TITLE OF THE INVENTION

TELOMERASE REVERSE TRANSCRIPTASE FUSION PROTEIN, NUCLEOTIDES ENCODING IT, AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/851,183, filed October 12, 2006, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the therapy of cancer. More specifically, the present invention relates to polynucleotides encoding fusion proteins wherein the fusion proteins comprise at least a portion of the tumor associated polypeptide telomerase reverse transcriptase (TERT). The present invention also provides recombinant vectors and hosts comprising said polynucleotides, purified fusion proteins and methods for eliciting or enhancing an immune response against the protein product of the TERT gene, using the compositions and molecules disclosed herein.

BACKGROUND OF THE INVENTION

Vaccination has become a standard procedure for the prevention of numerous infectious diseases. The application of vaccines to other diseases, such as cancer, is now an attractive possibility due to advances in molecular engineering and a better understanding of tumor immunology.

Cancer is one of the leading causes of mortality worldwide. Despite an abundance of cancer-related research, conventional therapies that combine surgery, radiation, and chemotherapy, often fail to effectively treat established cancers. Reliable methods of prevention also remain unavailable.

Cancer typically involves the malfunction of genes that contribute to the regulation of the cell cycle or cell proliferation, such as growth factors and their receptors, oncogenes, and tumor suppressor genes. The products of many of these genes are expressed on the surface of a wide variety of tumor cells and, hence, are designated tumor-associated antigens (TAAs). The introduction of genes encoding TAAs directly into a subject has been shown to generate a protective immune response against the TAA in many experimental models, making these molecules a target for vaccine therapy. However, because many of these gene products are also expressed in normal cells, albeit at lower levels, many immunological therapies targeting TAAs have proven ineffective due to self-tolerance.

Genes coding for several tumor-associated antigens (TAA) have been isolated, characterized and inserted in genetic vectors such as plasmid DNA and viral vectors. One tumor-associated antigen that has been implicated in the pathogenesis of cancer is telomerase (TERT).

Telomerase is a DNA polymerase that normally functions in maintaining telomere length at the ends of chromosomes. During normal cell growth, an RNA primer attaches to the 5' end of DNA and initiates replication. Upon removal of the RNA primer, a gap in length is introduced to the resulting daughter strand of DNA. Thus, replication of a linear strand of DNA with conventional polymerases leads to shortening of telomere length in each progressive round of replication. Such shortening of telomere length is responsible for cellular senescence or aging of normal human somatic cells.

Telomerase is a ribonucleoprotein comprising an RNA component and a catalytic protein component (telomerase reverse transcriptase). The catalytic component of human telomerase was described by Meyerson *et al.* (*Cell* 1197: 785-95 (1990) and Nakamura *et al.* (*Science* 277: 955-59 (1997)). The TERT enzyme uses its RNA component as a template for telomere DNA synthesis, thus allowing telomeres to maintain their length over successive generations of cell growth. Such maintenance of telomere length over numerous proliferative cycles allows a cell to escape normal senescence and become immortal, allowing a tumor to grow and metastasize over great lengths of time. Because telomerase confers replicative immortality to cells, telomerase activity has been detected in cancerous cell lines and a diverse range of tumor types (Kim *et al. Science* 266: 2011-15 (1994)). Conversely, telomerase is inactive or only transiently expressed at low levels in normal human tissues and normal somatic cell cultures. The combination of telomerase over-expression in most cancer types as well as low or absent expression in normal cells makes TERT a target for therapy and/or prophylaxis of diseases associated with aberrant cellular proliferation such as cancer.

The development of a telomerase-specific vaccine is now possible because the catalytic and RNA components of telomerase have been cloned and characterized (*see, e.g.*, U.S. Patent 6, 166,178). However, the development and commercialization of many vaccines have been hindered by difficulties associated with obtaining high expression levels of the desired immunogen in successfully transformed host organisms. The development of efficacious DNA-based vaccines has also been hindered by an inability to generate an immune response of sufficient magnitude in treated individuals to lead to tumor regression in a clinical setting. Therefore, despite the identification of the wild-type nucleotide sequences encoding telomerase proteins described above, it would be highly desirable to develop a vaccine which is capable of eliciting an enhanced TERT-specific immune response relative to a wild-type full-length TERT cDNA, when delivered to a mammal. It would also be desirable to develop methods for treating or preventing TERT-associated cancers which utilize nucleic acid molecules or proteins that safely and effectively potentiate a TERT-specific immune response.

SUMMARY OF THE INVENTION

As stated above, expression of the tumor-associated antigen telomerase reverse transcriptase (TERT) gene is commonly associated with the development or presence of adenocarcinomas, including colorectal carcinomas. To this end, the present invention relates to compositions and methods to elicit or enhance immunity to the protein products expressed by the human TERT (hTERT) gene. Specifically, the present invention provides polynucleotides encoding fusion proteins wherein the fusion proteins comprise the hTERT protein, or variant thereof, fused to a substantial portion of the B subunit of *E. coli* heat labile enterotoxin (LTB). The present invention also provides recombinant vectors, including but not limited to, adenovirus and plasmid vectors, comprising said polynucleotides and host cells comprising said recombinant vectors. Also provided herein are purified fusion proteins encoded by invention polynucleotides. The hTERT-LTB fusion proteins and polynucleotides which encode said fusion proteins are useful as vaccines for the prevention and/or treatment of telomerase-associated cancer. Said vaccines are useful as a monotherapy or as part of a therapeutic regime, said regime comprising administration of a second vaccine such as a polynucleotide, cell-based, protein, or peptide-based vaccine, or comprising radiotherapy or chemotherapy.

In preferred embodiments of the present invention, the sequence of nucleotides encoding hTERT and/or the sequence of nucleotides encoding LTB comprises codons that have been optimized for high levels of expression in a human host cell. In other words, in certain embodiments of the invention, the codon usage pattern of the polynucleotide sequence resembles that of highly expressed mammalian and/or human genes.

Another aspect of this invention is an expression construct comprising nucleotides encoding hTERT-LTB. In preferred embodiments of this portion of the invention, the construct comprises a TPA leader sequence before the coding sequence for the TERT gene to ensure that the TERT-LTB fusion protein is secreted.

In additional preferred embodiments of the present invention, telomerase catalytic activity of the telomerase antigen is inactivated so that the encoded TERT fusion protein is safer than wild-type TERT for vaccine purposes. The enzymatic activity of the TERT fusion protein can be inactivated by addition of mutations/deletions to the TERT-encoding nucleotide sequence. In specific exemplary embodiments of the invention, nucleotides have been mutated in order to change specific amino acids D712A and V713I in the human TERT protein sequence and D702A and V703I in the mouse TERT protein sequence.

The present invention further provides adenoviral and plasmid vectors comprising a nucleotide sequence that encodes an hTERT-LTB fusion protein. This invention also describes the use of the adenovirus and plasmid vectors in immunogenic compositions and vaccines for the prevention and/or treatment of hTERT-associated cancer.

Also provided are adenovirus vectors comprising a sequence of nucleotides that encode a human TERT protein or TERT protein variant. Variants useful in this aspect of the

invention comprise mutations that function to eliminate telomerase catalytic activity. In preferred embodiments of this portion of the invention, the adenovirus vector is an Ad6 vector. In other preferred embodiments the Ad vector is an Ad 5 vector.

The present invention further provides methods for preventing the development of a cancer in a patient or treating a patient with a telomerase-associated tumor by eliciting an immune response to the TERT protein by administering a vaccine or pharmaceutical composition comprising the TERT fusions or TERT fusion proteins provided by the invention. In preferred embodiments of the methods herein, the immune response is enhanced relative to the response elicited by a wild-type TERT.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers".

The term "cassette" refers to a nucleotide or gene sequence that is to be expressed from a vector, for example, the nucleotide or gene sequence encoding the hTERT(AI)-LTB fusion. In general, a cassette comprises a gene sequence that can be inserted into a vector, which in some embodiments, provides regulatory sequences for expressing the nucleotide or gene sequence. In other embodiments, the nucleotide or gene sequence provides the regulatory sequences for its expression. In further embodiments, the vector provides some regulatory sequences and the nucleotide or gene sequence provides other regulatory sequences. For example, the vector can provide a promoter for transcribing the nucleotide or gene sequence and the nucleotide or gene sequence provides a transcription termination sequence. The regulatory sequences that can be provided by the vector include, but are not limited to, enhancers, transcription termination sequences, splice acceptor and donor sequences, introns, ribosome binding sequences, and poly(A) addition sequences.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The term "first generation," as used in reference to adenoviral vectors, describes adenoviral vectors that are replication-defective. First generation adenovirus vectors typically have a deleted or inactivated E1 gene region, and preferably have a deleted or inactivated E3 gene region.

The designation 'pV1JnsA/TPA-mTERT(AI)-LTBopt" refers to a plasmid construct, disclosed herein, comprising the CMV immediate-early (IE) promoter with intron A, a full-length codon-optimized murine TERT gene fused to a codon-optimized LTB gene, and bovine growth hormone-derived polyadenylation and transcriptional termination sequences (see EXAMPLE 2). Additionally, a leader sequence encoding the human tissue plasminogen activator (TPA) signal sequence is present 5' to the nucleotide sequence encoding mTERT-LTB. The designation "AI" indicates that mutations were added to the TERT sequence to functionally eliminate telomerase catalytic activity. The designation 'pV1JnsA/mTERT(AI)opt" refers to a construct essentially as described above, except the construct comprises a murine optimized TERT nucleotide sequence that was not fused to LTB or to TPA.

The designation 'pV1JnsA/TPA-hTERT(AI)-LTBopt" refers to a plasmid construct, disclosed herein, comprising the CMV immediate-early (IE) promoter with intron A, a full-length codon-optimized human TERT gene fused to a codon-optimized LTB gene, and bovine growth hormone-derived polyadenylation and transcriptional termination sequences (see EXAMPLE 2). Additionally, a leader sequence encoding the human tissue plasminogen activator (TPA) signal sequence is present 5' to the nucleotide sequence encoding hTERT-LTB. The hTERT sequence in this construct comprises mutations to functionally eliminate telomerase catalytic activity.

The designations "Ad6/TPAmTERT(AI)-LTBopt" and "Ad6/hTERT(AI)" refer to two constructs, disclosed herein, which comprise an Ad6 adenoviral genome deleted of the E1 and E3 regions. In the "Ad6/TPAmTERT(AI)-LTBopt" construct, the E1 region is replaced by a codon-optimized murine TERT-LTB gene in an E1 parallel orientation under the control of a human CMV promoter without intron A, followed by a bovine growth hormone polyadenylation signal. The TERT-encoding sequence comprises mutations to eliminate telomerase catalytic activity. The construct further comprises sequences encoding the human tissue plasminogen activator (TPA) signal sequence 5' to the TERT(AI)-LTB encoding nucleotide sequence. The "Ad6/hTERT(AI)" construct is essentially as described above, except the E1 region of the Ad6 genome is replaced with a TERT cDNA sequence, said sequence comprising mutations to abolish enzymatic activity.

The abbreviation "LTB" refers generally to the B subunit of heat labile enterotoxin of *E. coli*, or substantial portion thereof, including subunits which are truncated on the C-terminal or N-terminal end but maintain adjuvant activity, as well as subunits that contain internal amino acid insertions, deletions, or substitutions but maintain adjuvant activity (Fingerut *et al. Vaccine* 23: 4685-4696 (2005)).

