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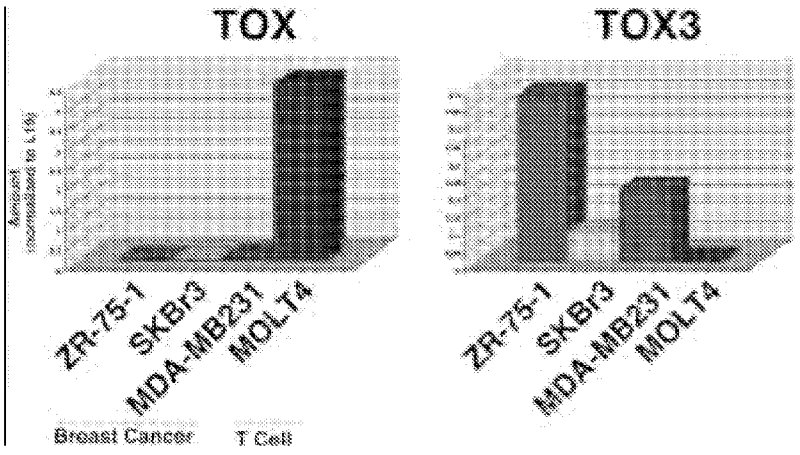
(54) Title: TOX3 AS A BIOMARKER FOR BREAST CANCER

FIGURE 2



(57) Abstract: The present invention relates to biomarkers useful for the diagnosis, prognosis and treatment of cancer. In an embodiment, the invention relates to the biomarker, TOX3. The inventive compositions and methods are also drawn to the use of anti-TOX3 antibodies and TOX3 nucleic acids and peptides for both detection and modulation of TOX3.

WO 2011/069014 A1

TOX3 AS A BIOMARKER FOR BREAST CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of the filing date of U.S. Provisional Application No. 61/266,918 filed December 4, 2009, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The invention relates in general to breast cancer biomarkers. More specifically, the invention provides compositions and methods for detecting and modulating TOX3 gene expression.

BACKGROUND

Breast cancer remains a serious public health problem. Aside from skin cancer, breast cancer is the most common form of cancer in women, with a lifetime incidence rate in the US population of approximately 13%. Breast cancer also remains one of the top ten causes of death for women in the US, and the second leading cause of cancer deaths in this population. Like all forms of cancer, breast cancer can be considered as a molecular reprogramming of the normal cell. Thus, understanding the gene regulatory networks that exist in breast cancer cells is of fundamental importance.

While mutations in BRCA1 or BRCA2 genes impart a very high risk for development of breast cancer, such mutations exist in the population at low frequency (and generally act as recessive cancer genes), and thus cannot account for the majority of breast cancers. Mutations in other genes, including PT53, PTEN, STK11, CDH1, also impart significantly increased risk of disease. However, even together with BRCA1 and BRCA2, these mutations may only account for 20% of familial disease (1). Thus, multiple additional genetic factors account for the observed disease incidence. In addition, the complexity of disease means that there can be additive and synergistic effects of changes in other mediators, even in the context of BRCA1 and BRCA2 mutations as discussed herein.

Using microarray analysis, the inventor has identified early changes in gene expression that take place in precursor thymocytes as they traversed a developmental checkpoint-termed

positive selection. These studies led to identification of a gene encoding a nuclear protein subsequently designated TOX (Thymocyte selection-associated HMG-box protein) (2). This protein contains a single centrally-located DNA binding motif known as an HMG-box, named after that found in canonical HMGB proteins. The HMG-box now defines a superfamily of proteins (which have 47 family members located in the human genome) that despite having diverse functions, share some general characteristics of DNA binding. HMG-box domains, including TOX, fold into three α-helices that form a concave L-shaped structure that binds the minor groove of DNA. HMG-box proteins also bind distorted DNA structures and often can induce bending and unwinding of the DNA helix to fit the protein domain structure. Two general classes of HMG-box proteins have been identified based on their mode of binding to DNA: (a) those that exhibit sequence-specific binding and (b) those that bind DNA in a sequence-independent but structure-dependent fashion. The latter class of proteins include the canonical HMGB proteins themselves, while the former include transcriptional regulators, such as LEF-1. Both kinds of proteins, however, play roles in regulating gene expression, often by inducing or stabilizing architectural changes in chromatin and facilitating nucleoprotein complex formation. HMG-box proteins may also augment other nuclear functions that benefit from architectural changes in DNA, including antigen receptor gene rearrangement (3) and chromatin remodeling (4). By inspection of key residues in the HMG-box domain (TOX-box), TOX is almost certainly a member of the sequence-independent DNA-binding family (5).

