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(54) Title: IDENTIFICATION OF SPECIFIC TUMOUR ANTIGENS BY SELECTION OF CDNA LIBRARIES WITH SERA

(57) Abstract: method is described for the identification of specific tumour antigens by means of the selection of cDNA libraries with sera, characterised in that said selection is accomplished with the phage display technique, and in particular said selection is accomplished by means of the SEREX technique (serological analysis of autologous tumour antigens through the expression of recombinant cDNA). The method according to the invention described herein advantageously combines the SEREX approach with the potency of the phage display technique defined above, at the same time avoiding the drawbacks characteristic of the SEREX techniques. The so identified antigens are useful for the preparation of medicaments for the treatment of tumors.

IDENTIFICATION OF SPECIFIC TUMOUR ANTIGENS BY SELECTION OF CDNA LIBRARIES WITH SERA

The invention described herein relates to a method for the identification of specific tumour antigens by means of selection with sera of cDNA libraries derived from tumor cell lines or from subjects suffering from tumours, and particularly for the therapy of tumours.

The invention described herein provides compounds, methods for their preparation, methods for their use, and compositions containing them, suitable for industrial application in the pharmaceutical field.

In particular, but not exclusively, the invention described herein relates to the field of tumor treatment.

Background to the invention

Tumor therapy is practised according to multiple approaches of tumor attack. Other then the use of cytotoxic substances, immunotherapeutic approach is gaining even higher interest.

In this context, tumor immunotherapy knows a constant increase of efforts in research, with the aim to find more effective methods for the identification of specific tumor antigens, useful for the preparation of medicaments for the treatment of tumors. In particular, antitumor vaccines constitute a kind of immunotherapy having the goal to stimulate immune system of the same patient to react against tumor antigens. For this reason, the research has

recently focused also on the target of identifying, isolating and cloning specific tumor-associated antigens, which can be recognized by the host immune system.

A review of arguments and related problems can be found, for example in EP 0 496 074 and WO 00/25813 and the references cited therein.

The identification of tumour antigens may then provide new and better target-specific therapeutic means and more effective methods for the treatment of tumors. More or less specific tumour antigens are known, which have been obtained using tumour cells as antigens-immunogens to stimulate antibodies in laboratory animals. Also known are a number of tumour antigens that stimulate the formation of antibodies in the patients themselves (for example, p53 mutants, HER-2/neu, CEA, PSA). Their identification, however, is difficult when using conventional methods.

The recent development of a method of analysing (screening) cDNA libraries with sera of patients suffering from various types of tumours, known as SEREX (serological analysis of autologous tumour antigens through the expression of recombinant cDNA, see P.N.A.S. 92, 11810-1995), has led to the identification of a large number of tumour antigens.

The SEREX technology is undoubtedly useful for identifying new tumour antigens, but it presents a number of drawbacks consisting in the very laborious nature of the library screening

operations, the high degree of background noise and the large amounts of material necessary.

Since 1993, the year the first tumour antigen (carbonic anhydrase) was characterised, more than 600 different proteins specifically expressed in tumours and to which an immune response is generated have been identified (M. Pfreundschuch et al. Cancer Vaccine Week, International Symposium, October 5-9, 1998, S03) and this number is destined to rise still further. It is interesting to note that 20-30% of the sequences isolated are as yet unknown gene products.

Further research, however, is necessary to improve the techniques for identifying specific tumour antigens for the treatment of tumours.

Abstract of the invention

It has now been found that a combination of the SEREX technique and phage display, a strategy based on the selection of libraries in which small protein domains are exposed on the surface of bacteriophages, within which the corresponding genetic information is contained, provides a method for the identification of specific tumour antigens by means of the selection of cDNA libraries with sera. Using this method it proves possible to identify antigens from very large libraries (i.e. which express a large number of different sequences). The antigens thus identified make it possible to obtain specific ligands, which in turn can be used as contrast media.

Therefore, one object of the invention described herein are specific tumour antigens obtainable by a method comprising the identification by means of the selection of cDNA libraries with sera, said method being characterised in that said selection is accomplished using the phage display technique.

The purpose of the invention described herein is to provide tumour antigens useful for the preparation of medicaments for the treatment of tumour.

Said medicaments are preferably in the form of vaccines.

In another embodiment of the present invention, said antigens are used for the preparation of specific ligands, which can be used for the preparation of medicaments, such as vaccines, or as carriers of antitumor drugs, for example cytotoxic agents or radionuclides.

Detailed description of the invention

The invention described herein comprises the construction of cDNA libraries from tumour cells, obtained both from biopsies (preferable fresh) and from cultured tumour lines, the selection (screening) of such libraries with autologous and heterologous patient sera to identify tumour antigens, including new ones, the characterisation of said antigens, the generation of specific ligands for said tumour antigens (for example, recombinant human antibodies or humanised recombinant murine antibodies), and the preparation of target-selective medicaments incorporating the ligands generated, optionally carrying antitumor active agents.

The method, according to the invention described herein, advantageously combines the SEREX approach with the potency of the phage-display technique defined above, at the same time avoiding the drawbacks characteristic of the SEREX technique, as outlined above.

What is meant by "phage display" is, as understood by the person of ordinary skill in the art, a strategy based on the selection of libraries in which small protein domains are exposed on the surface of bacteriophages within which is contained the corresponding genetic information.

The method implemented according to the invention described herein provides for the first time new and advantageous analysis possibilities:

- the use of smaller amounts of serum to identify tumour antigens, selecting, prior to screening, the library with sera of patients suffering from tumours, in such a way as to reduce their complexity, enriching it with those clones that express specific antigens;
- owing to technical problems, the direct screening of cDNA libraries, as realised with the state of the art technique, does not allow analysis of a large number of clones, and thus makes it impossible to exploit all the potential of recombinant DNA technology. With the method according to the invention, it is, in fact, possible to construct and analyse libraries 10-100 times

larger than those traditionally used in SEREX, thus increasing the likelihood of identifying even those antigens which are present to only a limited extent;

lastly, the possibility of effecting subsequent selection cycles using sera of different patients or mixtures of sera facilitates the identification of cross-reactive tumour antigens, which constitute one of the main objectives of the invention described herein.

