 3 (Figure 8-C). Finally, five of the DKK1⁺ tumors were scored stage III (28% of all DKK1⁺ tumors) which is in contrast with only three stage III for 52 DKK1⁻ tumors
15 (6%). The 5-year survival for stage III is about 50%; this illustrate again the fact that DKK1 is linked with the most aggressive breast tumors.

[0099] Altogether, there seems to be a correlation between the histological grade, the family history, and the ER/PR status with the expression of DKK1 in the most aggressive tumors.

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EXAMPLE 3

Expression Profile of DKK1 Gene in Lung Cancer Cells

Determination of DKK1 Gene Expression in Lung Tumor Cell Lines as Compared to that in Normal Cell Lines

[00100] DKK1 expression was revealed in all available lung cancer cell lines
25 (n=5) (5/5; Figure 4-B).

Expression Profile of DKK1 Gene in Clinical Samples of Lung Cancer

[00101] A total of eight clinical specimens were analyzed: three from the thoracic surgery department from the CHUM Notre-Dame, and five from the Lung cancer tissue library from "Fond de recherche en santé du Québec" (FRSQ) "Réseau en santé respiratoire" from Québec city. Freshly resected samples were analyzed as described above. DKK1 expression analyses revealed that five were clearly positives (63%). Interestingly, the patient with the highest DKK1 level expression had a metastatic disease at the time of surgery (four others did not have metastases, and for the remaining three the status was unavailable). The eight samples were stage II and higher. Stage II lung cancer survival for three years is under 40%.

EXAMPLE 4

Expression Profile of DKK1 Gene in Clinical Samples of Kidney Cancer

[00102] DKK1 expression was evaluated in a kidney cancer tissue library established in Dr Lapointe's group from a collaboration with Dr Simon Tanguay from McGill University (n=20 for availability of cDNA). Freshly resected samples were analyzed with similar methods described in Example 2. In this restricted panel of samples, 30% of cases were clearly DKK1-positive (Figure 7-C).

[00103] The occurrence of light renal cells carcinomas (VHL^{-/-}) is much higher (by 70 fold) than the occurrence of Wilms' tumors (about 500 cases per year in the USA). It is noted that although Wirths et al. (50) reported the presence of DKK1 in Wilms' tumors, Wirths did not disclose the absence or low expression of DKK1 in a sufficient number of normal cells to suggest that it would constitute a good TA. It further did not disclose its presence in other cancers..

EXAMPLE 5

Expression profile of DKK1 gene in Other Tumor Cell lines

[00104] As is apparent from Figure 4-B and C, DKK1 is expressed in melanomas (7/11), ovarian (Figure 4-C; SKOV3), and colon cancers (Figure 4-C;

HCT116). In addition, DKK1 expression was revealed in 2 prostate cancer cell lines derived from hormone-independent tumors (2/3; Figure 4-C).

EXAMPLE 6

Activation of T Lymphocytes Specific to DKK1-Derived Peptides :

5 Identification of HLA-A*0201-restricted epitopes from DKK1

[00105] DKK1 immunogenicity was evaluated by identifying T lymphocytes specific to putative human leukocyte antigens (HLA)-A2-restricted epitopes from DKK1 (Figure 2). Putative HLA-A*0201 nine- and ten-mer epitopes were predicted by exploiting both the SYFPEITHI⁴⁸ (<http://www.syfpeithi.de/>) tool and Dr. Kenneth Parker's algorithms⁴⁹ (http://bimas.dcrt.nih.gov/molbio/hla_bind/). Since these bio-informatic tools generate a substantial number of putative binders, the list was further shortened by predicting the generation of a valid epitope after proteasome cleavage with the PAPProC™ tool (<http://www.paproc.de/>)^{46,47}. The list of peptides prepared is presented in Table III. Only peptides that were predicted to be generated by the human proteasome, as approximated by the PAPProC™ tool were selected.

Table III List of selected HLA-A*0201 epitopes predicted for DKK1, using HLA-binding algorithms and a proteasome prediction tool (PAPProC™).

Decamers			
Position ^a	Sequence	Parker rank ^b	SYFPEITHI rank ^c
20	ALGGHPLLGV	1	1
36	VLNSNAIKNL	2	2
11	RVFVAMVAAA	7	14
18	AAALGGHPLL	28	7
40	NAIKNLPPPL	29	12
Nanomers			
182	KGQEGSVCL	8	20
25	PLLGVSATL	11	1
52	AAGHPGSAV	14	15
246	RIQKDHQA	17	43
191	RSSDCASGL	20	56
37	LNSNAIKNL	21	29
68	YPGGNKYQT	27	89
32	TLNSVLNSN	30	15
61	SAAPGILYP	>50	18
58	SAVSAAPGI	31	26

Legend: ^a – Starting position of the peptide in the Dkk1 protein sequence. ^b – Score ranking according to the predicted half-time of dissociation to HLA-A2 molecules from the Bioinformatics & Molecular Analysis Section (BIMAS) server (http://thr.cit.nih.gov/molbio/hla_bind/), based on Dr. Kenneth Parker's algorithm. ^c – Score ranking according to the peptide binding capacity to HLA-A2 molecules, from the SYFPEITHI server (<http://www.syfpeithi.de/>), based on Dr. Hans-Georg Rammensee's algorithm.

[00106] Polypeptides in Table III (polypeptides 20 (SEQ ID NO: 9), 36 (SEQ ID NO: 34), 11 (SEQ ID NO: 35), 18 (SEQ ID NO: 36), 40 (SEQ ID NO: 10), 182 (SEQ ID NO: 15), 25 (SEQ ID NO: 37), 52 (SEQ ID NO: 38), 246 (SEQ ID NO: 39), 191 (SEQ ID NO: 40), 37 (SEQ ID NO: 12), 68 (SEQ ID NO: 14), 32 (SEQ ID NO: 11), 61 (SEQ ID NO: 13), 58 (SEQ ID NO: 41)) were synthesized by "Le Service de Synthèse de Peptides de l'Est du Québec" (CHUL, Québec, Canada) and were >80% pure. Peptide quality was further confirmed by HPLC and MALDI-TOF analyses. Immunogenicity of these peptides was evaluated in *in vitro* T cell sensitization assays. PBMC were collected from breast cancer patients recruited by the Banque de cancer du sein FRSQ from the Hôpital Notre-Dame (CHUM) (Montréal, Québec, Canada). HLA-A2⁺ patients were identified by flow cytometry analyses using a FITC-labeled specific antibody (OneLabmda, Canoga Park, CA). The PBMC were prepared as mentioned earlier and frozen in fetal bovine serum (FBS, Invitrogen and Wisent) or Calf serum (Wisent) with 10% DMSO (Sigma; St-Louis, MO) at 5 to 10X10⁶ cells/ml in liquid nitrogen. For *in vitro* stimulations,

PMBC were thawed and resuspended at 1×10^6 cells/ml in complete medium supplemented with 500 ng/ml of CD40L, and aliquoted in 16 tubes for each DKK1 peptides (15) (Table III), and one peptide derived from the influenza M-1 protein (M1-FLU: GILGFVFTL) as a positive control. Peptides were added at a concentration of $1 \mu\text{M}$ and plated in flat bottom 96-well culture cluster (Corning; Corning, NY) at 5 wells per peptides (100 or $150 \mu\text{l/well}$). Individual cultures were restimulated with autologous peptide-pulsed CD40-B lymphocytes (CD40-B; method described above) seven to ten days later. CD40-B were pulsed with $1 \mu\text{M}$ peptides in ISCOVE (Invitrogen or Wisent) for three hours at room temperature.

10 Peptide-pulsed CD40-B were washed in PBS, and added to corresponding T cell cultures. Two days later, 150 U/ml of IL-2 was added to the cultures and repeated every three days after. Ten or 12 days after the restimulation, the specificity of T cell cultures was evaluated cells by IFN- γ ELISPOT™ assays. Co-cultures were performed in 96-well filtration Plates (MultiScreen™-HTS; Millipore; Bedford, MA) according to manufacturer's instructions. ELISPOT plates were coated with anti-IFN- γ mAb ($5 \mu\text{g/ml}$, Mabtech; Stockholm, Sweden) overnight at 4°C . After washing with 1X sterile PBS, plates were blocked with the complete medium (described earlier) and incubated for two hours at 37°C . Cultured T lymphocytes were washed and transferred (0.5×10^5 cells/well) into coated ELISPOT plates. HLA-A*0201⁺ TAP-deficient T2 cell lines (ATCC, Manassas, VA) or an HLA-A*0201⁺ EBV-B line established at the Surgery Branch (NCI/NIH), were pulsed with the relevant peptide or a control peptide at $1 \mu\text{M}$ for three hours. Peptide-pulsed T2 or EBV-B were washed and added to the stimulated T lymphocytes and incubated overnight at 37°C . Supernatants from ELISPOT recognition plates were harvested and