TOX may be targeted by recognizing structural features of chromatin or alternatively by binding to other proteins. The TOX-box also defines a subfamily of proteins that includes three additional members (TOX2, 3, and 4) (5). Based on a high degree of conservation of the TOX-box sequence, all family members are predicted to be sequence-independent DNA-binding factors. Outside of the DNA-binding domains, the N-terminal domains of family members are the next most similar, and this domain has transactivation activity. The C-terminal domains of the family members are quite distinct and there is reason to think that they may function as interaction domains (6). The C-terminal domain of TOX3 particularly stands out from the rest of the family, as it is glutamine-rich.

TOX expression is tissue- and stage-specific (although not T cell specific), with the greatest expression observed in the thymus and markedly reduced expression in peripheral lymphoid tissues (2). Detailed expression of other TOX family members, however, has been

characterized very little. TOX2 has been reported to be expressed in rat ovarian granulosa cells (7) and mouse retina (8). In addition, the inventor found expression of *Tox4* mRNA fairly widespread (5). Overall, it appears that despite some overlap in tissue expression, different TOX family members may play greater or lesser roles in specific tissues. The inventor previously discovered that even in the mouse brain, where *Tox* and *Tox3* mRNA are both expressed, they have non-overlapping patterns of expression.

The inventor characterized mice deficient in TOX and showed that this nuclear factor is required for development of a number of key aspects of the immune system including development of CD4 T cells, lymph nodes, and NK cells (9). Together the data indicate that TOX is a key regulator of precursor cell differentiation in various contexts, presumably by regulating gene expression. These results make it likely that other TOX family members will also be found to play important regulatory roles in various cellular contexts. The biological function of other TOX-family members *in vivo* has not been characterized. Recently, expression of TOX3 has been reported to link calcium signaling to c-fos regulation in isolated neurons (6).

SUMMARY OF THE INVENTION

In one embodiment, the invention includes methods of determining the susceptibility of cancer in a subject comprising: obtaining a sample from the subject, determining the expression level of a biomarker in the sample, comparing the expression level of the biomarker with the expression level of the biomarker from a healthy individual. If the biomarker expression level of the sample is greater than the level of expression of the same biomarker in the healthy individual, then it is indicative that the subject has an increased susceptiblity to cancer. The sample can be a tissue or cell. The biomarker is TOX3. Expression levels of TOX3 may be determined by analyzing the transcription level of TOX3 or by analyzing the protein level of TOX3. The method can be used to determine an increase in the susceptibility to breast cancer or more specifically a subset of breast cancer. Examples of subsets of breast cancer include but are not limited to luminal A, luminal B, Erbb2-enriched, basal, or normal-breast-like cancer.

In another embodiment, the invention includes methods of treating cancer in a subject comprising: administering a TOX3 modulator to the subject in an amount that is sufficient to reduce or inhibit TOX 3 expression in the cancer cells and inhibit the progression of the cancer. The TOX3 modulator can be an antisense molecule, antibody, antibody fragment, polypeptide,

or a small molecule. The cancer to be treated may be breast cancer. More preferably, the cancer is a subset of breast cancer, including but not limited to, luminal A, luminal B, Erbb2-enriched, basal, or normal-breast-like cancer.

In a related embodiment, the invention includes methods of reducing the likelihood of the development of cancer in a subject comprising: administering a TOX3 modulator to the subject in an amount that is sufficient to reduce or inhibit TOX 3 expression in the cancer cells and inhibit the development of cancer. The TOX3 modulator is an antisense molecule, antibody, antibody fragment, polypeptide, or a small molecule. The method can be used to reduce the likelihood of the development of breast cancer or more specifically a subset of breast cancer. Examples of subsets of breast cancer include but are not limited to luminal A, luminal B, Erbb2-enriched, basal, or normal-breast-like cancer.

In one embodiment, the invention includes isolated peptides that are capable of binding to TOX3 and modulating TOX3 expression. TOX3 expression can be decreased or inhibited. The peptide comprises SEQ. ID. NO: 1 or SEQ. ID. NO: 2. The isolated peptide are also used in a pharmaceutical composition that can be used to treat or prevent the likelihood of a subject developing cancer.