In a library of cDNA cloned in a non-directional manner, it is expected that approximately less than 6% of the proteins produced will be correct. The enrichment of this type of library with the true translation product is the real task of expression/display libraries. The invention described herein also provides a new vector for the expression of cDNA and the display of proteins as fusions with the amino-terminal portion of pD with limited expression of "*out-of-frame*" proteins. According to the vector project, the phage exposes the protein fragment on the surface only if its ORF ("*Open Reading Frame*") coincides with pD. The average size of the fragments of cloned DNA in our libraries is 100-600 b.p. (base pairs), and for statistical reasons, most of the "*out-of-frame*" sequences contain stop codons that do not allow translation of pD and display on the phage surface. In this case, the copy of the lambda genome of gpD supports the assembly of the capsid. The new expression/display vector (λKM4) for cDNA libraries differs from the one used in SEREX experiments (λgt11) in that the recombinant protein coded for by the

cDNA fragment is expressed as a fusion with a protein of the bacteriophage itself and thus is displayed on the capsid.

For each library, messenger RNA of an adequate number of cells, e.g. 10^6 cells, is purified, using common commercially available means, from which the corresponding cDNA has been generated. The latter is then cloned in the expression/display vector λKM4. The amplification of the libraries is accomplished by means of normal techniques known to the expert in the field, e.g. by plating, growth, elution, purification and concentration.

The libraries are then used to develop the conditions required for the selection, "screening" and characterisation of the sequences identified.

A library of the phage-display type, constructed using cDNA deriving from human cells, allows the exploitation of selection by affinity, which is based on the incubation of specific sera with collections of bacteriophages that express portions of human proteins (generally expressed in tumours) on their capsid and that contain within them the corresponding genetic information. Bacteriophages that specifically bind the antibodies present in the serum are easily recovered, in that they remain bound (by the antibodies themselves) to a solid support; the non-specific ones, on the other hand, are washed away.

The "screening", i.e. the direct analysis of the ability of the single phage clones to bind the antibodies of a given serum, is done

only at a later stage, when the complexity of the library (i.e. the different number of sequences) is substantially reduced, as a result of the selection.

The use of selection strategies allows faster analysis of a large number of different protein sequences for the purposes of identifying those that respond to a particular characteristic, for example, interacting specifically with antibodies present in the sera of patients with tumours.

Selection by affinity is based on the incubation of specific sera with collections of bacteriophages that express portions of human proteins (generally expressed in tumours) on their capsid and that contain within them the corresponding genetic information. The bacteriophages that specifically bind antibodies present in the serum are easily recovered in that they remain bound (by the antibodies themselves) to a solid support; the non-specific ones, on the other hand, are washed away.

The "screening", i.e. the direct analysis of the ability of the single phage clones to bind the antibodies of a given serum, is done only at a later stage, when the complexity of the library (i.e. the different number of sequences) is substantially reduced, as a result of the selection.

This makes it possible to reduce the work burden and, above all, to use a lower amount of serum for each analysis.

The direct "screening" of a classic cDNA library, in fact, entails the use of large amounts of serum, which are not always easy to procure; in order to analyse the whole complexity of a library (about 10^6 different sequences), one would have to incubate with the sera different filters onto which recombinant proteins, expressed in lysis plaques of infected bacteria, have been transferred: on each filter, it is possible to analyse no more than 10^4 plaques (therefore, at least 100-1000 filters having 15 cm diameter would be necessary) and a 5-10 ml serum volume is used for each filter at a 1:1000 serum dilution (0.5-10 ml total serum).

This strategy, moreover, does not allow the identification of antigens which are present in only slight amounts in the library or are recognised by antibodies present in low concentrations.

On the contrary, affinity selection allows the analysis of more than 10^{11} phage particles in a small volume (0.1-1.1 ml), thereby reducing the required amount of serum: with only 10 µl of serum for each reaction, one can work with a concentration of 10- to 100-fold greater than the one used direct screening, consequently increasing also the probability of identifying those antigens regarded as difficult (considering that one normally performs two selection cycles and one screening on 82 mm filters, the total overall consumption of serum in this case is only 40 µl).

It is thus possible to use selection strategies that favour the identification of antigens capable of interacting with the antibodies

present in sera of different patients affected by the same type of tumour (cross-reactive antigens).

For the purposes of obtaining an enrichment of specific sequences in relation to background noise, various protocols can be adopted based on the use of different solid supports. These protocols are known to experts in the field.

For the purposes of obtaining an enrichment of specific sequences in relation to background noise, various protocols can be used based on the use of different solid supports, such as, for example:

- sepharose: the serum antibodies with the bound phages are attached to a sepharose resin coated with protein A which specifically recognises the immunoglobulins. This resin can be washed by means of brief centrifuging operations to eliminate the aspecific component;
- magnetic beads: the serum antibodies with the bound phages are recovered using magnetic beads coated with human anti-IgC polyclonal antibodies. These beads are washed, attaching them to the test tube wall with a magnet;
- Petri dishes: the serum antibodies with the bound phages are attached to a Petri dish previously coated with protein A. The dish is washed by simply aspirating the washing solution.

The invention will now be illustrated in greater detail by means of examples and figures, Figure 1 representing the map of vector λKM4.