25 frozen at -20°C to evaluate the GM-CSF secretion by ELISA. ELISPOT plates were then washed with PBS/0.01% Tween 20 (Sigma) and a biotinylated anti-IFN- γ mAb ($2 \mu\text{g/ml}$, Mabtech) was added to each well. After a two-hour incubation at 37°C , plates were washed with the PBS-Tween and Streptavidine-HRP (Mabtech) was added. After a 45 min room temperature incubation, plates were washed with

30 PBS/0.01% Tween 20 and once with PBS. Spots were revealed with the AEC

substrate (0,1N acetic acid, 0,1M sodium acetate and H₂Odd) for five minutes and then washed with water. Spots were enumerated using an automated ELISPOT Counter (Immunospot 3A Analyser, CTL technologies, Cleveland OH). Only T cell cultures with >10 spots when co-cultured with the relevant peptide-pulse target, and with a ratio >2 (ratio of relevant peptide/control peptide) were considered positive cultures. GM-CSF secretion was evaluated by ELISA from supernatant from the ELISPOT co-culture. ELISA were performed in Flat bottom 96-well (Nunc, Apogent Technologies; Portsmouth, NH) with paired antibodies for GM-CSF (Endogen; Woburn, MA), according to manufacturer's procedure. Human recombinant GM-CSF (Endogen) was used as a standard with a concentration ranging between 16 to 1000 pg/ml. Revelation was done with Poly-HRP20-streptavidin (Research Diagnostic; Flanders, NJ) and TMB Substrate (Neogen; Lexington, KY). Plates were analyzed with a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA). To be considered as a positive culture, GM-SCF secretion must be >50 pg/ml and twice the amount secreted when co-cultured with the control peptide. As seen in Table IV, several peptides failed to generate specific T cells (11, 18 and 25) and some only in one patient (52, 246, 191, 58). Among the stimulatory peptides, seven (20, 32, 40, 37, 61, 68 and 182) had the capacity to generate specific T cells in two or more different patients.

TABLE IV PEPTIDE RECOGNITION BY PEPTIDE-STIMULATED T CELL LINES FROM HLA-A*02+ BREAST CANCER PATIENTS.

		Patient #10	Patient #18	Patient #37	Patient #40 ^b	Patient #52
5	Decamers					
	→ 20	+ ^a	-	+	-	+
	36	-	-	+	-	-
10	11	-	-	-	-	-
	18	-	-	-	-	-
	→ 40	+	+	-	-	-
	Nanomers					
	→ 182	+	-	-	-	+
15	25	-	-	-	-	-
	52	+	-	-	-	-
	246	-	+	-	-	-
	191	+	-	-	-	-
	→ 37	+	+	-	-	-
20	→ 68	+	+	+	-	-
	→ 32	+	-	-	-	+
	→ 61	+	-	-	+	+
	58	-	-	-	-	+

25

30

The specificity of individual cultures was determined by IFN- γ ELISPOT™ assays and GM-CSF ELISA, by co-culturing with T2 cells pulsed with the relevant peptide, or with an irrelevant control peptide. Legend: a – only lines with > 10 spots and with a ratio > 2 were considered positive for ELISPOT assays, and GM-CSF secretion must be >50 pg/ml and twice the amount secreted when co-cultured with the control peptide. b - only wells demonstrating proliferating cells were tested for this patient.

→ Peptides recognized by 2 and more patient lymphocytes.

[00107] Tumors expressing both HLA-A2 and DKK1 serve as targets for peptide-specific T lymphocyte line or clones to determine naturally-processed epitopes. Finally, peptide sequences of identified epitopes are optimized to enhance their binding capacity on anchor residues to HLA and T cell receptor (TCR) complexes, a procedure that frequently results in an increased immunostimulatory capacity in patient immunization.

[00108] Immunogenic peptides were aligned to define the most probable anchor residues (Figure 9). According to this alignment, immunogenic peptides from DKK1 can be defined as follows for the putative anchor residues: Position 2: Aliphatic residues (I or L), or a small residue (N, A, P or G); Position 6: Aliphatic residues (I or L), a polar residue (K or S) or a P; Position 9: Small hydrophobic residue (G or T), a small residue (N or P) or a L.

[00109] Even with these predictions, some peptides will not necessarily be immunogenic in a given patient while it could be in another one because different HLA sub-types will bind different peptides with different affinities. A putative immunogenic peptide will therefore be considered effective if it combines with the MHC complex of a given patient and if the lymphocytes are activated and capable of eliciting antibody production or killing activity towards the patient's cancer cells.

EXAMPLE 7

Immunization with a Recombinant DKK1 Protein

[00110] A recombinant DKK1 DNA is expressed in *E. coli* as a fusion protein with a protein D derived from *H. influenzae* at the N terminus, and a sequence of several histidine residues at the C terminus of the protein. The inclusion of the first 109 residues of the protein D as a fusion partner is expected to improve the immunogenicity and to provide the vaccine protein with additional bystander help properties, whereas the inclusion of a His affinity tail facilitates the purification of the fusion protein. The vaccination schedule comprises six vaccinations at three-week intervals. After a first cycle of six vaccinations, patients whose tumor have regressed, stabilized, or progressed slowly receive additional vaccinations at increasing intervals for a maximum of five years, or until major tumor progression or relapse requiring other treatments. Six additional vaccinations are first given at six-week intervals, followed by three-month intervals between the following ones. The day of each vaccination, a total dose of 300 µg of lyophilized recombinant DKK1 protein is reconstituted in 0,5 ml NaCl 0,9 % and divided between three

injection sites, one i.d. (75 μ g), one s.c. (75 μ g) and one bayonet i.d./s.c. site (75 μ g/75 μ g), where 75 μ g are injected i.d. and then, by changing the needle position, 75 μ g are injected s.c. Injections are given in the arms or the anterior aspect of the thighs. No injection is given into limbs where draining lymph nodes have been
5 surgically removed or irradiated, or in case draining lymph nodes are known to contain metastases. The sites are rotated on each vaccination. This procedure is adapted from a successful method of the prior art.

EXAMPLE 8

10 Immunization of Cancer Patients with HLA-A1-restricted DKK1 Immunogenic Polypeptides

[00111] An HLA-A*0101-restricted DKK1 immunogenic polypeptide is administered in sterile, endotoxin-free PBS, at a concentration of 100 or 300 μ g/ml, in three vaccination at one-month intervals. The vaccine is divided between two sub cutaneous (s.c.) sites and two intradermal (i.d.) sites distant from the tumor.
15 When possible, the injection sites is changed for each vaccination. For tumor-bearing patients who display tumor regression, additional immunizations are administered. This procedure is adapted from a successful method of the prior art.

EXAMPLE 9

20 Immunization of Cancer Patients with HLA-A2-restricted DKK1 Immunogenic Polypeptides and IL-2

[00112] Cancer patients receive DKK1 immunogenic polypeptides or modified version of these peptides (i.e. substitution in position two for I or M, six for V or nine for L to improve the binding to the HLA-A*0201 molecule) from the antigen DKK1. Patients receive one mg of peptide emulsified in incomplete
25 Freund's adjuvant in two to six immunizations at three weeks intervals. Some of the patients also received high-dose IL-2 at 720 000 IU/kg as an intravenous bolus over 15 minutes starting either one to five days after peptide injection. After two or four injections, all known sites of the disease are assessed. A response is considered complete if all measurable lesions disappear. A partial response is

defined as 50% or greater decrease of the sum of the products of the longest perpendicular diameters of all legions, lasting at least one month without increase of any tumor or the appearance of new lesions. This procedure is adapted from a successful method of the prior art.

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EXAMPLE 10**Immunization of Cancer Patients with HLA-A*0201-Restricted DKK1 Immunogenic Polypeptides and Fully Human Antagonizing Anti-CTLA-4 Antibodies**

[00113] Strategies seeking to decrease negative T cell regulators such as CTLA-4, are used to improve the immune response. Every 3 weeks, patients receive anti-CTLA-4 Ab at 3 mg/kg i.v. over 90 min, followed by 1 mg of a DKK1 immunogenic polypeptide emulsified in IFA injected s.c. in their extremities. The anti-CTLA-4 Ab (MDX-010 by Medarex) is a fully human IgG1 Ab derived from transgenic mice having human genes encoding heavy and light chains to generate a functional human repertoire. This Ab has been shown to bind to CTLA-4 expressed on the surface of human T cells and inhibit binding of CTLA-4 to B7 molecules. This procedure is adapted from a successful method of the prior art.