As used herein, a "fusion protein" refers to a protein having at least two heterologous polypeptides covalently linked in which one polypeptide comes from one protein sequence or domain and the other polypeptide comes from a second protein sequence or domain. The fusion proteins of the present invention comprise a TERT polypeptide or fragment or variant

thereof, and a second polypeptide, which comprises a substantial portion of LTB. The TERT polypeptide, fragment or variant thereof may be a human TERT or TERT homolog from another species, e.g. mouse. The polypeptides that comprise the fusion protein are preferably linked N-terminus to C-terminus. The TERT polypeptide and the LTB polypeptide can be fused in any order. In some embodiments of this invention, the C-terminus of the TERT polypeptide is fused to the N-terminus of LTB, as exemplified in FIGUREs 1 and 2. However, fusion proteins in which the LTB subunit is fused to the N-terminus of the TERT polypeptide are also contemplated.

The term "TERT fusion protein" or "TERT-LTB fusion protein" are used interchangeably herein as a general term which refers to a fusion as described above, which comprises a TERT polypeptide or fragment or variant thereof fused to an LTB polypeptide. The term "TERT-LTB fusion" refers to a nucleic acid sequence in which at least a portion of the TERT gene is fused to a substantial portion of LTB subunit of *E. coli* heat labile enterotoxin. The term "TERT-LTB fusion protein" refers to a polypeptide encoded by a TERT-LTB fusion as described. The terms "TERT-LTB fusion" and "TERT-LTB fusion protein" are also understood to refer to fragments thereof, homologs thereof, and functional equivalents thereof (collectively referred to as "variants"), such as those in which one or more amino acids is inserted, deleted or replaced by other amino acid(s). The TERT-LTB fusions of the present invention, upon administration to a mammal such as a human being, can stimulate an immune response by helper T cells or cytotoxic T cells, at least as well as a "wild-type" hTERT sequence. In preferred embodiments of the invention, the TERT-LTB fusion can enhance the immune response as compared to a wild-type hTERT.

The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented. As used herein, "treatment" also includes reduction of the likelihood of obtaining the disorder, as well as the reduction of the severity of the disorder in those already afflicted.

A "disorder" is any condition that would benefit from treatment with the molecules of the present invention, including the nucleic acid molecules described herein and the fusion proteins that are encoded by said nucleic acid molecules. Encompassed by the term "disorder" are chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. The molecules of the present invention are intended for use as treatments for disorders or conditions characterized by aberrant cell proliferation, including, but not limited to, breast cancer, colorectal cancer, and lung cancer.

The term "effective amount" means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that an immune response results. One skilled in the art recognizes that this level may vary.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

"hTERT" refers to a human telomerase reverse transcriptase antigen or nucleotides encoding the human telomerase reverse transcriptase antigen. "hTERTopt" refers to a codon-optimized nucleotide sequence encoding human telomerase reverse transcriptase antigen.

"Substantially similar" means that a given nucleic acid or amino acid sequence shares at least 75%, preferably at least 85%, more preferably at least 90%, and even more preferably at least 95% identity with a reference sequence. In the present invention, the reference sequence can be relevant portions of the wild-type human TERT nucleotide or amino acid sequence, as set forth in SEQ ID NOs:11 and 12, respectively, or the nucleotide or amino acid sequence of LTB, as set forth in SEQ ID NOs:13 (opt sequence) and 14, as dictated by the context of the text. The reference sequence may also be, for example, a wild-type rhesus monkey or murine TERT sequence. Thus, a TERT protein sequence that is "substantially similar" to the wild-type human TERT protein or fragment thereof will share at least 75% identity with the relevant fragment of the wild-type human TERT, along the length of the fragment, preferably 85% identity, more preferably 90% identity and even more preferably 95% identity. Whether a given mTERT, hTERT, or LTB amino acid sequence or nucleotide sequence is "substantially similar" to a reference sequence can be determined for example, by comparing sequence information using sequence analysis software such as the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482, 1981).

A "substantial portion" of a gene, variant, fragment, or subunit thereof, means a portion of at least 50%, preferably 75%, more preferably 90%, and even more preferably 95% of a reference sequence.

A "gene" refers to a nucleic acid molecule whose nucleotide sequence codes for a polypeptide molecule. Genes may be uninterrupted sequences of nucleotides or they may include such intervening segments as introns, promoter regions, splicing sites and repetitive sequences. A gene can be either RNA or DNA. A preferred gene is one that encodes the invention peptide.

The term "nucleic acid" or "nucleic acid molecule" is intended for ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), probes, oligonucleotides, fragment or portions thereof, and primers.

"Wild-type TERT" or "wild-type protein" or "wt protein" refers to a protein comprising a naturally occurring sequence of amino acids or variant thereof. The amino acid sequence of wild-type human TERT is set forth in SEQ ID NO:12. The amino acid sequence of

the wild-type human TERT was previously described (U.S. Patent No. 6,166,178; U.S. Patent 6,261,836; U.S. Patent No. 6,927,285; U.S. Patent Application 2003-0096344; Meyerson *et al.*, *Cell* 90: 785-95 (1997); Nakamura et al., *Science* 277: 955-59 (1997)).

"Wild-type TERT gene" refers to a gene comprising a sequence of nucleotides that encodes a naturally occurring TERT protein, including proteins of human origin or proteins obtained from another organism, including, but not limited to, other mammals such as rat, mouse and rhesus monkey. The nucleotide sequence of the human TERT gene is available in the art (*supra*).

The designation "TERT(AI)" refers to a telomerase reverse transcriptase sequence that comprises mutations to eliminate or reduce telomerase catalytic activity.

The term "mammalian" refers to any mammal, including a human being.

The abbreviation "Ag" refers to an antigen.

The abbreviation "ORF" refers to the open reading frame of a gene.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the nucleotide (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of an exemplary TPA-hTERT(AI)-LTBopt fusion.

FIGURE 2 shows the nucleotide (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) of an exemplary TPA-mTERT(AI)-LTBopt fusion.

FIGURE 3 shows results of an IFNγ ELIspot assay on BALB/c mice vaccinated with repeated injections of plasmid pV1J/TPA-mTERT(AI)-LTBopt. 5 mice of each group were used to monitor immune response directed against mTERT or LTB using peptide pools. Data plotted are from 6 individual mice (empty circle). Geometric mean values are indicated (filled circles). No T cell response to mTERT was detected in mock treated mice (not shown). See EXAMPLE 7.

FIGURE 4 shows results from IFNγ intracellular staining on splenocytes from vaccinated BALB/c mice. $CD4^+$ and $CD8^+$ T cell response to mouse TERT was measured using pools of overlapping peptides that cover the entire protein. Immune response to LTB was also monitored. Data plotted are from 6 individual mice (filled triangles). Geometric mean values are indicated (straight line). No T cell response to TERT was detected in mock treated mice (not shown). See EXAMPLE 7.

FIGURE 5 shows the results of ^{51}Cr Release CTL killing by effector T cells of 4T1 target cells pulsed with $CD8^+$T cells specific peptide mTERTaa167 (AYQVCGSPL; SEQ ID NO:20). Effector cells were prepared from splenocytes of two immunized BALB/c mice and were re-stimulated *in vitro* with the specific peptide. Results are expressed as percentage of specific killing at different Effectors /Target ratio. See EXAMPLE 8.

FIGURE 6 shows the induction of an immune response to mouse TERT. C57BL/6 mice were immunized with 5 weekly injections of plasmid pV1J/TPA-mTERT(AI)-

LTBopt. Immune response was assessed on mouse splenocytes by IFNγ ELIspot assay using mouse TERT peptide pools. Antigen specific $CD4^+$ and $CD8^+$ T cell responses are shown as geometric mean values obtained from 6 immunized mice, standard deviation is also indicated. See EXAMPLE 7.

FIGURE 7 shows the induction of an mTERT specific immune response in BALB/c mice. Mice were immunized with 5 weekly injections of the constructs pV1J-mTERT(AI)opt or pV1J-TPAmTERT(AI)-LTBopt. Immune response was determined by IFNγ intracellular staining on PBMC using the CD8+ epitope corresponding to the peptide sequence mTERTaa167 (AYQVCGSPL; SEQ ID NO:20). Data plotted are from 8 individual mice (filled triangles). Geometric mean values are indicated (straight lines).

FIGURE 8 shows the TERT specific immune response is enhanced upon injection of adenovirus vectors expressing mTERT(AI)-LTBopt. Groups of 10 BALB/c mice were subjected to 5 weekly injections of pV1J/TPA-mTERT(AI)-LTBopt (50μg/inj.). Two weeks after the last injection of plasmid DNA, mice were either boosted with one additional injection of plasmid DNA (DNA-EP) or with 10^{10} vp of Ad6/TPA-mTERT(AI)-LTBopt (Ad). Immune responses to TERT were monitored by IFNγ ICS on PBMC. Data plotted are from 10 individual mice (filled diamonds). Geometric mean values of each group are also shown (straight lines). See EXAMPLE 10.

FIGURE 9 depicts the protocol utilized to analyze DMH-induced carcinogenesis and vaccination in BALB/c mice. DMH was administered i.p. on a weekly basis. Vaccination with pV1J/TPA-mTERT(AI)-LTBopt was carried out at the indicated time points. Analysis of intestinal lesions was carried out at week 12. See EXAMPLE 11.

FIGURE 10 shows the anti-tumor effect of pV1J/TPA-mTERT(AI)-LTBopt on DMH-induced carcinogenesis. BALB/c mice were treated with DMH and DNA-EP as indicated in FIGURE 9. Analysis of ACF and adenoma formation in the colon was carried out at week 12 from the beginning of the treatment. The number of ACF and adenoma in vaccinated mice was compared to that detected in non vaccinated controls. See EXAMPLE 11.

FIGURE 11 depicts the protocol utilized to analyze DMH-induced carcinogenesis and vaccination in BALB/c mice. DMH was administered i.p. on a weekly basis. Vaccination with pV1J/TPA-mTERT-LTB DNA and Ad were carried out at the indicated time points. Analysis of the intestinal lesions was carried out at week 30. See EXAMPLE 11.

FIGURE 12 demonstrates the anti-tumor effect of pV1J/TPA-mTERT(AI)-LTBopt DNA prime/Ad boost on the number of tumor lesions. BALB/c mice were treated DMH and DNA-EP as indicated in FIGURE 9. Analysis of ACF and adenoma formation in the colon was carried out at week 30 from the beginning of the treatment. The number of ACF and adenoma in vaccinated mice was compared to that detected in non vaccinated controls. See EXAMPLE 11.

FIGURE 13 demonstrates the anti-tumor effect of pV1J/TPA-mTERT-LTB(AI)opt DNA prime/Ad boost on the volume and differentiation stage of tumor lesions. BALB/c mice were treated DMH and DNA-EP as indicated. The volume (Panel A) and histological differentiation stage (Panel B) of colon adenomas present in vaccinated and control mice were analyzed 30 weeks from the beginning of the treatment. Tumors were classified as follows: G1: well-differentiated adenocarcinoma; G2: moderately-differentiated adenocarcinoma; G3: poorly-differentiated adenocarcinoma). See EXAMPLE 11.