EXAMPLE

MATERIALS AND METHODS

Phages and plasmids:

Plasmid pGEX-SN was constructed by cloning the DNA fragment deriving from the hybridisation of the synthetic oligonucleotides K108 5'-GATCCTTACTAGTTTTAGTAGCGGCCGCGGG-3' and K109 5'-AATTCCCGCGGCCGCTACTAAAACTAGTAAG-3' in the BamHI and EcoRI sites of plasmid pGEX-3X (Smith D.B. and Johnson K.S. Gene, 67(1988) 31-40).

Plasmid pKM4-6H was constructed by cloning the DNA fragment deriving from the hybridisation of the synthetic oligonucleotides K106 5'-GACCGCGTTTGCCGGAACGGCAATCAGCATCGTTCACCACCACCAC CACCACTAATAGG-3' and K107 5'-AATTCCTATTAGTGGTGGTGGTGGTGGTGAACGATGCTGATTGCCGT TCCGGCAAACGCG-3' in the RsrII and EcoRI sites of plasmid pKM4.

Selection by affinity

Falcon plates (6 cm, Falcon 1007) were coated for one night at 4°C with 3 ml of 1 μg/ml of protein A (Pierce, #21184) in $NaHCO_3$ 50 mM, pH 9.6. After discarding the coating solution, the plates were

incubated with 10 ml of blocking solution (5% dry skimmed milk in PBS x 1, 0.05% Tween 20) for 2 hours at 37°C. 10 µl of human serum were preincubated for 30 minutes at 37°C under gentle stirring with 10 µl of BB4 bacterial extract, and 10 µl of $MgSO_4$ 1M in 1 ml of blocking solution. Approximately 10^{10} phage particles of the library were added to the serum solution for a further 1 hour incubation at 37°C under gentle stirring. The incubation mixtures were plated on plates coated with protein A and left to stir for 30 minutes at ambient temperature. The plates were rinsed several times with 10 ml of washing solution (1 x PBS, 1% Triton, 10 mM $MgSO_4$). The bound phages were recovered by infection of BB4 cells added directly to the plate (600 µl per plate). 10 ml of molten NZY-Top Agar (48-50°C) were added to the infected cells and immediately poured onto NZY plates (15 cm). The next day, the phages were collected from the incubation plate by stirring with 15 ml of SM buffer for 4 hours at 4°C. The phages were purified with PEG and precipitation by NaCl and stored in one tenth of the initial volume of SM with 0.05% sodium azide at 4°C.

Immunoscreening

The phage plaques of the bacterial medium were transferred onto dry nitrocellulose filters (Schleicher & Schuell) for 1 hour at 4°C. The filters were blocked for 1 hour at ambient temperature in blocking buffer (5% dry skimmed milk in PBS x 1, 0.05% Tween 20). 20 µl of human serum were preincubated with 20 µl of BB4 bacterial

extract, 10^9/ml of wild-type lambda phage in 4 ml of blocking buffer. After discarding the blocking solution, the filters were incubated with serum solution for 2 hours at ambient temperature under stirring. The filters were washed several times with PBS x 1, 0.05% Tween 20 and incubated with human anti-IgG secondary antibodies conjugated with alkaline phosphatase (Sigma A 2064) diluted 1:5000.

Preparation of lambda phage on large scale (from lysogenic cells)

The BB4 cells were grown under stirring up to OD_{600} = 1.0 in LB containing maltose 0.2%, recovered by centrifugation and resuspended in SM buffer up to OD_{600} = 0.2. 100 µl of cells were infected with lambda with a low multiplicity of infection, incubated for 20 minutes at ambient temperature, plated on LB with ampicillin and incubated for 18-20 hours at 32°C. The next day, a single colony was incubated in 10 ml of LB with ampicillin for one night at 32°C under stirring. 500 ml of fresh LB with ampicillin and $MgSO_4$ mM were inoculated with 5 ml of the overnight culture in a large flask and grown at 32°C up to OD_{600} = 0.6 under vigorous stirring. The flask was incubated for 15 minutes in a water bath at 45°C, then incubated under stirring at 37°C for a further 3 hours. 10 ml of chloroform were added to the culture to complete the cell lysis and the mixture was incubated in a shaker for another 15 minutes at 37°C. The phage was purified from the lysate culture according to standard procedures (Sambrook, J., Fritsch, E.F. & Maniatis, T.

(1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

The phage lysates for ELISA were prepared from the lysogenic cells by means of a similar procedure, but without the addition of chloroform. After precipitation with NaCl and PEG, the bacteriophage preparation was resuspended in one tenth of the starting volume of SM buffer with sodium azide (0.05%) and stored at 4°C.

Lambda ELISA

Multi-well plates (Immunoplate Maxisorb, Nunc) were coated for one night at 4°C with 100 µl/well of anti-lambda polyclonal antibodies at a 0.7 µg/ml concentration in $NaHCO_3$ 50 mM, pH 9.6. After discarding the coating solution, the plates were incubated with 250 µl of blocking solution (5% dry skimmed milk in PBS x 1, 0.05% Tween 20). The plates were washed twice with washing buffer (PBS x 1, Tween 20). A mixture of 100 µl of blocking buffer and phage lysate (1:1) was added to each well and incubated for 1 hour at 37°C. 1 ml of human serum was incubated for 30 minutes at ambient temperature with 10^9 plaque forming units (pfu) of phage λKM4, 1 µl of rabbit serum, 1 µl of BB4 extract, 1 µl of FBS in 100 µl of blocking buffer. The plates were washed after incubation with phage lysate and incubated with serum solution for 60 minutes at 37°C. The plates were than washed and goat antihuman HRP conjugated antibody was added (Jackson ImmunoResearch Laboratories), at a

dilution of 1:20000, in a blocking buffer/secondary antibody mixture (1:40 rabbit serum in blocking solution). After a 30 minute incubation, the plates were washed and peroxidase activity was measured with 100 µl of TMB liquid substrate system (Sigma). After 15 minutes' development, the reaction was stopped with 25 µl of H_2SO_4 2M. The plates were read with an automatic ELISA plate reader and the results were expressed as $A = A_{450nm}-A_{620nm}$. The ELISA data were measured as the mean values of two independent assays.