In one embodiment, the invention includes isolated antibodies that are capable of binding to TOX3 and modulating TOX3 expression. TOX3 expression can be decreased or inhibited. The antibodies are derived from peptides comprising SEQ. ID. NO: 1 or SEQ. ID. NO: 2. The isolated antibodies are also used in a pharmaceutical composition that can be used to treat or prevent the likelihood of a subject developing cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Microarray of TOX3 expression. **(A)** Data analyzing 51 breast cancer cell lines **(B)** data analyzing 118 aggressively-treated early stage breast tumors (see text). Both sets of data are shown as heat maps, ordered based on expression of the TOX3 gene, with high to low expression shown bottom to top.

Figure 2. Graphical representation of TOX and TOX3 expression in human cell lines using quantitative RT-PCR for expression of TOX or TOX3, as indicated, in T cell or breast cancer cell lines, normalized to expression of the MRPL19 housekeeping gene.

Figure 3. Graphical representation TOX3 expression in breast cancer. **(A)** Quantitative RT-PCR experiment for TOX3 expression in breast tumors, as in **Figure 2**. Normal breast tissue samples are in blue, breast cancer cell line ZR75-1 in yellow, and tumor samples in red. The normal sample derived from a non-cancer patient was arbitrarily set to 1. **(B)** Shows the same data plotted with a reduced scale on the y-axis.

Figure 4. TOX3 splice form variants that predominate in breast cancer. **(A)** The predicted amino acid sequences of the N-terminus encoded by variant forms of TOX3 mRNA. Gray indicates identity (variants are identical throughout subsequent C-terminal sequence, not shown). **(B)** Gel of variant forms of TOX3 from breast cancer cell lines ZR75-1 and BC.MFT.

DETAILED DESCRIPTION OF THE INVENTION

TOX3 is a member of the TOX family and is a sub-group of the HMG-box of proteins, which are involved in binding to chromatin and altering transcription. Based on several key observations, the inventor has realized that TOX3 (also known as TNRC9) has a role in breast cancer. Gene expression analysis was used to compare primary breast tumors from patients that were lymph node negative at the time of diagnosis but that had experienced relapse either to bone or to other parts of the body (10). Among the genes found to be upregulated in tumors that metastasized to bone was TOX3. More recently, two articles examined genome-wide association studies to identify breast cancer susceptibility loci, in particular searching for common low-penetrance alleles that would be associated with disease (11, 12). Both articles reported that SNPs linked to TOX3 were associated with increased breast cancer risk. Increased disease risk appears to be most associated with estrogen receptor positive tumors (11, 13). Estrogen positivity is a strong histopathological predictor of bone metastases, yielding a link between the two studies above (14). Among a European population the risk allele was present in a homozygous state at a 7% frequency and imparted a 1.64 greater disease risk (11). Among a small cohort of patients with familial breast cancer without BRCA1 and BRCA2 mutations, homozygotes for the TOX3 minor allele had a 2.4-fold increased cancer risk (15). However, TOX3 has not been associated with increased risk of ovarian cancer (17), suggesting the potential for tissue specificity in its mode of action.

A retrospective study of microarray data reported that by ANOVA analysis TOX3 mRNA was upregulated in luminal A, luminal B, and ErbB2+ molecular subtypes of breast

cancer tumors and downregulated in basal-like tumors (18), suggesting that TOX3 may not only play a biologically-relevant role in certain tumors but that expression may also have some value as a biomarker. However, only statistical analysis was reported and not quantitative data on levels of expression. In another analysis of breast cancer patients, individuals homozygous for the TOX3 locus variant were more likely to be diagnosed before the age of 60 than those not homozygous for the TOX3 locus variant (19). Interestingly, minor allele frequencies for the TOX3-associated SNP were elevated among 40 human breast cancer cell lines (20). Surprisingly, however, there was no correlation between the allele and actual expression of TOX3 mRNA, although the range of expression levels for TOX3 among individual cell lines was quite broad in this report. According to particular aspects, a lack of an association between haplotype and TOX3 expression in these cell lines is due to TOX3's role during induction but not maintenance of tumors or additional changes in these cell lines as a result of extensive propagation in culture.