FIGURE 14 shows the induction of an immune response to human TERT. HHD transgenic mice were immunized with two biweekly injections (DNA-DNA) of plasmid pV1J/TPA-hTERT(AI)-LTBopt or by one injection of plasmid pV1J/TPA-hTERT(AI)-LTBopt followed, after two weeks by one injection of Ad6hTERT(AI) 10^{10} pp (DNA/Ad). The immune response was assessed on mouse PBMC by IFNγ ICS assay. Mice PBMC were incubated with the CD8 immunodominant peptide for HLA-A2 allele hTERT865 (SEQ ID NO:22). Data plotted are from 10 individual mice (filled triangles). Geometric mean values are indicated (straight line). See EXAMPLEs 12 and 13.

FIGURE 15 shows the results of a CTL assay on Cr^{51} labeled target HeLa-HHD cells. CD8+ T cells obtained from one immunized mouse were tested in a CTL assay against HeLa-HHD cells, either exogenously loaded with the hTERT865 peptide or w/o peptide but in presence of the DMSO used for the peptide dilution. HeLa-HHD cells were also infected with an Ad vector coding for hTERT or an Ad coding for GFP as control. See EXAMPLE 14.

FIGURE 16 shows the induction of a cell-mediated immune response to hTERT in rhesus monkeys immunized by DNA-EP. ELIspot analysis was performed on PBMC from hTERT-immunized rhesus monkeys (pV1J/TPA-hTERT-LTB;DNA-EP/DNA-EP). The assay was performed in duplicate and the average value from the replicates is shown. Analysis performed after the second DNA-EP is shown (Panel A). Analysis performed after the fifth DNA-EP is also shown (Panel B). See EXAMPLE 15.

FIGURE 17 shows that the cell-mediated immune response to hTERT was amplified in rhesus monkeys that were vaccinated with DNA-EP and boosted with adenovirus expressing hTERT, compared to the CMI elicited in monkeys vaccinated by DNA-EP alone. ELIspot was performed on PBMC from hTERT-immunized rhesus monkeys at the end of the immunization program, which included five DNA-EP (pV1J/TPA-hTERT-LTB) injections followed by two Ad (Ad6-hTERT) injections. The assay was performed in duplicate and the average value from the replicates is shown. See EXAMPLE 15.

DETAILED DESCRIPTION OF THE INVENTION

Expression of the catalytic component of telomerase, known as telomerase reverse transcriptase (TERT) is commonly detected in a diverse range of tumor types. The combination of telomerase over-expression in most cancer types as well as low or absent expression in normal

cells makes TERT a target for therapy and/or prophylaxis of diseases associated with aberrant cellular proliferation such as cancer. To this end, the present invention relates to compositions and methods to elicit or enhance immunity to the protein product expressed by the TERT tumor-associated antigen, wherein aberrant TERT expression is associated with the carcinoma or its development. Association of aberrant TERT expression with a carcinoma does not require that the TERT protein be expressed in tumor tissue at all time points of its development, as abnormal TERT expression may be present at tumor initiation and not be detectable late into tumor progression or vice-versa.

Accordingly, the present invention provides polynucleotides, vectors, host cells, and encoded proteins comprising a TERT sequence or variant thereof for use in vaccines and pharmaceutical compositions for the treatment and/or prevention of a cancer. The polynucleotides of the present invention comprise a nucleotide sequence encoding a TERT protein or variant thereof, fused to a nucleotide sequence encoding the B subunit of *E. coli* heat labile enterotoxin (LTB), which is able to effectively adjuvant an immune response to the associated TERT antigen.

In some embodiments of the invention, the nucleotide sequences disclosed herein further comprise a TPA leader sequence before the coding sequence for the TERT gene to ensure that the TERT-LTB fusion protein is secreted. In preferred embodiments of this aspect of the invention, the TPA-hTERT(AI)-LTB fusion encodes an amino acid sequence as set forth in SEQ ID NO:2. A preferred nucleotide sequence is set forth in SEQ ID NO:1. In further preferred embodiments, the TPA-mTERT(AI)-LTB fusion encodes an amino acid sequence as set forth in SEQ ID NO:4. A preferred nucleotide sequence is set forth in SEQ ID NO:3.

The TERT nucleotide sequences of the present invention can be of human origin or can be a TERT homolog from another species, e.g. mouse. The wild-type human TERT nucleotide sequence is set forth in SEQ ID NO:12 and has been reported previously (*see, e.g.,* U.S. Patent No. 6,166,178; U.S. Patent 6,261,836; U.S. Patent No. 6,927,285; U.S. Patent Application 2003-0096344; Meyerson *et al.*, *Cell* 90: 785-95 (1997); Nakamura *et al.*, *Science* 277: 955-59 (1997)). The TERT portion of the TERT fusion may be full-length, or any variant sufficient to elicit a TERT-specific T-cell immune response in a mammal. TERT variants of the present invention include, but are not limited to sequences that are C- or N-terminally truncated, sequences with conservative substitutions, and sequences with internal deletions or insertions. Preferred TERT variants of the present invention comprise mutations that functionally eliminate telomerase catalytic activity. Encoded TERT fusion proteins of the present invention are capable of inducing a cell-mediated immune response when introduced into a vaccine recipient or patient in need thereof.

As stated above, in preferred embodiments of the present invention, telomerase catalytic activity of the telomerase antigen is inactivated (herein designated 'TERT(AI)') so that

the encoded TERT fusion protein is safer than wild-type TERT for vaccine purposes. The enzymatic activity of the TERT fusion protein can be inactivated by addition of mutations/deletions to the TERT-encoding nucleotide sequence. Therefore, the present invention includes nucleic acid molecules that encode a TERT-LTB fusion protein, wherein the sequence of nucleotides encodes a variant hTERT protein, wherein said variant comprises one or more mutations relative to the wild-type hTERT amino acid sequence as set forth in SEQ ID NO: 12, and wherein said mutations function to eliminate telomerase catalytic activity of the encoded hTERT protein. In specific exemplary embodiments of the invention, nucleotides have been mutated in order to change specific amino acids D712A and V713I in the human TERT protein sequence (as set forth in SEQ ID NO:10) and D702V and V703I in the mouse TERT protein sequence. A nucleotide sequence encoding the mutated human TERT sequence set forth in SEQ ID NO:10 is disclosed in SEQ ID NO:9.

In preferred embodiments of the present invention, the TERT portion of the TERT-LTB fusion is human TERT or a variant thereof, such as hTERT(AI). In other preferred embodiments, the TERT portion is a murine TERT, or variant thereof, such as mTERT(AI). The TERT variants and TERT-encoding nucleotide sequence variants of the present invention comprise mutations that abolish telomerase enzymatic activity.

Accordingly, the present invention relates to synthetic polynucleotides comprising a sequence of nucleotides encoding a TERT-LTB fusion protein, said fusion protein comprising a TERT protein or a biologically inactive fragment or mutant form of a TERT protein fused to an LTB protein or variant thereof, which can effectively enhance the immune response to the TERT protein. Said mutant forms of the TERT protein include, but are not limited to: conservative amino acid substitutions, amino-terminal truncations, carboxy-terminal truncations, deletions, or additions. Any such TERT variant, fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the immunological properties of the TERT protein as set forth in SEQ ID NO:12. Preferred TERT variants are catalytically inactive, for example, the TERT variant set forth in SEQ ID NO:10.

The synthetic polynucleotides of the present invention encode mRNA molecules that express a TERT(AI)-LTB fusion protein that is capable of stimulating or enhancing the immune response to the associated TERT protein, so as to be useful in the development of a therapeutic or prophylactic cancer vaccine. The LTB portion of the TERT-LTB fusions of the present invention has been shown to strongly potentiate the immunogenicity of co-delivered antigens (*See, e.g.* Simmons et al. *Scand. J. Immunol.* 53:218-26 (2001)).

Also contemplated for use in the present invention are nucleotide sequences encoding LTB variants or mutants including but not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. In some cases, it may be advantageous to add specific point mutations to the nucleotide sequence encoding LTB to reduce or eliminate toxicity of the encoded protein. In preferred embodiments

of the present invention the LTB sequence fused to the TERT sequence is truncated of its signal sequence.

The LTB portion, or variant thereof, of the TERT-LTB fusions of the present invention may be fused to the amino terminus or the carboxy terminus of the TERT sequence. Further, the LTB sequence and the TERT sequence can be fused N-terminus to N-terminus, C-terminus to C-terminus, C-terminus to N-terminus or N-terminus to N-terminus. In preferred embodiments of the present invention, the C-terminus of the TERT polypeptide is fused to the N-terminus of LTB.

The present invention relates to a synthetic nucleic acid molecule (polynucleotide) comprising a sequence of nucleotides which encodes mRNA that expresses a novel TERT-LTB fusion protein; for example, nucleotide sequences encoding the fusion proteins as set forth in SEQ ID NOs:6 and 8. A particularly preferred TERT fusion of the present invention is the hTERT(AI)-LTB fusion sequence set forth in SEQ ID NO:5. The nucleic acid molecules of the present invention are substantially free from other nucleic acids.

One of skill in the art will recognize that other nucleotide sequences are useful for vaccine purposes, such as nucleotide sequences that encode the hTERT(AI)-LTB protein sequence set forth in SEQ ID NO:6. The present invention also includes nucleotide sequences that encode inactive hTERT-LTB fusion protein that are substantially similar, to SEQ ID NO:6, but not exactly the same, especially due to amino acid differences at the junction of the TERT(AI) sequence and the LTB sequence that were created by the cloning strategy.

The present invention also relates to recombinant vectors and recombinant host cells, both prokaryotic and eukaryotic, which contain the nucleic acid molecules disclosed throughout this specification. The synthetic DNA molecules, associated vectors, and hosts of the present invention are useful for the development of a cancer vaccine.

Exemplary nucleic acid molecules of the present invention comprise a nucleotide sequence selected from the group consisting of: SEQ ID NOs:5 and 7, as shown in FIGUREs 1-2, which encode exemplary hTERT-LTB and mTERT-LTB fusion proteins of the present invention.

The present invention also includes biologically active fragments or mutants of SEQ ID NOs: 5 and 7, which encode mRNA expressing exemplary TERT-LTB fusion proteins. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the immunological properties of the hTERT protein, including but not limited to the hTERT protein as set forth in SEQ ID NO:12. Such polynucleotides include but are not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. Preferred mutated nucleotide sequences of the present invention encode mRNA molecules that express an enzymatically inactive TERT-LTB fusion protein in a eukaryotic cell, which is capable of eliciting a prophylactic or therapeutic immune response in a patient in need thereof so as to be useful in cancer vaccine development.

Also included within the scope of this invention are mutations in the DNA sequence that do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in the desired functionality of the polypeptide, such as the ability to elicit an immune response.

As stated above, the present invention further relates to recombinant vectors that comprise the nucleic acid molecules disclosed throughout this specification. These vectors may be comprised of DNA or RNA. For most cloning purposes, DNA vectors are preferred. Typical vectors include plasmids, modified viruses, baculovirus, bacteriophage, cosmids, yeast artificial chromosomes, and other forms of episomal or integrated DNA that can encode a TERT-LTB fusion protein. It is well within the purview of the skilled artisan to determine an appropriate vector for a particular gene transfer or other use.