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EXAMPLE 11**Immunization of Cancer Patients with Viral Vectors Encoding a DKK1 Immunogenic Polypeptide**

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[00114] Vaccination of cancer patients is performed with recombinant ALVAC virus bearing DKK1 sequences coding for HLA-A2-restricted DKK1 immunogenic polypeptides. ALVAC miniDKK1 clinical material is produced by cloning cDNA encoding a DKK1 immunogenic polypeptide, is then ligated into a donor plasmid downstream of a vaccinia H6 early/late promoter element. The recombinant plasmid, harboring this expression cassette, is transfected into primary chick embryo fibroblasts, which are then infected with wild-type ALVAC virus. After successive rounds of plaque purification and selection, a recombinant ALVAC virus containing the appropriate expression cassette inserted into the C6

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nonessential site, is isolated and amplified. The viral vaccine is formulated as a lyophilized powder corresponding to a viral dose of $1,23 \times 10^7$ CCID₅₀ (50% of the cell culture infectious dose). The vaccine vials are kept stored at 4°C, and are reconstituted before administration with one ml of water for injection. The vaccination schedule starts with four priming vaccinations with ALVAC miniDKK1 at three-week intervals. The fixed virus dose is determined by the titer of the available clinical batch. After reconstitution, the viral suspension is injected in two i.d. sites (0,1 ml each) and two subcutaneous sites (0,4 mL each), in the arms and the anterior aspect of the thighs. Unless the disease has progressed in such a way that the patient needs another treatment, the ALVAC vaccinations are followed after three weeks by three booster vaccinations with the DKK1 immunogenic polypeptides at three-week intervals. Each peptide is injected once i.d. (60 µg) and once subcutaneously (240 µg), also in the arms and thighs. This procedure is adapted from a successful method of the prior art.⁴⁵

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EXAMPLE 12

Immunization of Cancer Patients with Blood Monocytes-Derived DCs Pulsed with DKK1 Immunogenic Polypeptides

[00115] Blood monocytes are first differentiated in DCs. Peripheral blood mononuclear cells (PBMC) are subjected to Ficoll purification and monocytes are enriched following a two-hour adherence in tissue culture flasks in RPMI™ 1640 media supplemented with 10% fetal calf serum (FCS). Non-adherent cells are removed and adherent cells are cultured for seven days in complete medium supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml). DCs are then harvested and pulsed with HLA-A2, HLA-A1 or HLA-A3 binding DKK1 immunogenic polypeptides, depending on the HLA type of the patient. Peptides are pulsed at 50 µg/ml for two hours. Before injection, pulsed DCs are washed three times in sterile phosphate-buffered saline (PBS) and resuspended in a total volume of 0,5 ml of PBS (1×10^6 pulsed DCs). Patients receive four vaccinations at weekly intervals. The fifth vaccination is administered at week six, and subsequent vaccinations are performed monthly for up to 10 months. Vaccine preparation is

administered intra-lymphatically into an inguinal lymph node under ultrasound control or is injected in close proximity to the regional lymph node. This procedure is adapted from a successful method of the prior art.

EXAMPLE 13

5 Immunization of Cancer Patients with Autologous CD34-Derived DCs Pulsed with a combination of TAs Including DKK1 Immunogenic Polypeptides

[00116] HLA-A*0201 patients are immunized with DKK1 immunogenic polypeptides-pulsed autologous CD34-derived DCs. The patients receive recombinant granulocyte-CSF 10 µg/kg/day s.c. for five days, for peripheral blood
10 stem cell mobilization, and then undergo leukapheresis for two consecutive days to collect mobilized CD34⁺ HPCs. The cells are processed using the CEPRATE™ SC stem cell concentration system (CellPro Inc., Seattle, WA) to obtain an enriched population of CD34⁺ HPCs which are then cryopreserved. CD34-derived DCs are generated from CD34⁺ HPC by culture at a concentration of 0,5 x 10⁶/ml culture
15 medium (X-VIVO-15™; BioWhittaker) supplemented with autologous serum, 10⁻⁵ M 2-β-mercaptoethanol and 1% L-glutamine. The following human recombinant cytokines, approved for clinical use, are used: GM-CSF (50 ng/ml; Immunex Corp.), FLT3-L (100 ng/ml; Immunex Corp.), and TNF (10 ng/ml; CellPro, Inc.). On day eight of culture, all of the cells are pulsed overnight with KLH (2 µg/ml;
20 Intracell), 20% of the cells are pulsed separately with HLA-A*0201 restricted at 2,5 µg/ml, and 80% of the cells are pulsed overnight with a mix of various HLA-A*0201 restricted peptides (2,5 µg/ml) derived from tumor antigens (DKK1 immunogenic polypeptides). After overnight loading, all of the DCs are washed three times with sterile saline and counted and resuspended in 10 ml of sterile saline containing
25 melanoma peptides (1 µg/ml). After 2-h incubation at 22°C, the cells are centrifuged and resuspended in 9 ml of sterile saline for injection. Vaccination is administered s.c. in three injection sites (both thighs and the upper arm). Limbs from which draining lymph nodes has been surgically removed and/or irradiated are not injected. DCs are injected using a long spinal-cord needle and are spread
30 over a 6- to 8-cm distance. This procedure is adapted from a successful method of

the prior art.

EXAMPLE 14

Transduction of DCs with VSV-Pseudotyped Retroviruses Expressing DKK1

[00117] Retroviral vectors can be exploited to transduce the nucleic acid

5 coding for a DKK1 protein in DCs, derived from CD34 cells obtained as described in Example 13. For transduction with the VSV-pseudotyped retroviruses, retroviral supernatant is added to cultured CD34⁺ cells on days two and three at a ratio of 1:1 with culture medium. GM-CSF, SCF, TNF- α and polybrene are added and cells are spun in the plate at 1,000 X g for one hour. On day four, transduced DCs
10 are resuspended in fresh complete DC medium in a six well plate and the differentiation is completed with cytokines. Cells are utilized on day 14 and DC phenotype is confirmed by morphological and FACS analyses. The DKK1-VSV-pseudotyped retroviral system is prepared by first inserting the complete DKK1 sequence in the pCLNC retroviral plasmid. The pCLNC-DKK1 and pMDG-VSV
15 plasmids are co-transfected in 293-gag-pol packaging cells using Lipofectamine™ Plus (Life Technologies). The 293-gag-pol packaging cells are cultured in DMEM supplemented with 10% heat inactivated FBS and antibiotics. Medium is changed 16 hours and 48 hours after transfection. Culture supernatants are harvested on days three and four after transfection of the 293-gag-pol cells. Producer cells are
20 removed from retroviral supernatant by filtration with 0,2 μ m filter (Nalgene, Rochester, NY). Supernatants are immediately frozen at -70°C for future use. DKK1-transduced CD34-derived DCs have the capacity to present both MHC class I and class II epitopes, and are efficient in generating tumor-specific anti-DKK1 CD4⁺ and CD8⁺ T lymphocytes when co-cultured with autologous PBMC. This
25 procedure is adapted from a successful method of the prior art.

EXAMPLE 15

***Ex Vivo* T Lymphocyte Activation with Matured DKK1 Immunogenic Polypeptides Pulsed DCs**

[00118] Monocyte-derived DCs are cultured as described in Example 12

and are pulsed at 1 µg/ml for 90 minutes with DKK1 immunogenic polypeptides. DKK1 polypeptides pulsed DCs are then washed and co-cultured in the same plate with 2×10^6 purified autologous T lymphocytes in the presence of a combination of CD40Ls and LPS. The autologous T lymphocytes are isolated from cryopreserved
5 PBMC using a Human T Cell Enrichment Column (R&D Systems) according to the manufacturer's protocol. After five days of incubation, 50% of the medium is replaced with fresh medium and the cells are transferred to a new 24 well plate. IL-2 is then added to the culture at 10 CU/ml (Chiron, Emeryville, CA). This procedure is adapted from a successful method of the prior art.

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EXAMPLE 16

Immunization of Cancer Patients with DKK1 RNA transfected-DCs

[00119] DKK1 RNA transfection is carried out on the day of administration using cryopreserved and reconstituted monocyte-derived DCs as described in Example 12. Cryopreserved DCs are washed twice in PBS, counted,
15 and spun at 300 x g for 10 min. Subsequently, DCs are resuspended at a concentration of 1×10^7 cells/ml in AIM-V medium and are coincubated for 60 min with 50 µg/ml RNA in a humidified incubator at 37°C/5% CO₂. After transfection with "naked" DKK1 RNA, cells are washed twice in PBS, resuspended in normal saline solution, and administered to patients. DKK1 RNA-transfected DCs are
20 administered at three proposed, escalating dose levels with the highest dose to be tested corresponding to the largest number of DCs that could be generated from the PBMCs of healthy volunteers isolated by a routine leukapheresis. Dose escalation is performed through an i.v. route using three vaccination cycles with 1×10^7 (low dose), 3×10^7 (medium dose), or 5×10^7 (high dose) cells applied at study
25 weeks zero, two, and four. To optimize vaccination, a concomitant dose of 1×10^7 cells is given i.d. at each vaccination cycle. Patients are asked to undergo a second leukapheresis two weeks after the last dose to obtain sufficient numbers of cells for immunological monitoring. This procedure is adapted from a successful method of the prior art.

EXAMPLE 17**Immunization of Colon Cancer patients with DKK1 RNA transfected- DCs**

[00120] Patients with resected hepatic metastases of colon cancer are administered autologous DCs loaded with DKK1 mRNA. DCs are prepared as described in Example 12 and transfected as described in Example 16. Administration schedule is as described in Example 16. This procedure is adapted from a successful method of the prior art.