As mentioned above, BRCA1 or BRCA2 mutations can impart a very high risk for breast cancer. Interestingly, even among BRCA1 and BRCA2 mutation carriers, the minor allele SNP linked to TOX3 can impart an increased risk for disease, particularly for BRCA2 mutation carriers (15, 21). This highlights the potential for additive or synergistic effects of disease susceptibility loci. In addition, the fact that cancers with BRCA2 mutations are more likely to be estrogen receptor positive than those with BRCA1 mutations (22), is also consistent with the stronger association of TOX3 variation with estrogen receptor positive disease. At least in a cell line, however, expression of TOX3 itself was not estradiol responsive (23).

Based on the above studies, the inventors believe that TOX3 plays a role in breast cancer and has profound effects on regulation of cellular activity during initiation, maintenance, or spread of cancer.

One embodiment the present invention identifies the involvement of TOX3 in certain subtypes of breast cancer. These subtypes include, but are not limited to luminal A, luminal B, Erbb2-enriched, basal, and normal-breast-like cancers.

One embodiment the present invention provides for TOX3 biomarkers that can be used to determine a subject's susceptibility to certain types of cancer. Examples of biomarkers include nucleic acids and/or proteins of TOX3.

In another embodiment the present invention provides modulators of TOX3. Inventive modulators include, but are not limited to, antisense molecules, antibodies or antibody fragments, proteins or polypeptides as well as small molecules.

The present invention is also directed to a kit to treat cancer. The kit is also useful for practicing the inventive method of determining an increase in the susceptibility of cancer in a subject. The kit can also be useful for practicing the inventive method of reducing the likelihood of the development of cancer in a subject. The kit is an assemblage of materials or components, including at least one of the inventive compositions. Thus, in some embodiments the kit contains a composition including peptides and/or antibodies of the present invention.

The exact nature of the components configured in the inventive kit depends on its intended purpose. For example, some embodiments are configured for the purpose of treating cancer patients. In one embodiment, the kit is configured particularly for the purpose of treating mammalian subjects. In another embodiment, the kit is configured particularly for the purpose of treating human subjects. In further embodiments, the kit is configured for veterinary applications, treating subjects such as, but not limited to, farm animals, domestic animals, and laboratory animals.

Instructions for use may be included in the kit. "Instructions for use" typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as to treat ischemia. Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, ban daging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging material(s). As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as inventive compositions and the like. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. As used herein, the term "package" refers to a suitable solid matrix or material such as glass, plastic, paper, foil, and the

like, capable of holding the individual kit components. Thus, for example, a package can be a glass vial used to contain suitable quantities of an inventive composition containing a polyphenol analog. The packaging material generally has an external label which indicates the contents and/or purpose of the kit and/or its components.

According to additional aspects, the present invention provides for the use of TOX3 nucleic acids and/or proteins to detect an increase in susceptibility to certain types of cancers, e.g. breast cancer. Additional aspects relate to transgenic mice that either overexpress or inducibly express the TOX3 protein.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described. For purposes of the present invention, the following terms are defined below.

A "biomarker" as used herein refers to a molecular indicator that is associated with a particular pathological or physiological state. The "biomarker" as used herein is a molecular indicator, for cancer. More specifically, the biomarker is an indicator for subsets of breast cancer that can be linked to TOX3 expression. Examples of "biomarkers" include but or not limited to including a protein, polynucleotide, allele, or transcript of TOX3. A "biomarker" of the present invention may be detected in a tumor, tissue, or cell sample.

"Cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to breast, osteosarcoma, cancer, colon cancer, lung cancer, prostate cancer, hepatocellular cancer, gastric cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, and bladder cancer, cancer of the urinary tract, thyroid cancer, renal cancer, carcinoma, melanoma, head and neck cancer, brain cancer, or any TOX3 related cancer. Preferably, the cancer is breast cancer.

"Treatment" and "treating," as used herein refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent, slow down and/or lessen the disease even if the treatment is ultimately unsuccessful. Those in need of treatment include those already with cancer as well as those prone to have cancer or those in whom cancer is to be prevented. For example, in cancer treatment, a therapeutic agent or modulator may directly decrease the pathology of cancer cells, or render the tumor cells more susceptible to treatment by

other therapeutic agents or by the subject's own immune system.

"Therarpeutic agents" or "TOX 3 modulators" reduce or eliminate TOX3 expression in cells. Examples of therapeutic agents or modulators include, but are not limited to antisense molecules, antibodies or antibody fragments, proteins or polypeptides as well as small molecules.