Also provided by the present invention are purified TERT-LTB fusion proteins encoded by the nucleic acids disclosed throughout this specification, especially TERT(AI)-LTB fusion proteins. In exemplary embodiments of this aspect of the invention, the TERT-LTB fusion protein comprises a sequence of amino acids selected from the group consisting of: SEQ ID NOs: 6 and 8.

Included in the present invention are DNA sequences that hybridize to the complement of SEQ ID NOs: 5 and 7 under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows. Prehybridization of filters containing DNA is carried out for about 2 hours to overnight at about 65°C in buffer composed of 6x SSC, 5x Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for about 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for about 1 hour in a solution containing 2x SSC, 0.1% SDS. This is followed by a wash in 0.1x SSC, 0.1% SDS at 50°C for 45 minutes before autoradiography. Other procedures using conditions of high stringency would include either a hybridization step carried out in 5x SSC, 5x Denhardt's solution, 50% formamide at about 42°C for about 12 to 48 hours or a washing step carried out in 0.2x SSPE, 0.2% SDS at about 65°C for about 30 to 60 minutes. Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Edition; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989) or Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Plainview, NY (2001). In addition to the foregoing, other conditions of high stringency which may be used are also well known in the art.

An expression vector containing a TERT-LTB fusion protein-encoding nucleic acid molecule may be used for high-level expression of TERT-LTB fusion protein in a

recombinant host cell. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Also, a variety of bacterial expression vectors may be used to express recombinant TERT-LTB fusion sequences in bacterial cells if desired. In addition, a variety of fungal cell expression vectors may be used to express recombinant TERT-LTB fusion sequences in fungal cells. Further, a variety of insect cell expression vectors may be used to express recombinant protein in insect cells.

The present invention also relates to host cells transformed or transfected with vectors comprising the nucleic acid molecules of the present invention. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of bovine, porcine, monkey and rodent origin; and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Such recombinant host cells can be cultured under suitable conditions to produce a TERT-LTB fusion protein or a biologically equivalent form. In a preferred embodiment of the present invention, the host cell is human. As defined herein, the term "host cell" is not intended to include a host cell in the body of a transgenic human being, human fetus, or human embryo.

As noted above, an expression vector containing DNA encoding a TERT-LTB fusion protein may be used for expression of TERT fusion protein in a recombinant host cell. Therefore, another aspect of this invention is a process for expressing a TERT-LTB fusion protein in a recombinant host cell, comprising: (a) introducing a vector comprising a nucleic acid comprising a sequence of nucleotides that encodes a TERT-LTB fusion protein into a suitable human host cell, wherein the TERT-LTB fusion protein comprises a TERT protein or inactive variant thereof, fused to a substantial portion of the LTB protein, and wherein the fusion protein is capable of producing an immune response in a mammal; and, (b) culturing the host cell under conditions which allow expression of said TERT-LTB fusion protein.

In preferred embodiments of the process for expressing a TERT-LTB fusion protein described above, the LTB sequence is deleted of its signal sequence.

Following expression of a TERT-LTB fusion in a host cell, TERT-LTB fusion protein may be recovered to provide purified TERT-LT fusion protein. Several protein purification procedures are available and suitable for use. Recombinant protein may be purified from cell lysates and extracts by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography. In addition, recombinant TERT-LTB fusion protein can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for a TERT protein, or polypeptide fragments of a TERT protein.

The nucleic acid molecules comprising TERT-LTB fusions and the encoded fusion proteins of this invention were designed to enhance the TERT-specific immune response, relative to full-length wild-type cDNA encoding TERT, for use in vaccine development. To further enhance the immunogenic properties of the TERT-LTB fusion sequences of the present invention, in some embodiments described herein, the polynucleotides encoding TERT-LTB fusion proteins comprise optimized codons for further high level expression in a host cell, as described below. In these embodiments, at least a portion of the codons of the TERT-LTB fusions are designed so as to use the codons preferred by the projected host cell, which in preferred embodiments is a human cell. The optimized TERT-LTB fusions may be used for the development of recombinant adenovirus or plasmid-based DNA vaccines, which provide effective immunoprophylaxis against TERT-associated cancer through cell-mediated immunity. The synthetic molecules may be used as an immunogenic composition. This invention provides codon-optimized TERT-LTB fusion polynucleotides which, when directly introduced into a vertebrate *in vivo*, including mammals such as primates and humans, induce the expression of encoded proteins within the animal.

As stated above, in some embodiments of the present invention, the synthetic molecules comprise a sequence of nucleotides, wherein some of the nucleotides have been altered so as to use the codons preferred by a human cell, thus allowing for high-level fusion protein expression in a human host cell. The synthetic molecules may be used as a source of a TERT-LTB fusion protein, which may be used in a cancer vaccine to provide effective immunoprophylaxis against TERT-associated carcinomas through cell-mediated immunity. The nucleic acid molecules disclosed herein may also serve as the basis for a DNA-based cancer vaccine.

A "triplet" codon of four possible nucleotide bases can exist in over 60 variant forms. Because these codons provide the message for only 20 different amino acids (as well as transcription initiation and termination), some amino acids can be coded for by more than one codon, a phenomenon known as codon redundancy. For reasons not completely understood, alternative codons are not uniformly present in the endogenous DNA of differing types of cells. Indeed, there appears to exist a variable natural hierarchy or "preference" for certain codons in certain types of cells. As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG. Exhaustive analysis of genome codon frequencies for microorganisms has revealed endogenous DNA of *E. coli* most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally believed that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an *E. coli* host will depend to some extent on the frequency of codon use. For example, it is likely that a gene rich in TTA codons will be poorly expressed in *E. coli*, whereas a CTG rich gene will probably be highly expressed in this host. Similarly, a preferred codon for expression of a leucine-rich polypeptide in yeast host cells would be TTA.

The implications of codon preference phenomena on recombinant DNA techniques are evident, and the phenomenon may serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms--a less "preferred" codon may be repeatedly present in the inserted gene and the host cell machinery for expression may not operate as efficiently. This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide an optimal form of foreign genetic material for practice of recombinant DNA techniques. Thus, one aspect of this invention is a TERT-LTB fusion gene that is codon-optimized for expression in a human cell. In a preferred embodiment of this invention, it has been found that the use of alternative codons encoding the same protein sequence may remove the constraints on expression of exogenous TERT-LTB fusion protein in human cells.

In accordance with some embodiments of the present invention, the nucleic acid molecules which encode the TERT-LTB fusion proteins are converted to a polynucleotide sequence having an identical translated sequence but with alternative codon usage as described by Lathe, "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations" *J. Molec. Biol.* 183:1-12 (1985), which is hereby incorporated by reference. The methodology generally consists of identifying codons in the wild-type sequence that are not commonly associated with highly expressed human genes and replacing them with optimal codons for high expression in human cells. The new gene sequence is then inspected for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.). Undesirable sequences are eliminated by substitution of the existing codons with different codons coding for the same amino acid. The synthetic gene segments are then tested for improved expression.

It is understood that this procedure will not necessarily result in a polynucleotide sequence in which all of the codons are optimal codons according to the codon usage of highly expressed human and/or mammalian cells. However, it is preferred that, in embodiments of the invention wherein codon-optimized polynucleotide variants of TERT and/or LTB are contemplated, a substantial portion of the resulting codons resemble the codon usage of highly expressed human and/or mammalian genes.

The methods described above were used to create synthetic gene sequences which encode TERT-LTB fusion proteins, resulting in a gene comprising codons optimized for high level expression in human cells. While the above procedure provides a summary of a representative methodology for designing codon-optimized genes for use in cancer vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence.

One of skill in the art will also recognize that additional nucleic acid molecules may be constructed that provide for high levels of TERT-LTB fusion expression in human cells,

wherein only a portion of the codons of the DNA molecules are codon-optimized. For example, in some embodiments of the present invention, codons comprising the TERT portion of the TERT-LTB fusion are optimized for high-level expression in human cells, and codons comprising the LTB portion of the TERT-LTB fusion are substantially similar to the wild-type LTB. In other embodiments of the present invention, codons comprising the LTB portion of the TERT-LTB fusion are optimized for high-level expression in human cells, and codons comprising the TERT portion of the TERT-LTB fusion are substantially similar to a wild-type TERT gene. In still other embodiments of the present invention, both the TERT and LTB portions of the TERT-LTB fusion are codon-optimized for high-level expression in human cells, for example, the hTERT-LTBopt sequence as set forth in SEQ ID NO:5. TERT-LTB fusions in which only a subset of codons are optimized within the TERT and/or the LTB portion of the TERT-LTB fusion are also contemplated by this invention.

The nucleic acids of the present invention may be assembled into an expression cassette which comprises sequences designed to provide for efficient expression of the protein in a human cell. The cassette preferably contains TERT-LTB fusion protein-encoding gene, with related transcriptional and translations control sequences operatively linked to it, such as a promoter, and termination sequences. In a preferred embodiment, the promoter is the cytomegalovirus promoter with intron A sequence (CMV), although those skilled in the art will recognize that any of a number of other known promoters such as a strong immunoglobulin or other eukaryotic gene promoter may be used. A preferred transcriptional terminator is the bovine growth hormone terminator, although other known transcriptional terminators may also be used. The combination of CMV-BGH terminator is particularly preferred.

In accordance with this invention, the TERT-LTB fusion expression cassette is inserted into a vector. The vector is preferably an adenoviral or plasmid vector, although linear DNA linked to a promoter, or other vectors, such as adeno-associated virus or a modified vaccinia virus, retroviral or lentiviral vector may also be used.

In preferred embodiment of the invention, the vector is an adenovirus vector (used interchangeably herein with "adenovector"). Adenovectors can be based on different adenovirus serotypes such as those found in humans or animals. Examples of animal adenoviruses include bovine, porcine, chimp, murine, canine, and avian (CELO). Preferred adenovectors are based on human serotypes, more preferably Group B, C, or D serotypes. Examples of human adenovirus Group B, C, D, or E serotypes include types 2 ("Ad2"), 4 ("Ad4"), 5 ("Ad5"), 6 ("Ad6"), 24 ("Ad24"), 26 ("Ad26"), 34 ("Ad34") and 35 ("Ad35"). In particularly preferred embodiments of the present invention, the expression vector is an adenovirus type 6 (Ad6) vector.

If the vector chosen is an adenovirus, it is preferred that the vector be a so-called first-generation adenoviral vector. These adenoviral vectors are characterized by having a non-functional E1 gene region, and preferably a deleted adenoviral E1 gene region. In addition, first generation vectors may have a non-functional or deleted E3 gene region (Danthinne *et al. Gene*

Therapy 7:1707-1714 (2000); F.L. Graham, *Immunology Today* 21(9): 426-428 (2000)). Adenovectors do not need to have their E1 and E3 regions completely removed. Rather, a sufficient amount the E1 region is removed to render the vector replication incompetent in the absence of the E1 proteins being supplied *in trans*; and the E1 deletion or the combination of the E1 and E3 deletions are sufficiently large enough to accommodate a gene expression cassette.