Construction of λKM4

Plasmid pNS3785 (Hoess, 1995) was amplified with reverse PCR with the oligonucleotide sequences 5'-TTTATCTAGACCCAGCCCTAGGAAGCTTCTCCTGAGTAGGACAAATC C-3' bearing sites XbaI and AvrII (underlined) for subsequent lambda phage cloning and 5'-GGGTCTAGATAAAACGAAAGGCCCAGTCTTTC-3' bearing XbaI. In the reverse PCR, a mixture of Taq polymerase and Pfu DNA polymerase was used to increase the fidelity of the DNA synthesis. Twenty-five amplification cycles were done (95°C-30 sec, 55°C-30 sec, 72°C-20 min). The self-ligation of the PCR product, previously digested with XbaI endonuclease, gave rise to plasmid pKM3 (Figure 1). The lambda pD gene was amplified with PCR from plasmid pNS3785 using the *primers* 5'-CCGCCTTCCATGGGTACTAGTTTTAAATGCGGCCGCACGAGCAAAGA AACCTTTAC-3' and 5'-AGCTTCCTAGGGCTGGGTCTAG-3' containing

the restriction sites NcoI, SpeI, NotI and EcoRI, respectively (underlined). The PCR product was purified, digested with NcoI and EcoRI restrictase and re-cloned in the NcoI and EcoRI sites of pKM3, resulting in plasmid pKM4 bearing only the restriction sites SpeI and Not I at extremity 5' of gpD. The plasmid was digested with XbaI enzyme and cloned in the XbaI site of lambda phage λDam15imm21nin5 (Hoess, 1995).

Construction of cDNA libraries

mRNA was isolated from 10^7 MCF-7 cells (T1 library) or from 0.1 g of a solid tumour sample (T4 library) using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Double-stranded cDNA was synthesised from 5 μg of poly(A)$^+$ RNA using the TimeSaver cDNA Synthesis Kit (Amersham Pharmacia Biotech). Random tagged priming was performed as described previously (Santini, 1986). From 500 ng of first-strand cDNA a copy cDNA was synthesised with the random tagged primer 5'-GCGGCCGCTGG$(N)_9$-3', and second-strand CDNA with the primer 5'-GGCCGGCCAAC$(N)_9$-3'. The final cDNA product was amplified using oligonucleotides bearing SpeI with three reading sequences or NotI sites to facilitate cloning in the λKM4 lambda vector (5'-GCACTAGTGGCCGGCCAAC-3', 5'-GCACTAGTCGGCCGGCCAAC-3', 5'-GCACTAGTCGGGCCGGCCAAC-3' and 5'-GGAGGCTCGAGCGGCCGCTGG-3'). The PCR products were purified on Quiaquick columns (Quiagen) and filtered on

Microcon 100 (Amicon) to eliminate the small inserts, digested with SpeI, NotI restriction enzymes, and, after extraction with phenol, filtered again on Microcon 100.

Vector λKM4 was digested with SpeI/NotI and dephosphorylated, and 8 binding mixtures were produced for each library, each containing 0.5 mg of vector and approximately 3 ng of insert. After overnight incubation at 4°C the binding mixtures were packaged *in vitro* with a lambda packaging kit (Ready-To-Go™ Lambda Packaging Kit, Amersham Pharmacia Biotech) and plated for infection with BB4 cells. After overnight incubation, the phage was eluted from the plates with SM buffer, purified, concentrated and stored at -80°C in 7% DMSO SM buffer.

The complexity of the two libraries, calculated as total independent clones with inserts, was 10^8 for the T1 library and $3.6x10^7$ for the T4 library.

Selection by affinity

For the identification of specific tumour antigens two different affinity selection procedures were used. The first consisted of two panning cycles with a positive serum (i.e. deriving from a patient suffering from tumour pathology), followed by an immunological screening procedure carried out with the same serum, or, alternatively, by analysis of clones taken at random from the mixture of selected phages. A second procedure used a mixture of sera from different patients for the selection, both for panning and

for screening, for the purposes of increasing the efficacy of selection of cross-reactive antigens.

The T1 library was selected with 10 positive sera (B9, B11, B13, B14, B15, B16, B17, B18, B19, and B20), generating, after a single selection cycle, the corresponding mixtures $p9^{I}$, $p11^{I}$, $p13^{I}$, $p14^{I}$, $p15^{I}$, $p16^{I}$, $p17^{I}$, $p18^{I}$, $p19^{I}$, and $p20^{I}$. Each mixture was then subjected to a second affinity selection cycle with the same serum, according to the first strategy mentioned above, giving rise to a second series of mixtures (called $p9^{II}$, $p11^{II}$, $p13^{II}$, $p14^{II}$, $p15^{II}$, $p16^{II}$, $p17^{II}$, $p18^{II}$, $p19^{II}$, and $p20^{II}$). Characterisation by immune enzyme assay (ELISA) showed that some of the mixtures were more reactive with the corresponding serum used for the selection, thus confirming the efficacy of the library and of the affinity selection procedure. By means of immunological screening per plate of the more reactive mixtures ($p9^{II}$, $p13^{II}$, $p15^{II}$, $p19^{II}$, $p20^{II}$) several positive clones were identified.

The second procedure mentioned above was applied to the $p13^{II}$ mixture, subjecting it to a third selection cycle with a mixture of sera (B11, B14, B15, B16, B17, B18, B19, and B20), and thus excluding serum B13 for the purpose of selecting cross-reactive clones. The resulting preparation of selected phages ($p13^{III}$) was assayed by ELISA with the same mixture of sera used in the panning. The subsequent immunological screening procedure per

plate with this mixture of sera (B11, B14, B15, B16, B17, B18, B19, and B20) made it possible to isolate further positive clones.