TOX3 modulators and compositions comprising one or more TOX3 modulator as well as methods that employ these inventive inhibitors in *in vivo*, *ex vivo*, and *in vitro* applications where it is advantageous to reduce or eliminate the expression or activity of TOX3 or a functionally-downstream molecule. TOX3 modulators may find use as drugs for supplementing cancer therapeutics and other agents. TOX3 modulators may also find use in other diseases of hyperproliferation.

Compositions may be administered parenterally, topically, orally, or locally for therapeutic treatment. Preferably, the compositions are administered orally or parenterally, *i.e.,* intravenously, intraperitoneally, intradermally, or intramuscularly.

Inventive compositions will include one or more TOX3 modulator and may further comprise a pharmaceutically-acceptable carrier or excipient. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include other proteins for enhanced stability, such as albumin, lipoprotein, globulin, etc., subjected to mild chemical modifications or the like.

TOX3 modulators useful in the treatment or prevention of disease in mammals will often be prepared substantially free of naturally-occurring immunoglobulins or other biological molecules. Preferred TOX3 modulators will also exhibit minimal toxicity when administered to a mammal.

The compositions of the invention may be sterilized by conventional, well-known sterilization techniques. The resulting solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, and stabilizers (*e.g.*, 1-20% maltose, etc.).

The selection of the appropriate method for administering TOX3 modulators of the present invention will depend on the nature of the application envisioned as well as the nature of the TOX3 modulator. Thus, for example, the precise methodology for administering a TOX3 modulator will depend upon whether it is an antisense molecule, a protein and/or peptide, an antibody or antibody fragment, or a small molecule. Other considerations include, for example, whether the TOX3 modulator will be used to regulate tumor cell initiation, growth, invasion, or metastasis, or as an adjunct to other cancer therapeutics.

A variety of methods are available in the art for the administration of antisense molecules. Exemplary methods include gene delivery techniques, including both viral and non-viral based methods as well as liposome-mediated delivery methods.

Gene delivery methodologies will be effective to, for example, reduce tumor cell proliferation, or supplement radiation and/or chemotherapeutic treatment of tumors. *See*, Wheldon, T.E. et al., *Radiother Oncol 48(1)*:5-13 (1998) (gene delivery methodologies for enhancement of fractionated radiotherapy). By these methodologies, substantial therapeutic benefit may be achieved despite transfection efficiencies significantly less than 100%, transient retention of the transfected inhibitor, and/or existence of a subpopulation of target cells refractory to therapy.

Alternatively, gene delivery methodology may be used to directly knock-out endogenous TOX3 within tumor cells. For example, the TOX3 gene may be targeted by transfection of a gene delivery vector carrying a TOX3 modulator. Preferential transfection into or expression within tumor cells may be achieved through use of a tissue-specific or cell cycle-specific promoter, such as, *e.g.*, promoters for BRCA2 or for immunoglobulin genes (Vile, R.G. et al., *Cancer Res. 53*:962-967 (1993) and Vile, R.G., *Semin. Cancer Biol. 5*:437-443 (1994)) or through the use of trophic viruses that are confined to particular organs or structures, such as, *e.g.*, a replication selective and neurotrophic virus that can only infect proliferating cells in the central nervous system.

Thus, to achieve therapeutic benefit, TOX3 within the tumor cells should be preferentially modulated. This can be accomplished by transfecting a gene expressing a TOX3 inhibitor, a TOX3 antisense molecule, a TOX3 gene-specific repressor, or an inhibitor of the protein product of the TOX3 gene.

As used herein, the phrase "gene delivery vector" refers generally to a nucleic acid construct that carries and, within certain embodiments, is capable of directing the expression of an antisense molecule of interest, as described in, for example, *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, Ch. 21, pp. 555-590 (*ed.* B.P. Glick and J.J. Pasternak, 2nd ed. 1998); Jolly, *Cancer Gene Ther. 1*:51-64 (1994); Kimura, *Human Gene Ther. 5*:845-852 (1994); Connelly, *Human Gene Ther. 6*:185-193 (1995); and Kaplitt, *Nat. Gen. 6*:148-153 (1994).

A number of virus- and non-virus-based gene delivery vector systems have been described that are suitable for the administration of TOX3 modulators. Virus-based gene delivery systems include, but are not limited to, retrovirus such as Moloney murine leukemia virus, spumaviruses, and lentiviruses; adenovirus; adeno-associated virus; and herpes-simplex virus vector systems. Viruses of each type are readily available from depositories or collections such as the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, Virginia 20110-2209) or may be isolated from known sources using commonly available materials and techniques.