EXAMPLE 18**Immunization of Cancer Patients with DKK1 cDNA Transfected- DCs**

[00121] The plasmid pCMV DKK1, containing the cDNA of DKK1 under the control of the human CMV promoter, is produced under good manufacturing practice (GMP) conditions at Q-One™ Biotech Ltd (Glasgow, U.K.). DCs from the patients are prepared as described in Example 12 and transfected with pCMV DKK1 on day 5 of culture with cationic liposomes (Lipofectin; Gibco). One million of these DCs per vaccine are cryopreserved 24 h later using Gelifundol™ (Biotest, Dreieich, Germany) and DMSO at a final concentration of 10%. DCs are evaluated by morphology and flow cytometry using the monoclonal antibodies (mAb) against CD1a, CD86 (Pharmingen), CD80, CD83 and HLA-DR (Immunotech, Coulter). To evaluate the DC transfection rate, the expression of DKK1 epitopes is tested by flow cytometry analysis using a mAb against DKK1. One million gene-transfected DCs are injected s.c. into the upper limb close to the inguinal lymph nodes on days one, 21 and 42, respectively. Delayed-type hypersensitivity (DTH) reactions comparing the vaccine, untransfected DC, and physiological saline are performed before and after vaccination (5×10^5 cells are injected i.d. in the patients' backs). This procedure is adapted from a successful method of the prior art.

EXAMPLE 19**Adoptive Transfer of DKK1 Specific CD8⁺ T-Cells**

[00122] PBMCs are obtained and antigen-specific cytotoxic T

lymphocytes (CTLs) are generated from autologous blood monocytes-derived DCs and pulsed with the HLA-A2-restricted DKK1 immunogenic polypeptides as described in Example 12. After three cycles of stimulation at weekly intervals, T cells are cloned by and expanded for *in vitro* testing. CTL clones demonstrating
5 specific lysis of antigen-positive tumor targets in a chromium release assay are selected. Clones are expanded in 14-day cycles by using anti-CD3 antibody (OKT3, Orthoclone™; Ortho Biotech, Raritan, NJ) at 30 ng/ml, irradiated allogeneic PBMCs, at 10^6 cells/ml, irradiated allogeneic lymphoblastoid cell lines (2×10^5 cells/ml), and serial IL-2 (aldesleukin; Chiron) at 25–50 units/ml every two–three
10 days. All patients selected express HLA-A2. A total of four T cell infusions is planned, the first without low-dose IL-2 and subsequent infusions (second, third, and fourth) co-administered with increasing doses of s.c. IL-2 (0,25, 0,5, and 1,0 $\times 10^6$ units/m² twice daily for 14 days. Patients are monitored closely by physical examination and serum chemistries for evidence of toxicity. Stopping rules
15 included the appearance of serious (grade III toxicity by National Cancer Institute common toxicity criteria). This procedure is adapted from a successful method of the prior art.

EXAMPLE 20

**Immunization of Cancer Patients by Adoptive Transfer of DKK1
Immunogenic Polypeptides-Activated Autologous TIL**
20 [00123] Granulocyte colony-stimulating factor (G-CSF)-mobilized stem cells are obtained by leukapheresis from all patients and cryopreserved. Each patient has one or more deposits of metastatic melanoma excised, and multiple independent TIL cultures are started from each nodule. TIL cultures are initiated by
25 the explant of a small (2mm^3) tumor fragments or by plating 1×10^6 viable cells of a single cell suspension of enzymatically digested tumor tissue into 2 ml of complete medium (RPMI1640 based medium supplemented with 10% human serum) containing 6000 IU/ml of IL-2. The cultures are maintained at cell concentrations between 5×10^5 and 2×10^6 cells per ml until several million TIL cells are available.
30 Multiple independent cultures are screened by cytokine secretion assay for

recognition of autologous tumor cells (if available) and HLA-A2⁺ tumor cell lines. Two to six independent TIL cultures exhibiting the highest cytokine secretion are further expanded in complete medium with 6000 IU per ml IL-2 until the cell number is over 5×10^7 cells (this cell number was typically reached three-six
5 weeks after tumor excision). TIL cultures that maintain specific tumor cell recognition are expanded for treatment using one cycle of a rapid expansion protocol with irradiated allogenic feeder cells, OKT3 (anti-CD3) antibody, and 6000 IU per ml IL-2. This rapid expansion protocol typically results in 1000-fold
10 expansions of cells by the time of administration 14-15 days after initiation of the expansions. Patients who have DKK1-specific TIL receive vaccination with 1 mg DKK1 immunogenic polypeptide in incomplete Freund's adjuvant (IFA) injected subcutaneously. This procedure is adapted from a successful method of the prior art.

[00124] The present invention therefore relates the use of DKK1 or an
15 immunogenic polypeptide thereof as a TA for tumors expressing DKK1. In particular, it is directed to the use of a DKK1 protein or an immunogenic polypeptide thereof as a TA for tumors originating from the breast, the lung, the kidney, the skin, the ovary, the colon and the prostate. DKK1 is considered to be a useful tool to: 1. detect aggressive tumors; 2. serve as a TA to be targeted in
20 immunization strategies including preventive immunization of patients at risk for developing cancer, preventive immunization to prevent recurrences after surgical resection, or for immunotherapy of established tumors.

[00125] Although the present invention has been described hereinabove by
25 way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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- 20 51. Kawano Y. *et al.* Secreted antagonists of the Wnt signaling pathway (2003) *Journal of Cell Science* 116 (13) 2627-2634.

WHAT IS CLAIMED IS:

1. A use of a purified or recombinant DKK1 protein or of an immunogenic polypeptide comprising at least 8 contiguous amino acids of said DKK1 protein, for activating T cells.
- 5 2. The use as recited in claim 1, wherein the DKK1 protein comprises an amino acid sequence as set forth in any one of SEQ ID NO: 8 and residues 32 to 266 of SEQ ID NO: 8.
- 10 3. The use as recited in any one of claims 1 and 2, wherein the DKK1 immunogenic polypeptide consists of a maximum of 25 amino acid residues and comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15, 34 and 38-41.
- 15 4. The use as recited in any one of claims 1 and 2, wherein the DKK1 immunogenic polypeptide consists of a maximum of 25 amino acid residues and comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15.
- 20 5. The use as recited in any one of claims 1 to 4, wherein said purified or recombinant DKK1 protein or said immunogenic polypeptide is expressed on an antigen presenting cell (APC).
6. The use as recited in claim 5, wherein the APC is a dendritic cell transformed to express the purified or recombinant DKK1 protein or immunogenic polypeptide comprising at least 8 contiguous amino acids of the DKK1 protein.
7. A use of an isolated polynucleotide comprising the coding sequence of a DKK1 protein, or the coding sequence of an immunogenic polypeptide comprising at least 8 contiguous amino acids of the DKK1 protein, for activating T cells.
- 25 8. A use as recited in claim 7, wherein said isolated polynucleotide is comprised in an expression vector.

9. A use as recited in claim 8, wherein the vector further comprises a polynucleotide encoding an immunogenicity enhancing polypeptide.

10. The use as recited in any one of claims 7 to 9, wherein the coding sequence is as set forth in any one of SEQ ID NO: 7 and nucleotides 248 to 952 of SEQ ID NO: 6.

11. The use as recited in any one of claims 7 to 9, wherein the coding sequence encodes an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15, 34 and 38-41.

12. The use as recited in any one of claims 7 to 9, wherein the coding sequence encodes an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15.

13. A use of a purified or recombinant DKK1 protein or of an immunogenic polypeptide comprising at least 8 contiguous amino acids of said DKK1 protein, in the making of a medicament for activating T cells.

14. A use of an isolated polynucleotide comprising the coding sequence of a DKK1 protein, or the coding sequence of an immunogenic polypeptide comprising at least 8 contiguous amino acids of the DKK1 protein, in the making of a medicament for activating T cells.

15. A method for monitoring the progression of breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in a patient, with the proviso that the kidney cancer is not Wilms' tumor, comprising the steps of: (a) obtaining from the patient a biological sample susceptible of containing tumor cells; (b) contacting the biological sample with a ligand to a DKK1 protein or an immunogenic polypeptide thereof, or to a polynucleotide encoding said protein or said polypeptide to form a complex; (c) measuring the amount of the complex if any, d) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time, (e) comparing the amount of the complex

measured in step (c) with that in step (d) thereby monitoring the progression of breast, lung, kidney, colon, melanoma , prostate, ovarian or colon cancer in the patient.

16. A method for detecting the presence of breast, lung, kidney, colon,
5 melanoma, prostate, ovarian or colon cancer in a patient, with the proviso that the kidney cancer is not Wilms' tumor, comprising the steps of: (a) obtaining from the patient a biological sample susceptible of containing tumor cells; (b) contacting the biological sample with a ligand to a DKK1 protein or an immunogenic polypeptide thereof, or to a polynucleotide encoding said protein or said polypeptide to form a
10 complex; (c) determining the presence or absence of the complex, wherein the presence of the complex is an indication of the presence of a breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in the patient.