In some embodiments, the expression cassette is inserted in the position where the adenoviral E1 gene is normally located. In addition, these vectors optionally have a non-functional or deleted E3 region. It is preferred that the adenovirus genome used be deleted of both the E1 and E3 regions (ΔE1ΔE3). The adenoviruses can be multiplied in known cell lines which express the viral E1 gene, such as 293 cells, or PERC.6 cells, or in cell lines derived from 293 or PERC.6 cell which are transiently or stably transformed to express an extra protein. For examples, when using constructs that have a controlled gene expression, such as a tetracycline regulatable promoter system, the cell line may express components involved in the regulatory system. One example of such a cell line is T-Rex-293; others are known in the art.

For convenience in manipulating the adenoviral vector, the adenovirus may be in a shuttle plasmid form. This invention is also directed to a shuttle plasmid vector which comprises a plasmid portion and an adenovirus portion, the adenovirus portion comprising an adenoviral genome which has a deleted E1 and optional E3 deletion, and has an inserted expression cassette comprising a TERT-LTB fusion protein-encoding nucleotide sequence. In preferred embodiments, there is a restriction site flanking the adenoviral portion of the plasmid so that the adenoviral vector can easily be removed. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic cells.

In a preferred embodiment of the invention, the expression cassette is inserted into an Ad6 (ΔE1ΔE3) adenovirus plasmid (*See* Emini et al., US20040247615, which is hereby incorporated by reference). This vector comprises an Ad6 adenoviral genome deleted of the E1 and E3 regions. In other preferred embodiments of the invention, the expression cassette is inserted into the pMRKAd5-HV0 adenovirus plasmid (*See* Emini et al., US20030044421, which is hereby incorporated by reference). This plasmid comprises an Ad5 adenoviral genome deleted of the E1 and E3 regions. The design of the pMRKAd5-HV0 plasmid was improved over prior adenovectors by extending the 5' cis-acting packaging region further into the E1 gene to incorporate elements found to be important in optimizing viral packaging, resulting in enhanced virus amplification. Advantageously, these enhanced adenoviral vectors are capable of maintaining genetic stability following high passage propagation.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the adenoviruses, shuttle plasmids, and DNA immunogens of this invention.

It has been determined in accordance with the present invention that genetic vaccination with plasmid DNA coding for TPA-mTERT(AI)-LTBopt can break immune tolerance

in BALB/c and B6 mice (*See* EXAMPLE 7). It has also been shown herein that immunization with plasmid TPA-mTERT(AI)-LTBopt can elicit a cytotoxic immune response in BALB/c mice (*See* EXAMPLE 8). It has further been demonstrated that this construct is able to induce a stronger CD8+ immune response than mTERT(AI) alone (see EXAMPLE 9), and is able to control tumor growth (EXAMPLE 11). Thus, the data described herein demonstrate that fusion of the TERT coding sequence to the LTB cDNA results in an increase in the TERT-specific immune response.

It has also been shown in accordance with the present invention that hTERT(AI)-LTBopt can induce an HLA-A2 restricted CD8+ immune response (see EXAMPLE 12) and that this immune response can be amplified by boosting with Ad6-hTERT(AI).

Therefore, the vectors described above may be used in immunogenic compositions and vaccines for preventing the development of tumors associated with aberrant TERT expression and/or for treating existing cancers. The vectors of the present invention allow for vaccine development and commercialization by eliminating difficulties with obtaining high expression levels of exogenous TERT in successfully transformed host organisms and by providing a TERT-LTB fusion protein which can elicit an enhanced immune response when administered to a mammal such as a human being.

To this end, one aspect of the instant invention is a method of preventing or treating TERT-associated cancer comprising administering to a mammal a vaccine vector comprising a polynucleotide comprising a sequence of nucleotides that encodes a TERT-LTB fusion protein, wherein the TERT-LTB fusion protein comprises an inactive TERT protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal.

In accordance with the method described above, the vaccine vector may be administered for the treatment or prevention of a cancer in any mammal, including but not limited to: lung cancer, breast cancer, and colorectal cancer. In a preferred embodiment of the invention, the mammal is a human.

Further, one of skill in the art may choose any type of vector for use in the treatment and prevention method described. Preferably, the vector is an adenovirus vector or a plasmid vector. In a preferred embodiment of the invention, the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising: (a) a sequence of nucleotides that encodes a TERT-LTB fusion protein, wherein the TERT-LTB fusion protein comprises an inactive TERT protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal; and (b) a promoter operably linked to the polynucleotide.

The instant invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a sequence of nucleotides that encodes a TERT fusion protein, wherein the TERT fusion protein comprises an inactive TERT protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal; and (b) a promoter operably linked to the polynucleotide.

In a preferred embodiment of this aspect of the invention, the adenovirus vector is an Ad 6 vector.

In another preferred embodiment of the invention, the adenovirus vector is an Ad 5 vector.

In yet another preferred embodiment, the adenovirus vector is an Ad 24 vector.

Also contemplated for use in the present invention is an adenovirus vaccine vector comprising an adenovirus genome that naturally infects a species other than human, including, but not limited to, chimpanzee adenoviral vectors. A preferred embodiment of this aspect of the invention is a chimp Ad 3 vaccine vector.

In another aspect, the invention relates to a vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising: (a) a sequence of nucleotides that encodes a TERT fusion protein, wherein the TERT fusion protein comprises an inactive TERT protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal; and (b) a promoter operably linked to the polynucleotide. An example of a suitable plasmid would be the mammalian expression plasmid V1Jns as described (J. Shiver *et. al.* in *DNA Vaccines*, M. Liu et al. eds., N.Y. Acad. Sci., N.Y., 772:198-208 (1996), which is herein incorporated by reference).

In some embodiments of this invention, the recombinant adenovirus and plasmid-based polynucleotide vaccines disclosed herein are used in various prime/boost combinations in order to induce an enhanced immune response. In this case, the two vectors are administered in a "prime and boost" regimen. For example the first type of vector is administered one or more times, then after a predetermined amount of time, for example, 2 weeks, 1 month, 2 months, six months, or other appropriate interval, a second type of vector is administered one or more times. Preferably the vectors carry expression cassettes encoding the same polynucleotide or combination of polynucleotides.

In the embodiment where a plasmid DNA is also used, it is preferred that the vector contain one or more promoters recognized by mammalian or insect cells. In a preferred embodiment, the plasmid would contain a strong promoter such as, but not limited to, the CMV promoter. One of skill in the art will recognize that any of a number of other known promoters may be chosen for purposes of driving expression of the TERT-LTB nucleotide sequences of the present invention. Additional examples of promoters include naturally occurring promoters such

as the EF1 alpha promoter, Rous sarcoma virus promoter, and SV40 early/late promoters and the p-actin promoter; and artificial promoters such as a synthetic muscle specific promoter and a chimeric muscle- specific/CMV promoter (Li et al., *Nat. Biotechnol.* 17:241-245 (1999); Hagstrom et al., *Blood* 95:2536-2542 (2000)). The synthetic TERT-LTB fusion gene or other gene to be expressed would be linked to such a promoter.

As stated above, an adenoviral vector vaccine and a plasmid vaccine may be administered to a vertebrate as part of a single therapeutic regime to induce an immune response. To this end, the present invention relates to a method of protecting a mammal from a TERT-associated cancer comprising: (a) introducing into the mammal a first vector comprising: i) a sequence of nucleotides that encodes a TERT fusion protein, wherein the TERT fusion protein comprises an inactive TERT protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a sequence of nucleotides that encodes a TERT fusion protein, wherein the TERT fusion protein comprises an inactive TERT protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of protection described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid. In some embodiments of the present invention, the first vector is administered to the patient more than one time before the second vector is administered.

In the method described above, the first type of vector may be administered more than once, with each administration of the vector separated by a predetermined amount of time. Such a series of administration of the first type of vector may be followed by administration of a second type of vector one or more times, after a predetermined amount of time has passed. Similar to treatment with the first type of vector, the second type of vector may also be given one time or more than once, following predetermined intervals of time.

The instant invention further relates to a method of treating a patient suffering from a TERT-associated cancer comprising: (a) introducing into the mammal a first vector comprising: i) a sequence of nucleotides that encodes a TERT fusion protein, wherein the TERT fusion protein comprises a TERT(AI) protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the patient a second vector comprising: i) a sequence of nucleotides that encodes a TERT fusion protein, wherein the TERT fusion protein comprises a TERT(AI) protein or variant thereof, fused to a substantial portion of LTB and wherein the fusion

protein is capable of producing an immune response in a mammal; and ii) a promoter operably linked to the polynucleotide.

In one embodiment of the method of treatment described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid. In further preferred embodiments of the method described above, the first vector is administered to the patient more than one time before the second vector is administered to the patient.

In preferred embodiments of the methods described above, the vectors comprise a sequence of nucleotides that encode a TERT(AI)-LTB fusion protein, wherein the TERT fusion protein comprises an inactive TERT protein, fused to a substantial portion of LTB.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend partially on the strength of the promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 μg to 300 μg of a plasmid vaccine vector is administered directly into muscle tissue. An effective dose for recombinant adenovirus is approximately $10^6 - 10^{12}$ particles and preferably about 10^7—10^{11}particles. Subcutaneous injection, intradermal introduction, impression though the skin, and other modes of administration such as intraperitoneal, intravenous, intramuscular or inhalation delivery are also contemplated.

In preferred embodiments of the present invention, the vaccine vectors are introduced to the recipient through intramuscular injection.

The vaccine vectors of this invention may be naked, i.e., unassociated with any proteins, or other agents which impact on the recipient's immune system. In this case, it is desirable for the vaccine vectors to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, it may be advantageous to administer an agent which assists in the cellular uptake of DNA, such as, but not limited to calcium ion. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers. Those of skill in the art will be able to determine the particular reagent or pharmaceutically acceptable carrier as well as the appropriate time and mode of administration.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in

the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Construction of TERT Fusion Proteins

To determine if fusion of the telomerase reverse transcriptase (TERT) antigen to the LTB subunit of *E. coli* heat labile enterotoxin (Fingerut *et al.* *Vaccine* 23(38): 4685-96 (2005); Rigano *et al.* *Plant Cell Rep.* 22(7): 502-8 (2004)) could enhance the immunogenicity of TERT alone, vectors were constructed encoding the full-length telomerase reverse transcriptase, with modifications. First, the DNA sequence was codon-optimized to incorporate codon preferred by human host cells. Additionally, to ensure that the encoded antigen was safe for vaccine use, mutations were introduced to the TERT nucleotide sequence to inactivate telomerase catalytic activity of the encoded protein. Specifically, the mutations D712A and V713I were added to the human TERT sequence and the mutations D702A and V703I were added to the mouse TERT sequence (Arai *et al.* Two independent regions of human telomerase reverse transcriptase are important for its oligomerization and telomerase activity. *J. Biol. Chem.* 277 (10): 8538-44 (2002)).

TERT fusions were engineered by joining the C-terminal end of the modified TERT nucleotide sequences, described above, to a nucleotide sequence encoding a modified LTB (nt 64 to 375) in which the signal peptide coding sequence had been removed. Additionally, a leader sequence encoding the human tissue plasminogen activator (TPA) signal sequence was added 5' to the TERT-encoding nucleotide sequence to ensure the secretion of the TERT protein (Haddad *et al.* Comparative study of DNA-based immunization vectors: effect of secretion signals on the antibody responses in mice. *FEMS Immunol. Med. Microbiol.* 18(3): 193-202 (1997)). The entire sequence was codon-optimized to incorporate codons preferred by human host cells using computer algorithms. The synthetic codon optimized gene was assembled by synthetic oligonucleotides. In the final construct, two amino acids were added (S and R) between the TERT sequence and the LTB sequence due to the cloning strategy.