The gene delivery vector systems of the present invention will find applications both in *in vivo* as well as *ex vivo* therapeutic regimens. Methods for gene delivery systems are well known in the art (*e.g.* retroviral gene delivery vector systems, adeno-associated viral gene delivery vector systems, and non-viral gene delivery vectors).

"Therapeutically effective amount" as used herein refers to that amount which is capable of achieving beneficial results in a patient with cancer; in particular a patient with breast. A therapeutically effective amount can be determined on an individual basis and will be based, at least in part, on consideration of the physiological characteristics of the mammal, the type of delivery system or therapeutic technique used and the time of administration relative to the progression of the disease.

The term "overexpression," as used herein refers to overexpression of a gene and/or its encoded protein in a cell, such as a tumor cell. A tumor cell that "overexpresses" a protein is one that has significantly higher levels of that protein compared to a normal cell of the same tissue type.

As used herein a "polypeptide" or "peptide" comprising at least a part (i.e., the whole or a part) of the amino acid sequence may be used as an antigen. More specifically, the present

invention provides for a peptide which is capable of binding TOX3. The peptide may be prepared by chemical synthesis or biochemical synthesis using Escherichia coli or the like. Methods well-known in those skilled in the art may be used for the synthesis. In addition to the baculovirus expression system, other suitable bacterial or yeast expression systems may be employed for the expression of TOX3 protein or polypeptides thereof. As with the baculovirus system, it may be advantageous to utilize one of the commercially-available affinity tags to facilitate purification prior to inoculation of the animals. Thus, the TOX3 cDNA or fragment thereof may be isolated by, *e.g.*, agarose gel purification and ligated in frame with a suitable tag protein such as 6-His, glutathione-S-transferase (GST) or other such readily available affinity tag. *See, e.g., Molecular Biotechnology: Principles and Applications of Recombinant DNA*, ASM Press pp. 160-161 (*ed.* Glick, B.R. and Pasternak, J.J. 1998).

According to certain embodiments, TOX3 peptides can be used to produce antibodies or similar TOX3 binding proteins. TOX3 peptides useful in producing antibodies can be made from the TOX3 polypeptide of SEQ ID NO:1 containing amino acids from about position 1 to about 576, from about position 1 to about 238, from about position 1 to about 150, from about position 2 to about 238, and from about position 2 to about 150. TOX3 peptides useful in producing antibodies can be made from the N-terminal portion of TOX3 polypeptide (SEQ ID NO:2) containing between 5 to 10 consecutive amino acids, containing between 5 to 15 consecutive amino acids, containing between 5 to 20 consecutive amino acids, containing between 5 to 25 consecutive amino acids, containing between 5 to 30 consecutive amino acids, containing between 5 to 35 consecutive amino acids, containing between 5 to 40 consecutive amino acids, containing between 5 to 45 consecutive amino acids, containing between 5 to 50 consecutive amino acids, containing between 5 to 55 consecutive amino acids, containing between 5 to 60 consecutive amino acids, containing between 5 to 65 consecutive amino acids, containing between 5 to 70 consecutive amino acids, containing between 5 to 75 consecutive amino acids, containing between 5 to 80 consecutive amino acids, containing between 5 to 85 consecutive amino acids, containing between 5 to 90 consecutive amino acids, containing between 5 to 95 consecutive amino acids, containing between 5 to 100 consecutive amino acids, containing between 5 to 105 consecutive amino acids, containing between 5 to 110 consecutive amino acids, containing between 5 to 115 consecutive amino acids, containing between 5 to 120 consecutive amino acids, containing between 5 to 125 consecutive amino acids, containing

between 5 to 150 consecutive amino acids, containing between 5 to 175 consecutive amino acids, containing between 5 to 200 consecutive amino acids, and containing between 5 to 238 consecutive amino acids.