17. A method for monitoring the progression of breast, lung, kidney, colon, melanoma, prostate, ovarian or colon cancer in a patient, with the proviso
15 that the kidney cancer is not Wilms' tumor, comprising the steps of: (a) contacting a biological sample from the patient, susceptible of containing tumor cells, with at least two oligonucleotide primers under conditions wherein said oligonucleotide primers are effective for specifically amplifying a polynucleotide sequence of DKK1 in a reverse transcription polymerase chain reaction; (b) detecting in the sample an
20 amount of polynucleotide amplified in step (a); (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polynucleotide detected in step (c) to the amount detected in step (b) thereby monitoring the progression of breast, lung, kidney, colon, melanoma , prostate, ovarian or colon cancer in the patient.

25 18. A method for activating T cells specific for a DKK1 protein, or an immunogenic polypeptide thereof, comprising incubating the T cells with at least one component selected from the group consisting of: (i) a purified or recombinant DKK1 protein or an immunogenic polypeptide thereof; (ii) a polynucleotide comprising the coding sequence of a DKK1 protein or an immunogenic polypeptide

thereof; and (iii) antigen presenting cells transformed to express a recombinant DKK1 protein or an immunogenic polypeptide thereof, whereby T cells are activated.

19. A method for activating T cells specific for a DKK1 protein, or an immunogenic polypeptide thereof in a patient, comprising administering to the patient at least one component selected from the group consisting of: (i) a purified or recombinant DKK1 protein or an immunogenic polypeptide thereof; (ii) a polynucleotide comprising the coding sequence of a DKK1 protein or an immunogenic polypeptide thereof; and (iii) antigen presenting cells transformed to express a recombinant DKK1 protein or an immunogenic polypeptide thereof, whereby T cells are activated in the patient.

20. An expression vector comprising a polynucleotide encoding a DKK1 protein operably associated with an expression control sequence, and a polynucleotide encoding an immunogenicity enhancing polypeptide.

21. An expression vector comprising an immunogenic polypeptide derived from a DKK1 protein operably associated with an expression control sequence.

22. An expression vector as recited in claim 21, further comprising a polynucleotide encoding an immunogenicity enhancing polypeptide.

23. A host cell expressing the vector according to any one of claims 21 to 22.

24. A cell as recited in claim 23 which is an antigen-presenting cell (APC).

25. A fusion protein comprising a DKK1 protein or an immunogenic polypeptide thereof and an immunogenicity enhancing polypeptide.

26. A composition comprising a physiologically acceptable carrier, and a second component selected from the group consisting of: (a) a purified or recombinant DKK1 protein or an immunogenic polypeptide of DKK1; (b) a polynucleotide comprising the coding sequence of a DKK1 protein or an immunogenic polypeptide of DKK1; (c) an antibody specific to a DKK1 protein or to an immunogenic polypeptide thereof; (d) a fusion protein as recited in claim 25; (e) a T cell population activated by cells expressing a DKK1 protein or expressing an immunogenic polypeptide thereof; and (f) APC cells according to claim 24.

27. A method for activating T cells, comprising contacting the T cells with a composition as defined in claim 26.

28. An immunogenic polypeptide derived from a DKK1 protein.

29. A polypeptide as recited in claim 28, of up to 25 amino acid residues comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 9-15, 34 and 38-41.

30. An isolated polynucleotide encoding a polypeptide as recited in claim 29.

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1   gcagagctct gtgctccctg cagtcaggac tctgggaccg cagggggctc ccggaccctg
61  actctgcagc cgaaccggca cggtttcgtg gggaccagg cttgcaaagt gacggtcatt
      M M A L G A A G A
121 ttctctttct ttctccctct tgagtccttc tgagatgatg gctctgggag cagcggggagc
      T R V F V A M V A A A L G G H P L L G V
181 taccgggggtc tttgtcgcga tggtagcggc ggctctcggc ggccaccctc tgctggggagt
      S A T L N S V L N S N A I K N L P P P L
241 gagcgccacc ttgaactcgg ttctcaattc caacgctatc aagaacctgc cccaccgct
      G G A A G H P G S A V S A A P G I L Y P
301 gggcggcgct gcggggcacc caggctctgc agtcagcgcc gcgcgggaa tcctgtaccc
      G G N K Y Q T I D N Y Q P Y P C A E D E
361 gggcgggaaat aagtaccaga ccattgacaa ctaccagccg taccggtgcg cagaggacga
      E C G T D E Y C A S P T R G G D A G V Q
421 ggagtgcggc actgatgagt actgcgctag tcccaccgcg ggaggggacg cagggtgca
      I C L A C R K R R K R C M R H A M C C P
481 aatctgtctc gcctgcagga agcgccgaaa acgctgcatg cgtcacgcta tgtgctgccc
      G N Y C K N G I C V S S D Q N H F R G E
541 cggaattac tgcaaaaatg gaatatgtgt gtcttctgat caaaatcatt tccgaggaga
      I E E T I T E S F G N D H S T L D G Y S
601 aattgaggaa accatcactg aaagctttgg taatgatcat agcaccttgg atgggtattc
      R R T T L S S K M Y H T K G Q E G S V C
661 cagaagaacc accttgtctt caaaaatgta tcacacaaa ggacaagaag gttctgtttg
      L R S S D C A S G L C C A R H F W S K I
721 tctccggtca tcagactgtg cctcaggatt gtgttgtgt agacacttct ggtccaagat
      C K P V L K E G Q V C T K H R R K G S H
781 ctgtaaacct gtctgaaaag aaggtcaagt gtgtaccaag cataggagaa aaggctctca
      G L E I F Q R C Y C G E G L S C R I Q K
841 tggactgaa atattccagc gttgttactg tggagaaggc ctgtcttgcc ggatacagaa
      D H H Q A S N S S R L H T C Q R H *
901 agatcaccat caagccagta attcttctag gcttcacact tgtcagagac actaaaccag
961 ctatccaaat gcagtgaact ctttttatat aatagatgct atgaaaacct tttatgacct
1021 tcatcaactc aatcctaagg atatacaagt tctgtgggtt cagttaagca ttccaataac
1081 accttccaaa aacctggagt gtaagagctt tgtttcttta tggaactccc ctgtgattgc
1141 agtaaattac tgtattgtaa attctcagtg tggcacttac ctgtaaatgc aatgaaactt
1201 ttaattatct ttctaaaggc gctgcactgc ctatttttcc tcttgttatg taaatttttg
1261 tacacattga ttgttatctt gactgacaaa tattctatat tgaactgaag taaatcattt.
1321 cagcttatag ttcttaaaaag cataaccctt taccocattt aattotagag tctagaacgc
1381 aaggatctct tggaatgaca aatgataggc acctaaaatg taacatgaaa atactagctt
1441 attttctgaa atgtactatc ttaatgctta aattatattt ccctttaggc tgtgatagtt
1501 tttgaaataa aatttaacat ttaatatcat gaaatgttat aagtagacat acattttggg
1561 attgtgatct tagaggtttg tgtgtgtgta cgtatgtgtg tgttctacaa gaacggaagt
1621 gtgatatggt taaagatgat cagagaaaag acagtgtcta aatataagac aatattgato
1681 agctctagaa taactttaaa gaaagacgtg ttctgcattg ataaactcaa atgatcatgg
1741 cagaatgaga gtgaatctta cattactact ttcaaaaata gtttccaata aattaataat
1801 acctaaaaaa aaaaa

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Notes : Signal peptide 155...247
 Mature protein 248...952
 PolyA signal 1506..1511
 PolyA site 1529
 PolyA signal 1787..1792
 PolyA site 1805

Figure 1

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1   MMALGAAGAT RVFVAMVAAA LGGHPLLGVS ATLNSVLNSN AIKNLPPPLG GAAGHPGSAV
61  SAAPGILYPG GNKYQTIDNY QPYPCAEDEE CGTDEYCASP TRGGDAGVQI CLACRKRKR
121 CMRRHAMCCPG NYCKNGICVS SDQNHFRGEI EETITESFGN DHSTLDGYSR RTTLSSKMYH
181 TKGQEGSVCL RSSDCASGLC CARHFWKIC KPVLEKEQVC TKHRRKGSYG LEIFQRCYCG
241 EGLSRIQKD HHQASNSSRL HTCQRH
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Figure 2