Affinity selection experiments were also conducted with the T4 library (and also with the T1 library using different sera) according to the same methodology described here.

Multiple immunological screening (pick-blot analysis)

The individual plates which were positive in the immunological screening were isolated and the eluted phages were transferred on a bacteria mat to various 15 cm diameter Petri dishes according to an ordered scheme. The lysis area grids derived from the above-mentioned procedure were transferred onto nitrocellulose membranes and subjected to analysis with different positive sera. For the purposes of making the method more reliable and reproducible, a Genesys Tekan robotic station was used for transfer of the phages onto the dishes, which allowed analysis of up to a maximum of 396 individual clones on a membrane measuring 11 x 7.5 cm, or a lower number by means of repeated transfer of the same clone and subsequent cutting of the membrane into smaller pieces prior to incubation with the sera.

Characterisation of positive clones

The clones that presented multiple reactivity, or a greater specificity for the sera of tumour patients as compared to that of healthy donors, were subsequently sequenced and compared with different databases of sequences currently available (Non-Redundant

Genbank CDS, Non-Redundant Database of Genbank Est Division, Non-Redundant Genbank+EMBL+DDBJ+PDB Sequences).

The sequences obtained can be classified in six groups:

- sequences that code for epitopes of known breast tumour antigens;
- known sequences that code for epitopes of tumour antigens other than those of breast tumour;
- sequences that code for autoantigens;
- sequences that code for known proteins which are, however, not known to be involved either in tumours or in autoimmune diseases;
- sequences that code for unknown proteins (e.g. EST);
- new sequences not yet present in the databases.

Eighty-one different sequences were identified from the T1 library (called T1-1 to T1-115), 13% of which were unknown proteins and 16% were not present in the databases. Twenty-one sequences were identified from the T4 library (called T4-1 to T4-38), 40% of which were not to be found in the databases. The following table shows, by way of an example, the sequences of some of the clones selected:

Name of clone	Sequence	Identification	Classification

T1-2	ATGGGTACTAGTCGGCCGGCCAAC ATCACTCCCACCAATACAATGACT TCTATGAGAACTACAACCTATTGG CCCACAGCCACAATGATGGAACCA CCTTCATCCACTGTATCAACTACA GGCAGAGGTCAGACCACCTTTCCA GCTCTACAGCCACATTCCCCAATA CCAAACACCCCAGCGGCCGC	Intestinal mucin	Tumour antigen
T1-17	ATGGGTACTAGTCGGGCCGGCCAA CTTGTTGAAGAACTGGATAAAGTG GAATCTCAAGAACGAGAAGATGTT CTGGCTGGAATGTCTGGAAAATCC TCTTTCCAAAGATCTGAAGGAGAT TTTCTTTTAAGATCATTGACCAGC GGCCGC	DNA-topo-isomerase II beta	Tumour antigen (malignant mesothelioma)
T1-8	ATGGGTACTAGTGGCCGGCCAACA AGGCAGCTGGAAGAGGTTCTCAAA TTAGATCAAGAAATGCCTTTAACA GAAGTGAAGAGTGAACCTGAGGAA AATATCGATTCAAACAGTGAAAGT GAAAGAGAAGAGATAGAATTAAAA TCTCCGAGGGGACGAAGGAGAATT GCTCGAGATCCCAGCGGCCGC	RBP-1	Tumour antigen (cancer of the breast)
T1-6	ATGGGTACTAGTCGGGCCGGCCAA CTTGAGGAGCTGCAGAAGAAATAC CAGCAAAAGCTAGAGCAGGAGGAG AACCCTGGCAATGATAATGTAACA ATTATGGAGCTACAGACACAGCTA GCACAGAAGACGACTTTAATCAGT GATTCGAAATTGAAAGAGCAAGAG TTCAGAGAACAGATTCACAATTTA GAAGACCGTTTGAAGAAATATGAA AAGAATGTATATGCAACAACTGTG GGGACACCTTACAAAGGTGGCAAT TTGTACCATACGGATGTCTCACTC TTTGGAGAACCTACCAGCGGCCGC	Golgin p245	Autoantigen
T1-101	ATGGGTACTAGTCGGCCGGCCAAC TTCGTGGAAATCAGTGAAGATAAA ACTAAAATCAGAAGGTCTCCAAGC AAACCCCTACCTGAAGTGACTGAT GAGTATAAAAATGATGTAAAAAAC AGATCTGTTTATATTAAAGGCTTC CCAACTGAAGCCAGCGGCCGC	Human lupus La protein	Autoantigen