According to certain embodiments, TOX3 peptides can be used to produce antibodies or similar TOX3 binding proteins. TOX3 peptides useful in producing antibodies can be made from the TOX3 polypeptide containing amino acids. Examples of inventive peptides of length X (in amino acids), as indicated by polypeptide positions with reference to, *e.g.*, SEQ ID NO:1, include those corresponding to sets of consecutively overlapping peptides of length X, where the peptides within each consecutively overlapping set (corresponding to a given X value) are defined as the finite set of Z peptides from amino acid positions:

n to (n + (X-1));

where n=1, 2, 3,…(Y-(X-1));

where Y equals the length (amino acid or base pairs); where X equals the common length (in amino acid) of each peptide in the set (*e.g.*, X=10 for a set of consecutively overlapping 10-mers); and where the number (Z) of consecutively overlapping oligomers of length X for a given sequence of length Y is equal to Y-(X-1).

Examples of inventive 10-mer peptide within a sequence of length 576 amino acid residues include the following set of 576 oligomers, indicated by polypeptide positions 1-20, 2-21, 3-22, 4-23, 5-24 to 566-576.

The present invention encompasses, for each of SEQ ID NOS:1 and SEQ ID NO:2, multiple consecutively overlapping sets of peptides or modified peptides of length X, where, *e.g.*, X= 9, 10, 17, 20, 22, 23, 25, 27, 30, or 35 amino acids.

Preferred sets of such peptides or modified peptides of length X are those consecutively overlapping sets of oligomers corresponding to SEQ ID NOS:1 and SEQ ID NO:2.

According to preferred aspects, the inventor has developed an assay to detect the presence and level of TOX3, either an allele, a transcript, or protein as it relates to certain subsets of cancers (*e.g.* breast cancers). The inventor has determined a link between TOX3 and certain subsets of cancers (*e.g.* breast cancers). According to particular aspects, the current invention encompasses a kit for detecting TOX3 polynucleotides and/or proteins.

The term “antibody,” as used herein, refers to one which binds specifically to TOX3 meaning that it does not significantly cross-react with other proteins. “Antibodies” (Abs) is used

in the broadest sense and specifically covers, without limitation, intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

According to certain embodiments, peptides can be used to produce antibodies or similar TOX3 binding proteins. According to further embodiments, antibodies are useful to detect the presence of TOX3 in cells and tissue samples.

The present invention includes antibodies and/or antibody fragments that are effective in binding to TOX3. Suitable antibodies may be monoclonal or polyclonal antibodies. Antibodies may be derived by conventional hybridoma-based methodology, from antisera isolated from TOX3 inoculated animals or through recombinant DNA technology. Alternatively, inventive antibodies or antibody fragments may be identified *in vitro* by use of one or more of the readily available phage display libraries. Exemplary methods are well known in the art.

One embodiment of the present invention includes monoclonal antibodies that may be produced as follows. TOX3 protein may be produced, for example, by expression of TOX3 cDNA in a Baculovirus-based system. By this method, TOX3 cDNA or a fragment thereof is ligated into a suitable plasmid vector that is subsequently used to transfect Sf9 cells to facilitate protein production. In addition, it may be advantageous to incorporate an epitope tag or other moiety to facilitate affinity purification of the TOX3 protein. Clones of Sf9 cells expressing TOX3 are identified, *e.g.*, by enzyme-linked immunosorbant assay (ELISA), lysates are prepared and the TOX3 protein purified by affinity chromatography and the purified protein is injected, intraperitoneally, into BALB/c mice to induce antibody production. It may be advantageous to add an adjuvant, such as Freund's adjuvant, to increase the resulting immune response.

Serum is tested for the production of specific antibodies and spleen cells from animals having a positive specific antibody titer are used for cell fusions with myeloma cells to generate hybridoma clones. Supernatants derived from hybridoma clones are tested for the presence of monoclonal antibodies having specificity against TOX3. For a general description of monoclonal antibody methodology, *see, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988).

Polynucleic Acid Detection. There are many techniques readily available in the field for detecting the presence, absence, and/or level of an allele, transcript, or other biomarker,

including mRNA microarrays. For example, enzymatic amplification of nucleic acid from an individual may be used to obtain nucleic acid for subsequent analysis (*e.g.*, polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT PCR)). The presence or absence of allele, transcript or other biomarker may also be determined directly from the individual's nucleic acid without enzymatic amplification.

Analysis of the nucleic acid from an individual, whether amplified or not, may be performed using any of various techniques. Useful techniques include, without limitation, PCR-based analysis, sequence analysis, and electrophoretic analysis. As used herein, the term "nucleic acid" means a polynucleotide such as a single- or double-stranded DNA or RNA molecule including, for example, genomic DNA, cDNA, and mRNA. The term nucleic acid encompasses nucleic acid molecules of both natural and synthetic origin as well as molecules of linear, circular, or branched configuration representing either the sense or antisense strand, or both, of a native nucleic acid molecule.