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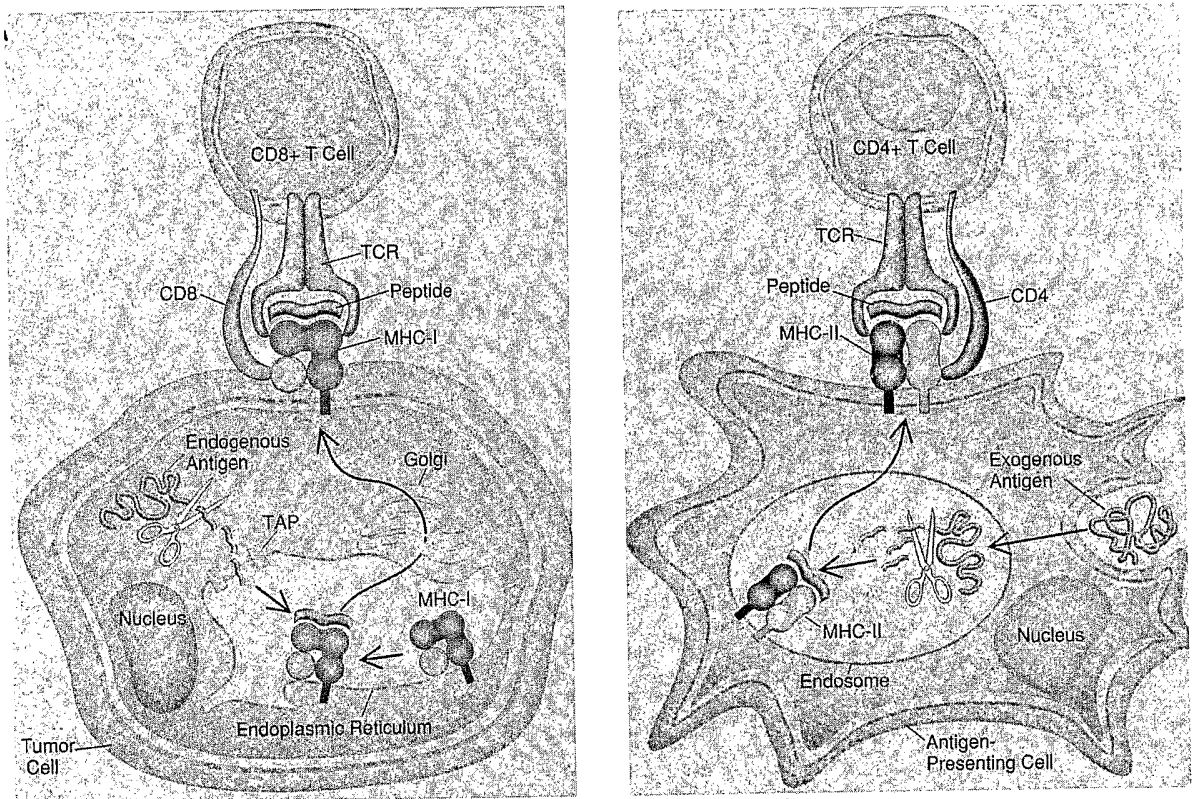


Figure 3

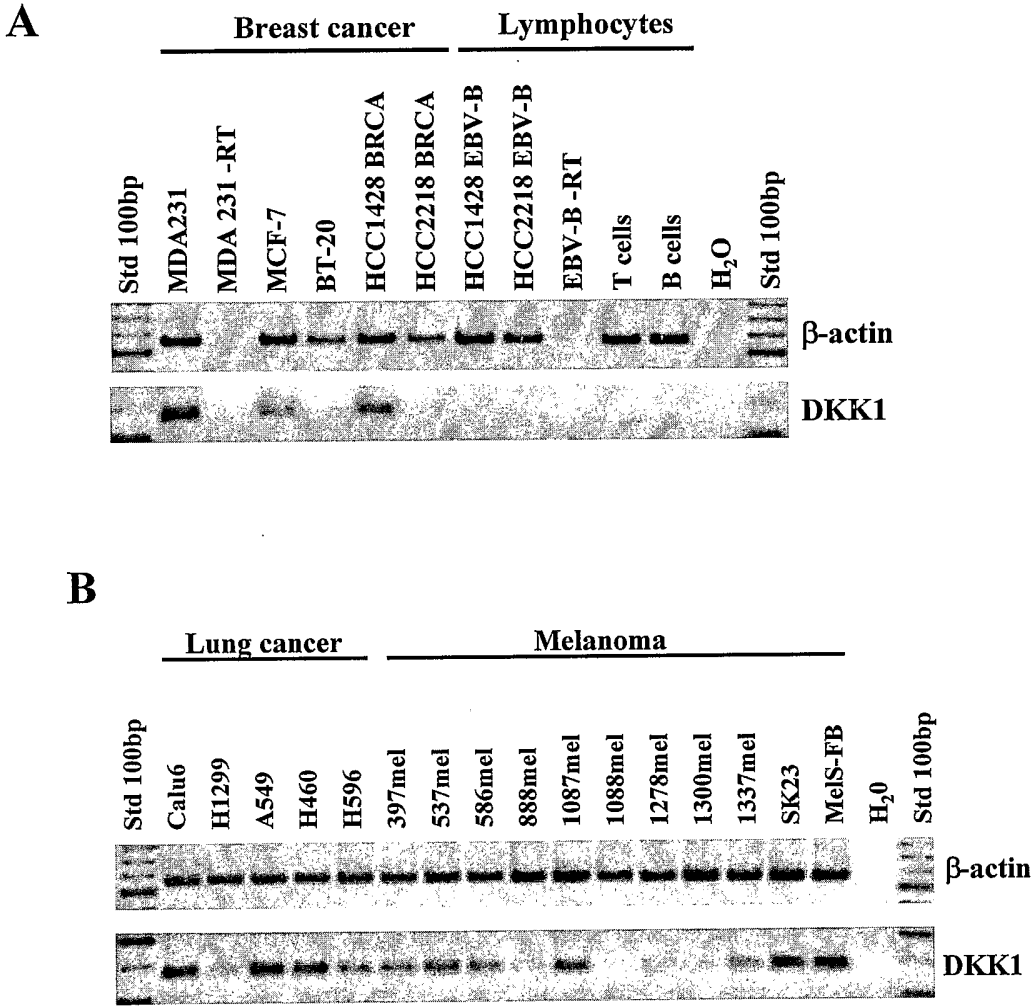


Figure 4

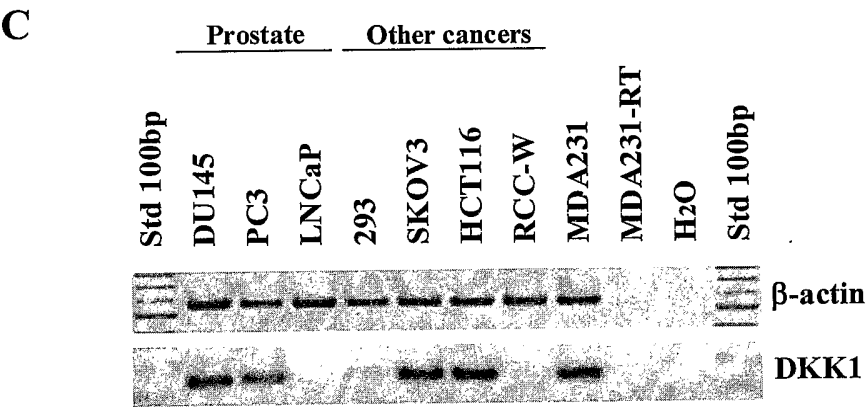


Figure 4 (continued)