The nucleotide sequences encoding the TERT-fusions were cloned into the vector pV1JnsA under the control of the human cytomegalovirus (CMV)/intron A promoter plus the bovine growth hormone (BGH) polyadenylation signal. Plasmid pV1J/TPA-hTERT-LTBopt carries the codon-optimized, inactivated, cDNA of human TERT fused to the coding sequences of the TPA signal sequence on the N-terminal end and LTB on the C-terminal end. Similarly, plasmid pV1J/TPA-mTERT-LTBopt carries the codon-optimized, inactivated, cDNA of mouse TERT fused to TPA and LTB.

EXAMPLE 2

Plasmid and Adenovirus Constructs

pV1JnsA/TPA-mTERT (AI)-LTBopt: Plasmid 041046pucKana containing TPA-mTERT(AI)-LTB sequence was obtained from GENEART (Geneart GmbH, Regensburg, Germany). The plasmid was digested with *Bgl*II and *Sal*I and the resulting fragment was cloned into the *Bgl*II/*Sal*I site in the plasmid pV1JnsA (Montgomery *et al.*, *DNA Cell Biol.*, 12(9):777-83(1993)).

pV1JnsA/mTERT(AI)opt: pV1JnsA/TPA-mTERT(AI)-LTB was digested *Xba*I to remove LTB coding sequence (present between two *XbaI* sites) the resulting construct pV1JnsA/TPA-mTERT(AI) had 3 aa (S;R;N) after the last mTERT aa and before the stop codon. Removal of the TPA coding sequence was performed on pV1JnsA/TPA-mTERT(AI) by *BamH*I and *EcoR*V digestion. The TPA coding sequence was replaced by a PCR product obtained amplifying mTERT sequence with the sense primer (5'- G A T C T G A T G A T A T C G C C A C C A T G A C C A G A G C C C C C A G A T G -3; SEQ ID NO: 15) and anti-sense primer (5'-A G G G G G G A T C C G C A C A C C T G G T A G G C G C A G C T G G G G -3'; SEQ ID NO:16) and cloned back into the *BamH*I and *EcoR*V digested vector.

pV1JnsA/TPA-hTERT(AI)-LTBopt: The synthetic fragment corresponding to TPA-hTERT(AI)-LTB obtained by GENEART was cloned into pV1JnsA using *Bgl*II /*Sal*I restriction sites.

Ad6-TPAmTERT(AI)-LTBopt: Plasmid 041046pucKana containing TPA-mTERT(AI)-LTBopt sequence was obtained from GENEART. The plasmid was digested with *Bgl*II and *Sal*I and the fragment cloned *Bgl*II /*Sal*I into the plasmid pNEBAd6-CMVpA shuttle vector. The plasmid pNEBAd6-CMVpA-TPA-mTERT(AI)-LTB digested with *Eco*RI /*Hind*III was recombined to *Cla*I-linearized plasmid pMRK Ad6 ΔE1 ΔE3. The plasmid was cut with *Pac*I to release the Ad ITRs and transfected in Perc-6 cells. Ad6 vector amplification was carried out by serial passages and purified trough standard CsCL gradient purification.

Ad6-hTERT(AI): Plasmid pCRsript containing wild type sequence of hTERT (hTERT wild type sequence was rescued by reverse transcription of mRNA of human tumor cells) was digested *Bgl*II/ *Xba*I filled with Klenow enzyme and cloned in pV1JnsA digested with *EcoR*V and *Bgl*II. pV1JnsA-hTERT was mutagenized by using an oligonucleotide (5'- C T G T A C T T T G T C A A G G T G G C T A T C A C G G G C G C G T A C G -3'; SEQ ID NO:17) and the Stratagene quikchange multi site-directed mutagenesis kit (Stratagene, LA Jolla, CA; Cat: 200513) to obtain pV1JnsA-hTERT(AI). The plasmid was digested with *Bgl*II *and Sal*I and the fragment cloned *Bgl*II /*Sal*I into the plasmid pNEBAd6-CMVpA shuttle vector. The plasmid pNEBAd6-CMVpA-hTERT(AI) was digested *Pac*I/*Pme*I and recombined to *Cla*I-linearized plasmid pMRK Ad6 ΔE1 ΔE3. The plasmid was cut with *Pac*I to release the Ad ITRs and transfected in Perc-6 cells. Ad6 vector amplification was carried out by serial passages and purified trough standard CsCL gradient purification.

EXAMPLE 3

IFN-γ ELISPOT Assay

Mouse splenocytes secreting IFN-γ in an antigen-specific manner were detected using a standard enzyme-linked immunospot (ELISPOT) assay (Miyahira *et al. J Immunol Methods* 181(1): 45-54 (1995)). Ninety-six wells MAIP plates (Millipore Corp., Billerica, MA) were coated with 100 µl/ well of purified rat anti-mouse IFN-γ (IgG1, clone R4-6A2, Pharmingen) diluted to 2.5 µg/ml in sterile PBS. After washing with PBS, blocking of plates was carried out with 200 µl/well of R10 medium for 2 hrs at 37°C.

Splenocytes were obtained by removing the spleen from the euthanized mice in a sterile manner, followed by spleen disruption through grating on a metal grid. Red blood cells were removed by osmotic lysis by adding 1 ml of 0.1X PBS to the cell pellet and vortexing for approximately 15s. One ml of 2x PBS was then added and the volume was brought to 4 ml with 1x PBS. Cells were pelleted by centrifugation at 1200 rpm for 10 min at RT, and the pellet was resuspended in 1 ml R10 medium. Viable cells were counted using Türks staining.

Splenocytes were plated at $5x10^5$ and $2.5x10^5$ cells/well in duplicate and incubated for 20h at 37°C with 1 µg/ml suspension of each peptide. Concanavalin A (ConA) was used as positive internal control for each mouse at 5 µg/ml. After washing with PBS, 0.05% Tween 20, plates were incubated O/N at 4°C with 50 µl/well of biotin-conjugated rat anti-mouse IFNγ (RatIgG1, clone XMG 1.2, PharMingen) diluted to 1:2500 in assay buffer. After extensive washing, plates were developed by adding 50 µl/well NBT/B-CIP (Pierce) until development of spots was clearly visible. The reaction was stopped by washing plates thoroughly with distilled water. Plates were air dried and spots were then counted using an automated ELISPOT reader.

EXAMPLE 4

Intracellular Cytokine Staining.

One to two millions mouse splenocytes or PBMC obtained by retroorbital bleeding in EDTA were resuspended in 1ml RPMI 10% FCS were incubated with pool of peptides (5-6 µg/ml final concentration of each peptide) and brefeldin A (1 µg/ml; BD Pharmingen cat #555028/2300kk) at 37°C and 5% CO_2 for 12-16 hours. Cells were then washed with FACS buffer (PBS 1% FBS, 0.01% NaN_3) and incubated with purified anti-mouse CD16/CD32 Fc block (BD Pharmingen cat # 553142) for 15 min at 4°C. Cells were then washed and stained with surface antibodies: CD4-PE conjugated anti-mouse (BD Pharmingen, cat.# 553049), PercP CD8 conjugated anti mouse (BD Pharmingen cat# 553036) and APC- conjugated anti-mouse CD3e (BD Pharmingen cat# 553066) for 30 minutes at room temperature in the dark. After the washing cells were fixed and permeabilized with Cytofix-Cytoperm Solution (BD Pharmingen cat #555028/2300kk) for 20 min at 4°C in the dark. After washing with PermWash Solution (BD Pharmingen cat #555028/2300kk) cells were incubated with the IFNγ-FITC antibodies (BD

Pharmingen). Cells were then washed, fixed with formaldehyde 1% in PBS and analyzed on a FACS-Calibur flow cytometer, using CellQuest software (Becton Dickinson, San Jose, CA).

EXAMPLE 5

Cytotoxic T Lymphocyte (CTL) Assay

Splenocytes were obtained by removing the spleen from the euthanized mice in a sterile manner, followed by spleen disruption through grating on a metal grid. Red blood cells were removed by osmotic lysis by adding 1 ml of 0.1X PBS to the cell pellet and vortexing for approximately 15s. Spleen cells at 2 x 10^6 cells/ml in R10 were plated in a 24 well cell culture plate (2 ml of cells/well) in presence of the immunogenic peptide at the final concentration of 10μg/ml. Plates were incubated at 37°C, 95% humidity, 5% CO_2 for 6 days. At day 3 of culture, recombinant human IL-2 at a final concentration of 10U/ml was added.

Target cells growing at exponential phase were harvested and brought to 1 x 10^6 cells/ml/tube in the presence of the immunogenic peptide at 10μg/ml final concentration. Cells were labeled with 50-100μCi ^{51}Cr/tube at 37° for 2 hours. Target cells were washed three times with 10 ml medium by centrifuging at 250 g, room temperature for 5 min, and brought at 1 x 10^5 cells/ml.

CTL assay was performed by plating Effector/Target cells with E/T ratio at 100:1, 50:1, 25:1 and 12.5:1. After 4 hours of incubation, plates were centrifuged at 1200 rpm for 5 min and the supernatant was harvested (30 μl/well). Plates were dried overnight and counted using a Beta-plate counter.

EXAMPLE 6

Mice Immunization

Female C57BL/6 mice (H-2^b) were purchased from Charles River (Lecco, Italy). HLA-A2.1 mice (HHD) were kindly provided by F. Lemmonier (Institute Pasteur, Paris, France). BALB/c mice (H-2^d) were purchased from Charles River (Lecco, Italy). Mice were immunized at 8 weeks of age. Fifty micrograms of plasmid DNA were electroinjected in a 50 μl volume in mice quadriceps as previously described (Rizzuto *et al. Proc. Natl. Acad. Sci. U.S.A.* 96(11): 6417-22 (1999)). Electroporation (EP) was performed as previously described (Zucchelli *et al. J. Virol.* 74(24): 11598-607 (2000); Rizzuto et al., *supra*). Briefly, electrical shock consisted of 10 trains with 1000 square bipolar pulses (90V/cm, 75mA, 200μs/phase). Ad injections were carried out in mice quadriceps in 50 μl volume. Cell mediated immune response was analyzed at the indicated time.

EXAMPLE 7

Genetic Vaccination with Plasmid DNA Coding for TPA- mTERT(AI)-LTB Breaks Immune Tolerance.

To determine if vaccination of mice with TPA- mTERT(AI)-LTB could break immune tolerance, groups of 20 BALB/c mice were immunized with 5 weekly injections of plasmid pV1J/TPA-mTERT(AI)-LTB followed by electroporation, as described in EXAMPLE 6. Eleven days after the last injection, splenocytes were obtained from individual mice, which were used to analyze the cell-mediated immune response by IFN-γ ELISPOT assay and by intracellular cytokine staining (ICS).