T1-52	GTGGCCGGCCAACGTTATCAGAGT AGAAGTGGGCATGATCAGAAGAAT CATAGAAAGCATCATGGGAAGAAA AGAATGAAAAGTAAACGATCTACA TCATTGTCATCTCCCAGAAACGGA ACCAGCGGCCGC	Binding protein p53	Unknown as tumour antigen
T1-35	ATGGGTACTAGTCGGGCCGGCCAA CAAATTAGGCAGATTGAGTGTGAC AGTGAAGACATGAAGATGAGAGCT AAGCAGCTCCTGGTTGCCTGGCAA GATCAAGAGGGAGTTCATGCAACA CCTGAGAATCTGATTAATGCACTG AATAAGTCTGGATTAAGTGACCTT GCAGAAAGTCCCAGCGGCCGC	Nuclear matrix protein	Unknown as tumour antigen
T1-10	ATGGGTACTAGTGGCCGGCCAACG GCAGTAGTTCTGGAAAAGCCACTG GGGACGAGACAGGTGCTAAAGTTG AACGAGCTGATGGAGCTTCATGGT GAAGGCAGTAGTTCTGGAAAAGCC ACTGGGGACGAGACAGGTGCTAAA GTTGAACGAGCTGATGGAATGACC CCCAGCGGCCGC	Ribosomal protein s3a	Unknown as tumour antigen
T1-39	ATGGGTACTAGTGGCCGGCCAACG AATTATTCGAGTGCTATAGGCGCT TGTCAGGGAGGTAGCGATGAGAGT AATAGATAGGGCTCAGGCGTTTGT TGATGAGATATTTGGAGGTGGGGA TGATGCACATAATTTGAATCAACA CAACTCCAGCGGCCGC		
T1-12	ATGGGTACTAGTCGGGCCGGCCAA CGTGGTATTATTTAAAAATAGCTA AAAAGGTAAACAATCCAAATGCCA TTAAACAGAGAATTTTAAAAAATG AGATACTACACAGCAACAAAAACC TATGAGCTAATGCTAGATGCAACA ACACAGACCAGCGGCCGC	No data	
T1-32	ATGGGTACTAGTCGGGCCGGCCAA CTACACGCCTTTCCACTC CACTCTACTACACTCTACTACACT ACACCCAGCGGCCGC	No data	
T1-74	ATGGGTACTAGTCGGCCGGCCAAC AGAGAAGCTAAGCAACTGCATCAT CAGCCACATTCAATCGAATTAATA CAGTCCAGCGGCCGC	EST	
T4-2	ATGGGTACTAGTCGGCCGGCCAAC TCAGAGGTGTATAAGCCAACATTG CTCTACTCCAGCGGCCGC	EST	

T4-11	ATGGGTACTAGTGGCCGGCCAACG GTTGGTTTTACTCTAGATTTCACT GTCGACCCACCCAGCGGCCGC	EST	
T4-19	ATGGGTACTAGTCGGGCCGGCCAA CTATACCGTACAACCCTAACATAT ACCAGCGGCCGC	No data	

Clone T1-52 is known as an epitope of binding protein p53 (Haluska P. et al. *NAR, 1999, v.27, n.12, 2538-2544)*, but has never been identified as a tumour antigen. Said clone has the sequence VLVAGQRYQSRSGHDQKNHRKHHGKKRMKSKRSTSLSSPRNGTSGR and its use as a tumour antigen is part of the invention described herein.

Clone T1-32, hitherto unknown, has the following sequence MGTSRAGQLHAFPLHSTTLYYTTPSGR; it is a tumour antigen and as such is part of the invention described herein.

Clone T1-74, hitherto unknown, has the following sequence MGTSRPANREAKQLHHQPHSIELIQSSGR; it is a tumour antigen and as such is part of the invention described herein.

Clone T4-2, hitherto unknown, has the following sequence MGTSRPANSEVYKPTLLYSSGR; it is a tumour antigen and as such is part of the invention described herein.

Clone T4-11, hitherto unknown, has the following sequence MGTSGRPTVGFTLDFTVDPPSGR; it is a tumour antigen and as such is part of the invention described herein.

Clone T4-19, hitherto unknown has the following sequence MGTSRAGQLYRTTLTYTSGR; it is a tumour antigen and as such is part of the invention described herein.

The phage clones characterised by means of pick-blot analysis and for which specific reactivity had been demonstrated with sera from patients suffering from breast tumours were amplified and then analysed with a large panel of positive and negative sera. After this ELISA study, the cDNA clones regarded as corresponding to specific tumour antigens were cloned in different bacterial expression systems (protein D and/or GST), for the purposes of better determining their specificity and selectivity. To produce the fusion proteins each clone was amplified from a single plaque by PCR using the following oligonucleotides: K84 5'-CGATTAAATAAGGAGGAATAAACC-3' and K86 5'-CTCTCATCCGCCAAAACAGCC-3'. The resulting fragment was then purified using the QIAGEN Purification Kit, digested with the restriction enzymes SpeI and NotI and cloned in plasmid pKM4-6H to produce the fusion protein with D having a 6-histidine tail, or in vector pGEX-SN to generate the fusion with GST. The corresponding recombinant proteins were then prepared and purified by means of standard protocols (Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor).

The following table gives, by way of an example, the reactivities with negative and positive sera of a number of selected clones, assayed in the form of phage or fusion protein preparations:

Name of clone	Lambda phage reactivity with positive sera (number positive/ total number assayed)	Lambda phage reactivity with negative sera	Reactivity of fusion protein D with positive sera (* for GST fusion)	Reactivity of fusion protein D with negative sera (* for GST fusion)
T1-2	1/20	0/9		
T1-17	1/10	0/0	* 2/16	* 0/15
T1-8	1/10	0/0	1/13	0/15
T1-6	1/10	0/0		
T1-101	/20	0/1		
T1-52	7/41	0/20	13/53	3/24
T1-35	4/10	14/21		
T1-10	1/10	0/0		
T1-39	11/34	0/26	Non-reactive	
T1-12	23/72	0/31	Non-reactive	
T1-32	17/72	0/31	* 10/72	* 1/31
T1-74	29/72	2/27	* 21/72	* 4/32
T4-2	11/18	0/17	9/28	1/31
T4-11	4/21	0/26	8/70	0/30
T4-19	5/20	0/26	12/70	0/30

For the purposes of demonstrating the efficacy of the tumour antigens selected for recognising tumour cells and thus for the detection and diagnosis of pathological abnormalities, mice were immunised to induce an antibody response to a number of the clones selected.