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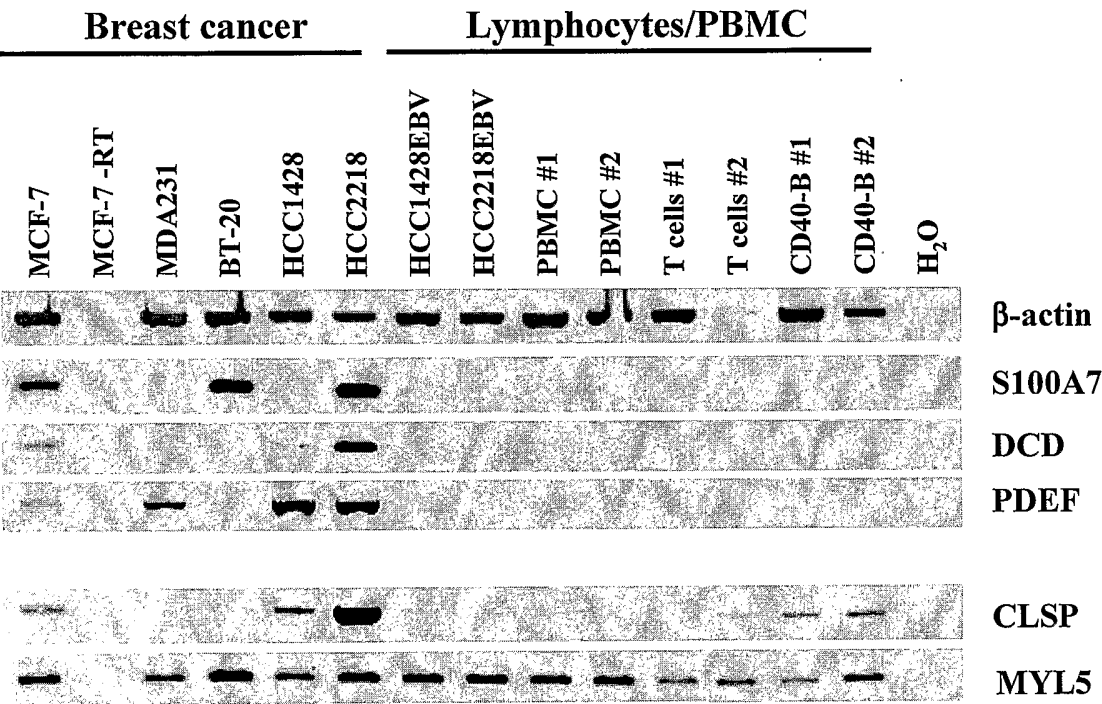
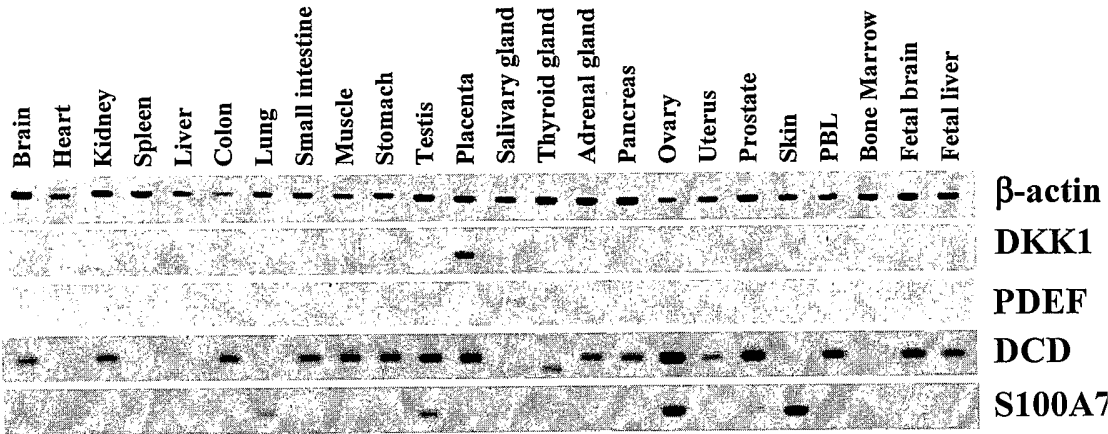


Figure 5

A



B

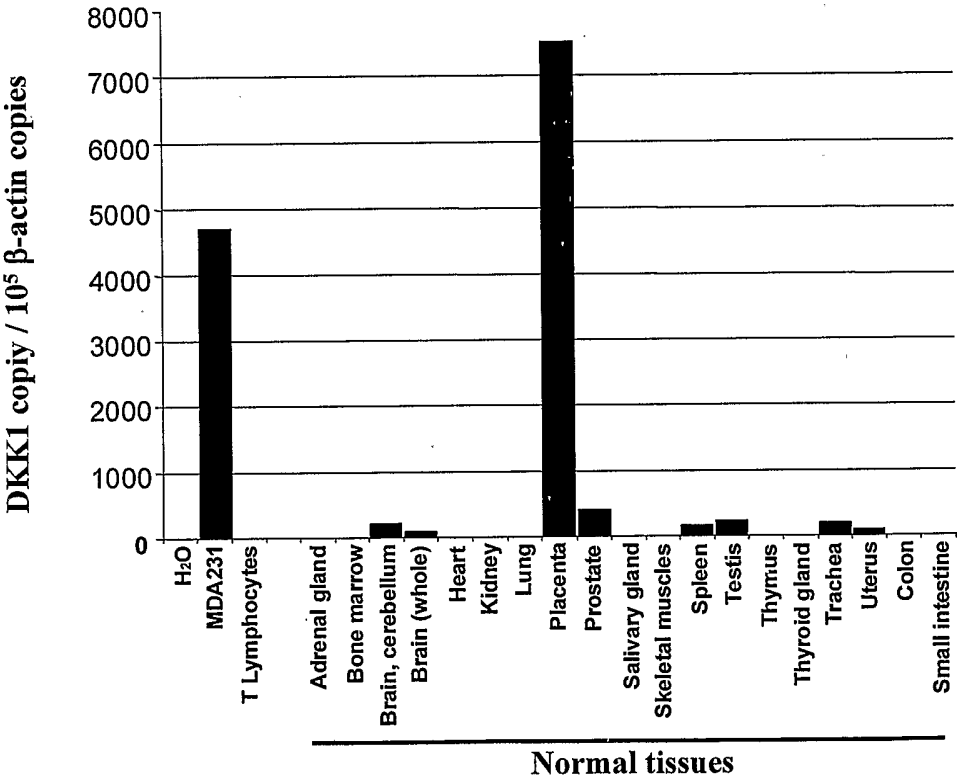


Figure 6

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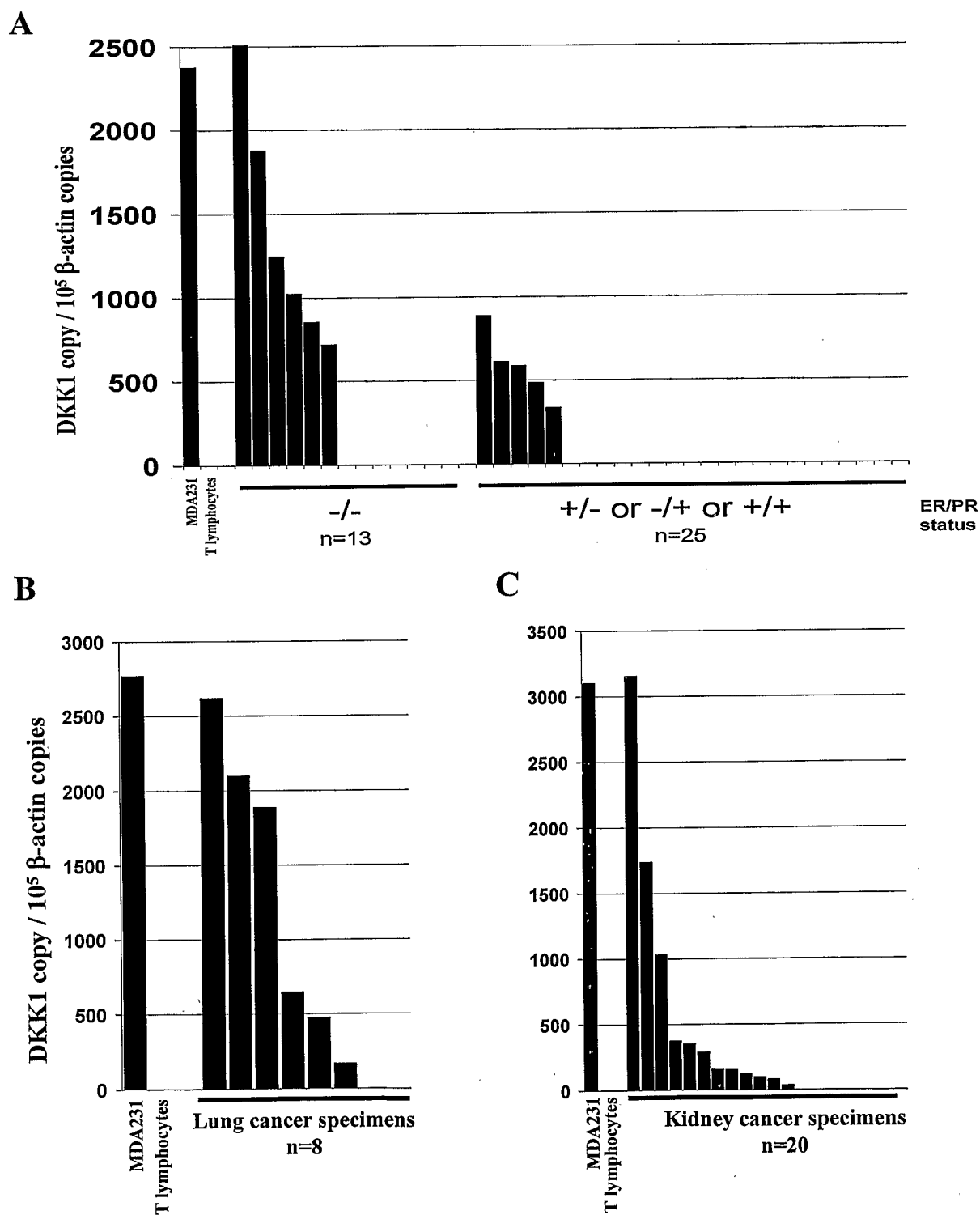
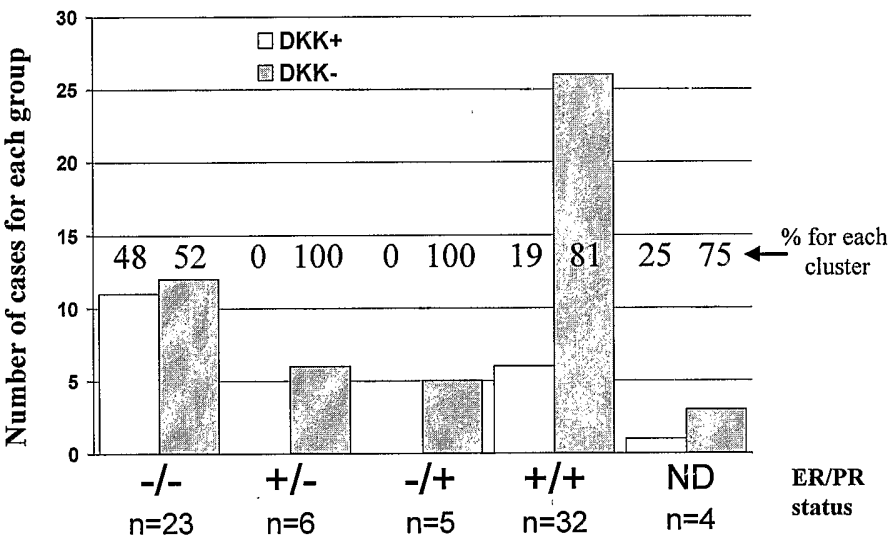