Antigen-specific IFNγ secretion from stimulated splenocytes was measured using three pools of 15mer mTERT peptides overlapping by 11 aa and encompassing the entire mTERT protein. The mTERT-1 pool was composed of 94 individual peptides covering the mTERT region from aa 1 to 388. The mTERT-2 pool was composed of 106 individual peptides covering the mTERT region from aa 377 to 811. The mTERT-3 pool was composed of 78 individual peptides covering the mTERT region from aa 801 to 1122. As a negative control, cytokine production was also measured upon stimulation of the splenocytes with DMSO at the same concentration utilized to solubilize the TERT peptides. The immune response induced against the LTB adjuvant was also detected by using a pool of 24 individual 15-mer peptides overlapping by 11 aa and covering the entire LTB sequence. ELIspot results indicate that vaccination of mice with TPA-mTERT(AI)-LTBopt elicited a cell mediated immune response (CMI) against the N-terminal and central regions of the TERT protein, contained within the TERT-1 and TERT-2 peptide pools (FIGURE 3). CMI against LTB was also detected by ELIspot (FIGURE 3).

To better characterize the T cell response elicited by the plasmid pV1J/TPA-mTERT(AI)-LTBopt and to identify the CD8+ and CD4+ T cell subset responsible for IFN-γ production, IFN-γ intracellular staining was carried out on vaccinated mice splenocytes and analyzed by FACS. Data obtained by ICS demonstrate that genetic immunization of BALB/c mice vaccinated with TPA-mTERT(AI)-LTBopt elicited a significant $CD8^+$ T cell response against the N-terminal region of the TERT protein (contained within mTERT-1 peptide pool, *see* FIGURE 4). The $CD4^+$ T cell response was mapped in the central region of TERT (mTERT-2 peptide pool). In contrast, no CMI could be detected in these mice against the C-terminal region of the protein (mTERT-3 peptide pool). A $CD4^+$ T cell response against LTB was also detected (FIGURE 4).

To further map the CD8+ T-cell response, a midi pool of mTERT 15mer peptides was used. Each midi pool contained ten 15mers, which were each individually contained in a separate midi-pool. Therefore, a positive reaction of 2 midi pools would unequivocally identify one individual 15mer as being immunogenic. Because CD8+ epitopes are always 9aa long, any CD8+ peptide is contained in two adjacent 11mer peptides. Results demonstrated that the CD8+ T-cell immune response was localized to the TERT region included in the following 15mer mTERT peptides: mTERT-41 (TERTaa161; LVPPSCAYQVCGSPL (SEQ ID NO:18) and mTERT-42 (TERTaa165; SCAYQVCGSPLYQIC; SEQ ID NO:19). By overlapping the two 15mer peptides (mTERT41 and mTERT-42), three possible reactive nonamers were synthesized

and tested in ICS. Finally, the immunodominant $CD8^+$ epitope in BALB/c mice was identified in the following sequence (mTERTaa167; AYQVCGSPL; SEQ ID NO:20). These results demonstrate that vaccination of BALB/c mice with pV1J/TPA- mTERT-LTB could break immune tolerance to the mTERT antigen.

To confirm data obtained in BALB/c mice, C57BL/6 (B6) mice were also immunized with 5 weekly injections of plasmid pV1J/TPA-mTERT(AI)-LTBopt. IFN-γ intracellular staining was carried out on vaccinated mice splenocytes and analyzed by FACS. Similar to results obtained with BALB/c mice, tolerance against mTERT was broken in all B6 mice. Additionally, both $CD4^+$ and $CD8^+$ T cells producing IFNγ were detected by ICS upon stimulation with TERT peptides (see FIGURE 6).

The CD8+ immune response elicited by DNA vaccination in C57BL/6 mice was primarily biased towards the central region of the TERT protein (peptide pool mTERT-2). A weaker CD8+ immune response was also detectable in the mTERT peptide pool-1. The CD8+ immune response was mapped in B6 mice using midi pools as described above. Two CD8+ epitopes were identified in B6. The first immunogenic sequence was mapped to the aa sequence mTERT198 (VGRNFTNL; SEQ ID NO:21) and the second CD8+ epitope was mapped to the sequence mTERT486 (SLGKYGKL; SEQ ID NO:22) Additionally, the CD4+ immune response was localized to the N-terminal region of TERT (peptide pool mTERT-1). No immune response was detected against the C-terminal region of mTERT.

Similar to the results obtained with BALB/c mice described above, immunization and analysis of the subsequent immune response of B6 mice confirm that TPA-mTERT(AI)-LTBopt immunization can break immune tolerance to the mTERT antigen. Therefore, taken together, results obtained with BALB/c and B6 mice indicate that the TPA-mTERT(AI)-LTBopt fusion is a potent immunogen in the self context independently from the mice strain used.

EXAMPLE 8

Cytolytic Activity of TERT-Specific $CD8^+$ T Cells.

A cytotoxic T-lymphocyte (CTL) assay was used to detect the cytolytic activity of TERT-specific $CD8^+$ T cells elicited by immunization of BALB/c mice with pV1J/TPA-mTERT(AI)-LTBopt. Mice were immunized with 5 weekly injections of plasmid pV1J/TPA-mTERT(AI)-LTBopt followed by electroporation. Mice splenocytes obtained from 2 individual mice were collected 10 days after the last immunization. These splenocytes were stimulated for one week with the immunogenic peptide mTERTaa167 (AYQVCGSPL; SEQ ID NO:20) to obtain *in vitro* activated T cells. The BALB/c syngenic tumor cell line 4T1 (Aslakson *et al.* *Cancer Res.* 52(6): 1399-405 (1992)) was used as a target. 4T1 cells were loaded with the immunoreactive peptide and labeled with Cr^{51} to be used as target for the CTL assay. The activated T cells were co-incubated with the target 4T1 cells at different effectors/target ratio. Activated T cells obtained both from mouse# 1 and mouse #2 showed cytolytic activity on target

cells when loaded with the immunogenic peptide mTERTaa167 (AYQVCGSPL; SEQ ID NO:20). The two mice showed different degrees of cytotoxic activity ranging from 80% (mouse #1) to 25% (mouse #2) at effectors /target ratio of 50/1.

Results indicate that a cytotoxic immune response was induced upon DNA immunization with TPA-mTERT(AI)-LTBopt as self antigen in BALB/c mice (FIGURE 5).

EXAMPLE 9

Comparative Immunogenicity of TPA-mTERT(AI)-LTBopt Construct.

To compare the immunogenicity of the secreted mTERT(AI) protein fused to LTB encoded by pV1J/TPA-mTERT(AI)-LTBopt, to a non-secreted version of mTERT(AI) without fusion to LTB, a plasmid pV1J derivative carrying mTERT(AI) was constructed, as described in EXAMPLE 2.

Groups of 6 BALB/c mice were immunized with 5 weekly injections of plasmids pV1J/TPA-mTERT(AI)-LTBopt or pV1J/mTERT(AI)opt. The immune response was monitored by IFNγ intracellular staining using the mTERTaa167 CD8+ immunodominant epitope sequence previously identified for BALB/c mice (AYQVCGSPL (SEQ ID NO:20).

Results indicate that TPA-mTERT(AI)-LTBopt is a better construct for the induction of a CD8+ immune response against murine telomerase than mTERT(AI) (FIGURE 7). The difference observed in $CD8^+$ T cell response induced in the group immunized by TPA-mTERT-LTB(AI)opt is statistically different from that obtained using the mTERT(AI)opt in the context of DNA immunization ($p=0.04$ student t test).

EXAMPLE 10

Comparison of Immunization Regimens

The anti-mTERT cell-mediated immune response elicited by immunization of BALB/c mice with DNA TPA-mTERT(AI)-LTBopt plus electrical stimulation alone was compared to the immune response induced by a diversified prime/ boost immunization regimen using DNA TPA-mTERT(AI)-LTBopt plus electrical stimulation as prime and Ad6 TPA-mTERT(AI)-LTBopt as final booster of the immune response.

Groups of 10 mice were immunized with 5 weekly injections of plasmid pV1J/TPA-mTERT(AI)-LTBopt. One week later, a booster injection was given consisting of either one additional injection of 50µg of DNA plus electroporation (EP) or with 10^{10} vp of the Ad6/TPA-mTERT (AI)-LTBopt. IFNγ intracellular staining was performed on mouse PBMCs to assess the resulting cell-mediated immune response. A peptide encompassing a $CD8^+$ T cell specific epitope mTERT 167 (AYQVCGSPL; SEQ ID NO:20) which was previously mapped to the N-terminal region of TERT, was used as antigen. Results indicate that the amplitude of the immune response elicited by the DNA-EP/Ad combination was 4.6 fold greater than that which was observed in mice that had received only a DNA-EP regimen (see FIGURE 8).

EXAMPLE 11

Vaccination with TPA-mTERT-LTB Controls Tumor Growth.

To determine if the mTERT-specific immune response could generate an anti-tumor effect, BALB/c mice were treated with the chemical carcinogen dimethyl hydrazine by intraperitoneal (i.p.) injection prior to vaccination. Treatment of mice with this carcinogen leads to progressive tumor development in the colon characterized by aberrant crypt formation, adenoma, and finally carcinoma. Chemical treatment of these mice did not render them immunocompromized, as immunization with TPA-mTERT(AI)-LTBopt elicited an immune response comparable to that previously found in non-treated mice (data not shown).

To assess the impact of genetic immunization on early stages of DMH induced carcinogenesis, BALB/c mice were treated with DMH and immunized by DNA followed by electroporation (DNA-EP) with pV1J/TPA-mTERT(AI)-LTBopt according to the schedule described in FIGURE 9.

Twenty mice were euthanized 12 weeks after the beginning of DMH treatment to count the number of ACF (aberrant crypt foci) and the number of adenoma which had formed. A significant reduction of both the number of ACF and the number of adenoma present was observed in vaccinated mice compared to non-vaccinated mice (FIGURE 10).

The efficacy of mTERT vaccination on a later stage of tumor development was also monitored. To enhance and sustain the immune response to mTERT, mice were subjected to repeated DNA-EP TPA-mTERT(AI)-LTBopt and Ad6TPA-mTERT(AI)-LTBopt (FIGURE 11). The immune response elicited in these mice was comparable to that detected in BALB/c mice vaccinated with the same DNA/Ad regimen and not treated with DMH.

Twenty mice were euthanized 30 weeks after the beginning of DMH treatment to count the number of ACF (aberrant crypt foci) and of adenoma formed. Like results seen with early stage tumor development, a significant reduction in the number of ACF and adenomas was observed in vaccinated mice compared to non-vaccinated mice (FIGURE 12).

Finally, tumors found in vaccinated mice were smaller in volume compared to those found in non-vaccinated mice (FIGURE 13A). Moreover, histological analysis showed that the tumors present in vaccinated mice were at a less advanced stage compared to tumors in non-vaccinated animals (FIGURE 13B).

EXAMPLE 12

Characterization of pV1JTPA-hTERT(AI)-LTBopt.