The mice were immunised by giving seven administrations of the antigen over a period of two months, using as immunogens the fusion proteins D1-52, D4-11 and D4-19, corresponding to the fusions of the sequences of clones T1-52, T4-11 and T4-19 with protein D. Each time, 20 μg of protein were injected

(intraperitoneally or subcutaneously) per mouse in CFA, 20 μg in IFA, 10 μg in PBS and four times 5 μg in PBS for each of the three proteins. For the purposes of checking the efficacy of immunisation to the sequence of the tumour antigen, the sera of the immunised animals were assayed against the same peptide sequences cloned in different contexts, in order to rule out reactivity to protein D.

In the case of D1-52, the sera of the immunised mice were assayed with the fusions with GST (GST1-52), whereas in the cases of D4-11 and D4-19 the corresponding peptide sequences were cloned in vector pC89 (Felici et al. 1991, J. Mol. Biol. 222:301-310) and then tested as fusions with pVIII (main protein of the capsid of filamentous bacteriophages). The results of ELISA with the sera of the immunised animals showed that effective immunisation was obtained in the cases of D1-52 and D4-11, and thus the corresponding sera were assayed for the ability to recognise tumour cells. To this end, the cell line MCF7 was used, and analysis by FACS demonstrated that antibodies present in both sera (anti-D1-52 and anti-D4-11) are capable of specifically recognising breast tumour MCF7 cells, and not, for instance, ovarian tumour cells, while this recognition capability is not present in preimmune sera from the same mice.

CLAIMS

1. Specific tumour antigens obtainable by selection of cDNA libraries with sera, characterised in that said selection is accomplished with the phage display technique.
2. Tumour antigens according to claim 1, in which said selection is accomplished by means of the SEREX technique (serological analysis of autologous tumour antigens through expression of recombinant cDNA).
3. Tumour antigens according to claim 1 or 2, in which said selection is accomplished by means of the affinity selection technique.
4. Tumour antigens according to claim 1, in which said libraries are obtained from tumour biopsies.
5. Tumour antigens according to claim 1, in which said libraries are obtained from cultured tumour cell lines.
6. Antigen according to claim 1 with the following sequence MGTSRPANREAKQLHHQPHSIELIQSSGR.
7. Antigen according to claim 1 with the following sequence MGTSRPANSEVYKPTLLYSSGR.
8. Antigen according to claim 1 with the following sequence MGTSGRPTVGFTLDFTVDPPSGR.
9. Antigen according to claim 1 with the following sequence MGTSRAGQLYRTTLTYTSGR.

10. Antigen according to claim 1 with the following sequence MGTSRAGQLHAFPLHSTTLYYTTPSGR.

11. Use of the sequence VLVAGQRYQSRSGHDQKNHRKHHGKKRMKSKRSTSLSSPRN GTSGR as a tumour antigen.

12. Use of antigens of claims 1-11 as active agents useful for the preparation of medicaments for the treatment of tumours.

13. Specific ligand for an antigen of any of claims 1-11.

14. Anti-antigen antibody of one of claims 1-11.

15. Use of a ligand of claim 13 or of an antibody of claim 14 as active agent for the preparation of medicaments for the treatment of tumours.

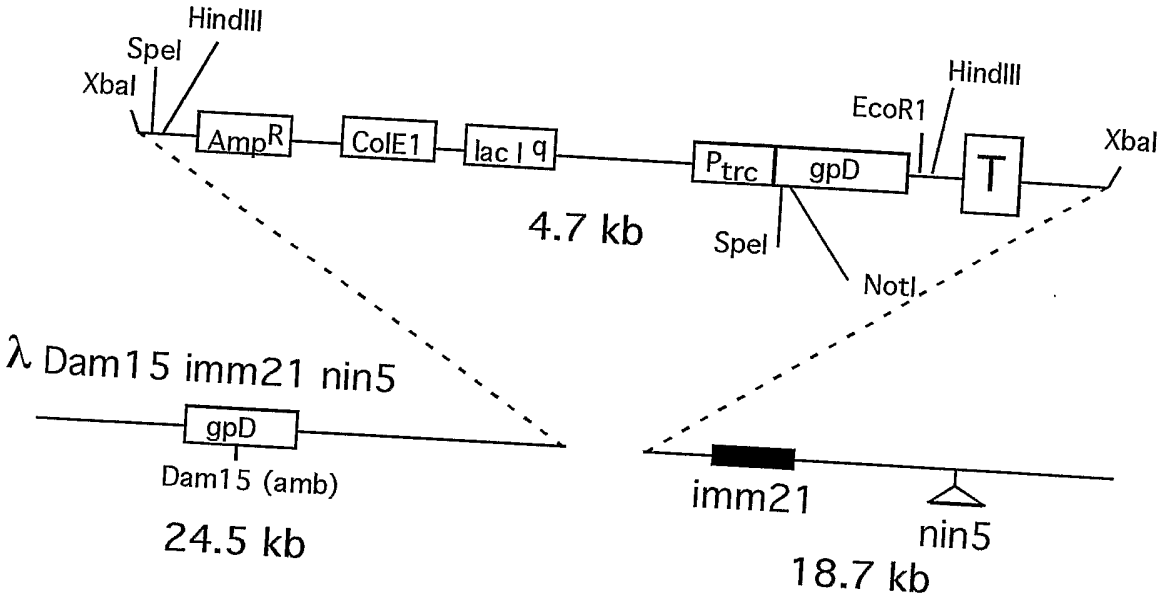
16. Use of a ligand of claim 13 or of an antibody of claim 14 as carrier for an active agent for the treatment of tumors.

17. Use of the expression/display vector (λKM4) for obtaining antigens of claims 1-11.

18. Antitumor vaccine comprising at least an antigen of claims 1-11.

19. Antitumor medicament comprising a ligand of claim 13.

20. Antitumor medicament comprising an antibody of claim 14.


HindIII
SpeI
XbaI
AmpR
ColE1
lac I q
Ptrc
gpD
EcoR1
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T
XbaI
4.7 kb
SpeI
NotI
λ Dam15 imm21 nin5
gpD
Dam15 (amb)
24.5 kb
imm21
nin5
18.7 kb

Figura 1

SEQUENCE LISTING

<110> KENTON s.r.l.