Figure 7

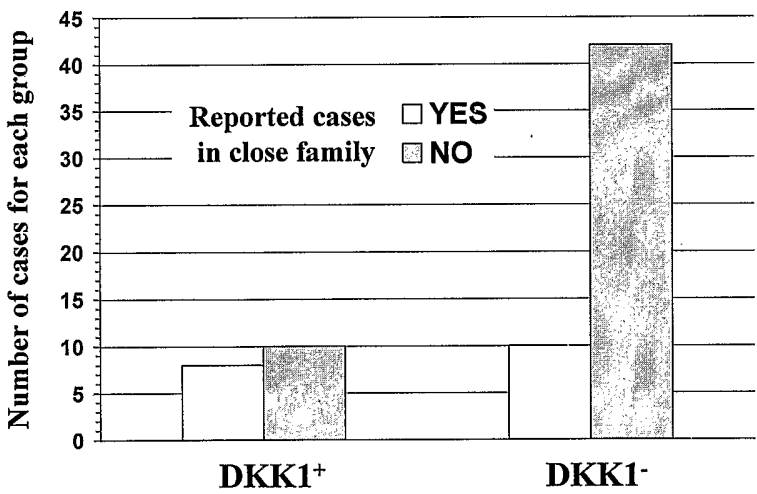
9/10

Figure 8

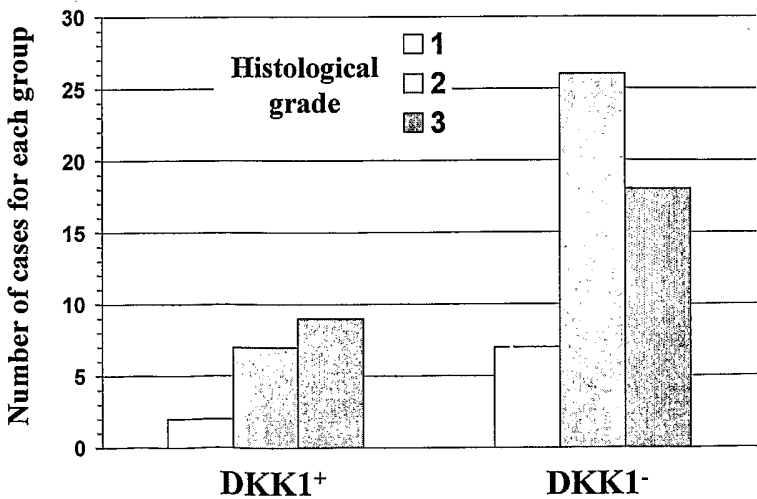
A



B



C



10/10

pos	123456789	
20	ALGGHPLLGV	
40	NAIKNLPPPL	
32	TINSVLNSN	
37	LNSNAIKNL	
61	SAAPGILYP	
68	YPGGNKYQT	
182	KGQEGSVCL	
pos	123456789	
	L V V	Anchor RESIDUES for HLA-A*0201
	M L	
pos	123456789	
	E K	Preferred residues
	K	
pos	123456789	
	I AGIIAE	Other residues
	L YPKLYS	
	F FDYTH	
	K PTN	
	M M G	
	Y S F	
	V R V	
	H	

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001251

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7): C12N 15/12, A61K 38/17, A61K 38/08, A61K 48/00, A61K 31/7088, A61K 39/00, G01N 33/574, C12Q 1/68, C07K 19/00, A61K 39/395, C07K 14/705

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(7): C12N 15/12, A61K 38/17, A61K 38/08, A61K 48/00, A61K 31/7088, A61K 39/00, G01N 33/574, C12Q 1/68, C07K 19/00, A61K 39/395, C07K 14/705

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

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

Databases: STN (BIOSIS, MEDLINE, CAPLUS), DELPHION, CANADIAN PATENT DATABASE, GENOMEQUEST

KEYWORDS/SEARCH PARAMETERS: Structure search of SEQ ID Nos:9-15, 34 and 38-41, DKK-1, Dickkopf-1, cancer, tumour, tumour antigen, immune, T-cell, T-lymphocyte, fusion, hybrid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/083921 A2 AGENSYS INC. [US/US] 24 October 2002 Figures 2D and 28-31 Table I Tables V-VIII (pages 187-201) AND XXIII-XX (pages 508-512) also, see e.g. pages 4-6, 28-29, 36-37, 40-44, 50-52, 56-57 and 66-68	1-14, 17-30
P,X	FORGET, M-A., "Characterization of DKK1 as a Breast Cancer Tumor Antigen." J. Immunotherapy, November-December 2004, Vol. 27, No. 6, p. S59 ISSN 1524-9557 see whole document	1-14, 17-30
X	WO 02/066509 A2 GENENTECH INC. [US/US] 29 August 2002 page 41	20

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

[X] See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 November 2005 (20-11-2005)

Date of mailing of the international search report

7 December 2005 (07-12-2005)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001(819)953-2476

Authorized officer

Ralph Salvino (819) 997-3031

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001251

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 18, 19 and 27
because they relate to subject matter not required to be searched by this Authority, namely :

Claims 18, 19 and 27, directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search under Rule 39.1(iv) of the PCT. Regardless, this Authority has carried out a search based on the alleged effects of the product defined in claims 18, 19 and 27.
2. ☒ Claim Nos. : 1, 2, 5-10, 13-16, 18, 19 and 21-28
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

See extra sheet, Box No. II
3. ☐ Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001251

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/03990 A1 HUMAN GENOME SCIENCES INC. [US/US] 28 January 1999 pages, 46, 47, 82 and 96-98	20
X	WO 02/092015 A2 GENOME THERAPEUTICS CORPORATION [US/US] and WYETH [US/US] 21 November 2002 example 8, Figure 2A	20
X	TATE, G. and MITSUYA, T., "Human Dickkopf as well as DAN family members, Cerberus and Gremlin, are preferentially expressed in epithelial malignant cell lines." J. Biochem. Mol. Biol. & Biophys., 1999, Vol. 3, Nos. 3-4, pp. 239-242 ISSN 1025-8140 Abstract, Figure 2, page 241	17
X	WIRTHS, O. et al., "Overexpression of human Dickkopf-1, an antagonist of wntless/WNT signalling, in human hepatoblastomas and Wilm's tumors." Lab. Invest., March 2003, Vol. 83, No. 3, pp. 429-434, ISSN 0023-6837 Abstract, Figures 1 and 2, Table 1, pages 431 and 432	17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001251

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		ZA200306232 A	17-11-2004

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001251

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
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The full scope of the subject matter defined in claims 1, 2, 5-10, 13-16, 18-19 and 21-28 was not searched because it does not comply with the prescribed requirements to such an extent that no meaningful search could be done. Specifically, the term "immunogenic polypeptides" as it relates to the claimed Dickkopf-1 protein (DKK-1) defined in claims 1, 2, 5-10, 13-14, 18-19 and 21-28 encompass an infinite number of products. A meaningful search over the whole of the claimed scope is impossible. Further, the description provides support within the meaning of Article 6 of the PCT and sufficient disclosure within the meaning of Article 5 of the PCT for only a limited number of said "immunogenic polypeptides". Consequently, as it relates to these "immunogenic DKK-1 polypeptides", the search has been established for the parts of the application which appear to be clear and supported, namely the amino acid sequences defined by SEQ ID Nos: 9-15, 34 and 38-41.

With respect to claims 15 and 16, the term "ligand" likewise encompasses an infinite number of products. Page 22, lines 5-10 defines said term as including any structurally undefined molecule with a specific function. Further, the description provides inadequate support within the meaning of Article 6 of the PCT and sufficient disclosure within the meaning of Article 5 of the PCT for any products that fall under the scope of the term "ligand". As such, no search was done on claims 15 and 16.

Last, no search was done on the subject matter of claim 26(c) as the present application also provides insufficient support and enabling disclosure for the subject matter defined therein.