The immunogenic potential of pV1J/TPA-hTERT(AI)-LTBopt was evaluated in a non–self context since mice transgenic for hTERT are not available. C57BL/6 mice transgenic for the human HLA-A2 (HHD transgenic mice) were utilized so that TERT immunogenic peptides would be presented in the context of human HLA class I. HHD transgenic mice were vaccinated

by DNA-EP with 2 biweekly injections of 50 μg of plasmid pV1J/TPA-hTERT(AI)-LTBopt. The induced immune response was evaluated by ICS on mice PBMC. The hTERT immunogenic peptide corresponding to the hTERT 865 epitope RLVDDFLLV (SEQ ID NO:23) was used as antigen. This epitope sequence was previously described as a strong binder for HLA-A2 and inducer of CTL activity in T cells *in vitro* priming experiments (Dupont et al. *Cancer Research* 65(12): 5417-27 (2005)).

Results obtained from 10 mice showed that on average, 12% of CD8+ cells produced IFN-γ when stimulated with the immunogenic hTERT 865 peptide (FIGURE 14). Results indicate that the pV1JTPA-hTERT(AI)-LTBopt vaccine candidate is immunogenic and induced a HLA-A2 restricted CD8 immune response.

EXAMPLE 13

Characterization of Immune Response in Vaccinated Mice after Ad6-hTERT(AI) Boost.

An Ad6 hTERT(AI) construct was assembled as described in EXAMPLE 2. This construct comprises wild-type codons, with the exception of two mutations that were added to abrogate telomerase catalytic activity. An Ad6 hTERT-LTB fusion construct was also made, but could not be rescued, so further testing was performed only with Ad6hTERT(AI).

The Ad6 hTERT(AI) construct was tested for its boosting capability on the immune response induced by DNA–EP in HHD transgenic mice. Groups of 10 mice were vaccinated by DNA-EP with 1 injection of 50 μg of plasmid pV1J/TPA-hTERT(AI)-LTBopt and boosted either with a second DNA injection or with Ad6 hTERT 10^{10} pp two weeks after the first DNA injection. The induced immune response was measured by using the immunodominant peptide for HLA-A2 allele hTERT865 previously identified. Reactive CD8+ T cells producing IFN-γ were detected with high frequencies. As shown in FIGURE 14, the amplitude of immune response elicited by the DNA-EP/Ad combination was 3 fold greater than what observed in mice that had received only a DNA-EP regimen.

The results demonstrated that hTERT specific immune response primed by injection of pV1J/TPA-hTERT(AI)-LTBopt DNA was amplified by boosting it with Ad6-hTERT(AI).

EXAMPLE 14

Characterization of CTL Activity Induced by Immunization with hTERT.

The $CD8^+$ T cell response to hTERT elicited in vaccinated mice was further characterized by testing the cytotoxic activity (CTL) of activated T cells against tumor target cells. Splenocytes from immunized mice were stimulated in culture for 6 days in the presence of the hTERT865 peptide (SEQ ID NO:23; see EXAMPLE 12) and IL-2. Thereafter, activated CD8+ T cells were tested against target cells for their lytic activity in a Cr^{51} release assay. HeLa cells stably transfected to express the chimeric MHC class I molecule present on HHD mice (HeLa-HHD) were used as target cells. HeLa–HHD target cells were either loaded exogenously with the immunogenic peptide hTERT865 or infected 24 hours before with an Ad6 vector coding for hTERT, in order to endogenously over-express the TERT antigen. As a control, target cells were infected with an Ad6 vector coding for GFP. The results indicate that CD8+ T cells induced by the vaccination described were able to kill target cell over-expressing the hTERT antigen (FIGURE 15).

EXAMPLE 15

Characterization of Immunogenicity of Human-TERT Vaccine in Rhesus Monkeys.

The immunogenicity of the genetic vaccination platform based on DNA prime, Ad boost, with hTERT was evaluated in rhesus monkeys. Because the homology of human TERT and rhesus TERT sequence is 96%, tolerance was expected to play a major role in determining vaccination efficacy.

The vaccination protocol consisted of five injections of DNA-EP (pV1J/TPA-hTERT-LTB, 5mg/injection), given every two weeks. Four weeks after the last DNA-EP treatment, monkeys were boosted with an Ad vector expressing hTERT (Ad6-hTERT, 10^{11} vp) 2 times with a two week interval. Data on body weight and clinical signs were collected every week. Negative effects on animal body weight or occurrence of clinical signs were not observed.

The induced immune response was monitored by IFN-γ ELISPOT assay of PBMC using hTERT peptide pools similar to those described in EXAMPLE 7 and a peptide pool covering the LTB sequence. Immune responses were scored positive if the signal obtained was at least four times higher than the background reactivity (DMSO). ELIspot analysis of the induced CMI indicated that there was a detectable immune response after the first 2 DNA-EP treatments in four out of four vaccinated monkeys (FIGURE 16A). A slight increase of the response was observed after the fifth DNA-EP (FIGURE 16B). The immune response was also measured at the end of the complete immunization protocol, after the two Ad boosts. Results indicate that the Ad boosts induced a consistent increase in the amplitude of CMI (FIGURE 17). Therefore, the power of a heterologous prime/boost modality was further confirmed by this experiment.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a sequence of nucleotides that encodes a human telomerase reverse transcriptase (hTERT) fusion protein, wherein the hTERT fusion protein comprises an hTERT protein or variant thereof, fused to a substantial portion of the B subunit of *E. coli* heat labile enterotoxin (LTB) protein and wherein the fusion protein is capable of producing an immune response in a mammal.

2. The nucleic acid molecule of claim 1, wherein the sequence of nucleotides encodes a variant hTERT protein, wherein said variant comprises one or more mutations relative to the wild-type hTERT amino acid sequence as set forth in SEQ ID NO: 12, and wherein said mutations function to eliminate or reduce telomerase catalytic activity of the encoded hTERT protein.

3. The nucleic acid molecule of claim 2, wherein the mutations consist of D712A and V713I.

4. The nucleic acid molecule of claim 3, wherein the sequence of nucleotides encoding hTERT has been codon-optimized for high-level expression in a human cell.

5. The nucleic acid molecule of claim 3, wherein the encoded LTB protein is truncated of its signal sequence.

6. The nucleic acid molecule of claim 5, wherein the sequence of nucleotides encoding LTB has been codon-optimized for high-level expression in a human cell.

7. The nucleic acid molecule of claim 6, wherein the sequence of nucleotides encoding LTB and the sequence of nucleotides encoding hTERT have been codon-optimized for high-level expression in a human cell.

8. The nucleic acid molecule of claim 7, wherein the sequence of nucleotides comprises a sequence as set forth in SEQ ID NO:5.

9. The nucleic acid molecule of claim 3, wherein the sequence of nucleotides encodes an hTERT-LTB fusion protein as set forth in SEQ ID NO:6.

10. A purified fusion protein comprising a human telomerase reverse transcriptase (hTERT) protein, or variant thereof, fused to a B subunit of *E. coli* heat labile enterotoxin (LTB) protein.

11. The purified fusion protein of claim 10, wherein the C-terminal end of the hTERT protein is fused to the N-terminal end of the LTB protein.

12. The purified fusion protein of claim 11, wherein the hTERT protein comprises mutations that function to eliminate telomerase catalytic activity.

13. The purified fusion protein of claim 12, wherein the protein comprises a sequence of amino acids as set forth in SEQ ID NO:6.

14. A vector comprising the nucleic acid molecule of claim 1.

15. A vector comprising the nucleic acid molecule of claim 9.

16. The vector of claim 15, wherein the vector is a plasmid vector or an adenovirus vector.

17. The vector of claim 16, wherein the vector is plasmid pV1J.

18. A host cell comprising the vector of claim 15.

19. The nucleic acid molecule of claim 7, wherein the sequence of nucleotides further comprises a leader sequence encoding the human tissue plasminogen activator (TPA) signal sequence.

20. The nucleic acid molecule of claim 19, wherein the sequence of nucleotides comprises a sequence as set forth in SEQ ID NO:1

21. A vector comprising the nucleic acid molecule of claim 20.

22. An adenovirus vaccine vector comprising a sequence of nucleotides encoding human telomerase reverse transcriptase (hTERT); wherein the sequence of nucleotides comprises mutations that function to eliminate telomerase catalytic activity.

23. The adenovirus vaccine vector of claim 22, wherein the sequence of nucleotides encodes the hTERT protein as set forth in SEQ ID NO:10.

24. The vector of claim 23, wherein the vector is an Ad 5 vector or an Ad 6 vector.

25. A process for expressing an hTERT-LTB fusion protein in a recombinant host cell, comprising:

(a) introducing a vector comprising the nucleic acid molecule of claim 1 into a suitable host cell; and,

(b) culturing the host cell under conditions which allow expression of said hTERT-LTB fusion protein.

26. A method of preventing or treating cancer comprising administering to a patient in need thereof a vaccine vector comprising the nucleic acid molecule of claim 2.

27. A method according to claim 26 wherein the vector is an adenovirus vector or a plasmid vector.

28. A method according to claim 27 wherein the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising:

(a) a polynucleotide comprising sequence of nucleotides that encodes a hTERT-LTB fusion protein, wherein the hTERT-LTB fusion protein comprises an hTERT protein variant; said hTERT protein variant comprising mutations to eliminate telomerase catalytic activity, fused to a substantial portion of *E.coli* heat labile enterotoxin subunit B (LTB) and wherein the fusion protein is capable of producing an immune response in a mammal; and,

(b) a promoter operably linked to the polynucleotide.

29. A method according to claim 27 wherein the vector is a plasmid vaccine vector, which comprises a plasmid portion and an expressible cassette comprising

(a) a polynucleotide comprising sequence of nucleotides that encodes a hTERT-LTB fusion protein, wherein the hTERT-LTB fusion protein comprises an hTERT protein variant; said hTERT protein variant comprising mutations to eliminate telomerase catalytic activity, fused to a substantial portion of *E.coli* heat labile enterotoxin subunit B (LTB) and wherein the fusion protein is capable of producing an immune response in a mammal; and,

(b) a promoter operably linked to the polynucleotide.

30. An adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising:

(a) a polynucleotide comprising a sequence of nucleotides that encodes a human telomerase reverse transcriptase variant as set forth in SEQ ID NO:10; and

(b) a promoter operably linked to the polynucleotide.

31. An adenovirus vector according to claim 30 which is an Ad 5 or an Ad 6 vector.

32. A method of treating a patient suffering from or predisposed to a TERT-associated cancer comprising:

(a) introducing into the patient one or more times a first vector comprising:

(i) a polynucleotide comprising sequence of nucleotides that encodes a hTERT-LTB fusion protein, wherein the hTERT-LTB fusion protein comprises an hTERT protein variant; said hTERT protein variant comprising mutations to eliminate telomerase catalytic activity, fused to a substantial portion of *E.coli* heat labile enterotoxin subunit B (LTB) and wherein the fusion protein is capable of producing an immune response in a mammal; and,

(ii) a promoter operably linked to the polynucleotide;

(b) allowing a predetermined amount of time to pass; and

(c) introducing into the patient a second vector comprising:

(i) a polynucleotide comprising a sequence of nucleotides that encodes a human telomerase reverse transcriptase variant as set forth in SEQ ID NO:10; and

(ii) a promoter operably linked to the polynucleotide;

wherein the first vector and the second vector are not the same.

33. A method according to claim 32 wherein the first vector is a plasmid and the second vector is an adenovirus vector.

34. A method according to claim 33 wherein the plasmid vector is introduced into the patient more than one time before the adenovirus vector is introduced.