<120> Identification of specific tumor antigens by selection of cDNA libraries with sera and use of said antigens in the treatment of tumors

<130> 015_KT_01_PCT

<140> not yet known
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<305> 12
<306> 2538-2544
<307> 1999-06-15
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1 5 10 15

Lys Asn His Arg Lys His His Gly Lys Lys Arg Met Lys Ser Lys Arg
20 25 30

Ser Thr Ser Leu Ser Ser Pro Arg Asn Gly Thr Ser Gly Arg
35 40 45

INTERNATIONAL SEARCH REPORT

ial Application No
PCT/IT 01/00413

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C07K16/18 A61K39/00 A61K39/395

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

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

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

BIOSIS, EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIOUD MOULDY ET AL: "Profiling the immune response in patients with breast cancer by phage-displayed cDNA libraries." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 31, no. 3, March 2001 (2001-03), pages 716-725, XP002193380 ISSN: 0014-2980	1-5, 12-16, 18-20
Y	the whole document	17

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"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)

"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

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 2002	24.07.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Morawetz, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int[illegible]nal Application No
PCT/IT 01/00413

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	JAEGER D ET AL: "CANCER-TESTIS ANTIGENS AND ING1 TUMOR SUPPRESSOR GENE PRODUCT ARE BREAST CANCER ANTIGENS: CHARACTERIZATION OF TISSUE-SPECIFIC ING1 TRANSCRIPTS AND A HOMOLOGUE GENE" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 59, 15 December 1999 (1999-12-15), pages 6197-6204, XP000986298 ISSN: 0008-5472	1-5, 12-16, 18-20
Y	the whole document	17
X	JAEGER D ET AL: "IDENTIFICATION OF A TISSUE-SPECIFIC PUTATIVE TRANSCRIPTION FACTOR IN BREAST TISSUE BY SEROLOGICAL SCREENING OF A BREAST CANCER LIBRARY" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 61, no. 5, 1 March 2001 (2001-03-01), pages 2055-2061, XP001053562 ISSN: 0008-5472	1-5, 12-16, 18-20
Y	the whole document	17
X	TURECI O ET AL: "SEROLOGICAL ANALYSIS OF HUMAN TUMOR ANTIGENS: MOLECULAR DEFINITION AND IMPLICATIONS" MOLECULAR MEDICINE TODAY, ELSEVIER, CAMBRIDGE, GB, vol. 3, no. 8, August 1997 (1997-08), pages 342-349, XP000985587 ISSN: 1357-4310	1-5, 12-16, 18-20
Y	the whole document	17
Y	MIKAWA Y G ET AL: "Surface display of proteins on bacteriophage lambda heads." JOURNAL OF MOLECULAR BIOLOGY, vol. 262, no. 1, 1996, pages 21-30, XP002203952 ISSN: 0022-2836 the whole document	17
Y	STERNBERG NAT ET AL: "Display of peptides and proteins on the surface of bacteriophage lambda." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 5, 1995, pages 1609-1613, XP002203953 1995 ISSN: 0027-8424 the whole document	17

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IT 01/00413

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	SANTINI CLAUDIA ET AL: "Efficient display of an HCV cDNA expression library as C-terminal fusion to the capsid protein D of bacteriophage lambda." JOURNAL OF MOLECULAR BIOLOGY, vol. 282, no. 1, 1998, pages 125-135, XP002203954 ISSN: 0022-2836 the whole document	17
Y	YANG FAN ET AL: "Novel fold and capsid-binding properties of the lambda-phage display platform protein gpD." NATURE STRUCTURAL BIOLOGY, vol. 7, no. 3, March 2000 (2000-03), pages 230-237, XP001063314 ISSN: 1072-8368 the whole document	17
Y	WO 00 61790 A (MERRIL CARL R ;US HEALTH (US)) 19 October 2000 (2000-10-19) example 1	17
A	HALUSKA PAUL JR ET AL: "Interaction between human topoisomerase I and a novel RING finger/arginine-serine protein." NUCLEIC ACIDS RESEARCH, vol. 27, no. 12, 15 June 1999 (1999-06-15), pages 2538-2544, XP002193382 ISSN: 0305-1048 cited in the application the whole document	11
A	SAHIN UGUR ET AL: "Human neoplasms elicit multiple specific immune responses in the autologous host." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 25, 1995, pages 11810-11813, XP002193381 1995 ISSN: 0027-8424 the whole document	

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	BEGHETTO ELISA ET AL: "Identification of a human immunodominant B-cell epitope within the GRA1 antigen of Toxoplasma gondii by phage display of cDNA libraries." INTERNATIONAL JOURNAL FOR PARASITOLOGY, vol. 31, no. 14, December 2001 (2001-12), pages 1659-1668, XP001063313 ISSN: 0020-7519 page 1660, left-hand column, paragraph 3 -right-hand column, paragraph 1	17

INTERNATIONAL SEARCH REPORT

national application No.
PCT/IT 01/00413

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of 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. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 16 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
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:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

see additional sheet

1. [X] 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 an additional fee, this Authority did not invite payment of any additional fee.

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 claims 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 claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-16, 18-20

   Specific tumour antigens, and subject-matter related thereto.

2. Claim : 17

   Use of the expression/display vector (lambdaKM4) for obtaining antigens of claims 1-11.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 01/00413

Patent document ted in search report	Publication date	Patent family member(s)	Publication date
WO 0061790 A	19-10-2000	AU 4461200 A	14-11-2000
		EP 1169473 A1	09-01-2002
		WO 0061790 A1	19-10-2000