Protein Detection and/or Biomarker Detection. There are many techniques readily available in the field for detecting the presence or absence of polypeptides or other biomarkers, including protein microarrays. For example, some of the detection paradigms that can be employed to this end include optical methods, electrochemical methods (voltametry and amperometry techniques), atomic force microscopy, and radio frequency methods, *e.g.*, multipolar resonance spectroscopy. Illustrative of optical methods, in addition to microscopy, both confocal and non-confocal, are detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (*e.g.*, surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method, or interferometry).

Similarly, there are any numbers of techniques that may be employed to isolate and/or fractionate biomarkers. For example, a biomarker may be captured using biospecific capture reagents, such as antibodies, aptamers, or antibodies that recognize the biomarker and modified forms of it. This method could also result in the capture of protein interactors that are bound to the proteins or that are otherwise recognized by antibodies and that, themselves, can be biomarkers. The biospecific capture reagents may also be bound to a solid phase. Then, the captured proteins can be detected by SELDI mass spectrometry or by eluting the proteins from the capture reagent and detecting the eluted proteins by traditional MALDI or by SELDI. One

example of SELDI is called "affinity capture mass spectrometry," or "Surface-Enhanced Affinity Capture" or "SEAC," which involves the use of probes that have a material on the probe surface that captures analytes through a non-covalent affinity interaction (adsorption) between the material and the analyte. Some examples of mass spectrometers are time-of-flight, magnetic sector, quadrupole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer, and hybrids of these.

Alternatively, for example, the presence of biomarkers such as polypeptides may be detected using traditional immunoassay techniques. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the analytes. The assay may be designed to specifically distinguish protein and modified forms of protein, which can be done by employing a sandwich assay in which a first antibody captures more than one form and a second distinctly-labeled antibody specifically binds and provide distinct detection of the various forms. Antibodies can be produced by immunizing animals with the biomolecules. Traditional immunoassays may also include sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays.

Prior to detection, biomarkers may also be fractionated to isolate them from other components in a solution or blood that may interfere with detection. Fractionation may include platelet isolation from other blood components, sub-cellular fractionation of platelet components, and/or fractionation of the desired biomarkers from other biomolecules found in platelets using techniques such as chromatography, affinity purification, 1D and 2D mapping, and other methodologies for purification known to those of skill in the art. In one embodiment, a sample is analyzed by means of a biochip. Biochips generally comprise solid substrates and have a generally planar surface to which a capture reagent (also called an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound there.

Microarray analysis. The inventor examined existing microarray data and determined the presence of TOX3 transcript in breast cancer cell lines and tumors. This analysis is a critical first step to take these studies beyond SNP associations. It should be noted that the expression of TOX3 appears to be relatively restricted in normal tissues, with highest levels of expression in fetal brain (BioGPS; http://biogps.gnf.org). As shown in **Figure 1**, the inventor analyzed two microarray studies (24, 25), organizing heat maps based on expression of TOX3 (UCSC Cancer

Genomics Browser). Interestingly, expression of TOX3 dramatically subsets both cell lines and tumors into high and low expressers. The inventor determined that TOX3 may act as a novel marker to subset tumors. The inventor compared this same ordered data set with expression of other select genes, including TOX itself. Results from this analysis include a number of interesting and surprising points. In both tumors and cell lines there appears to be an inverse correlation between expression of TOX and TOX3. According to particular aspects, based on the near identity of the DNA-binding domains and the differences elsewhere in these proteins, these two family members in essence act as dominant negative mutants of each other (i.e. compete for DNA binding but have different functions). In addition, there is a positive, although not absolute, correlation between estrogen receptor (ESR1) expression and TOX3 expression. This would be consistent with a more dominant role for TOX3 in ER+ disease, as discussed above. The transcription factor GATA3 is often coexpressed with estrogen receptor alpha in breast cancer cells and is one molecular marker of the luminal A subtype of breast cancer (26, 27). In addition, there is an overall positive correlation between GATA3 and TOX3 expression in the cell lines studied. In general, there is a positive correlation between TOX3 expression and ERBB2 and GRB7 expression (the latter analyzed for tumors). GRB7 is an SH2-domain adaptor protein that binds to receptor tyrosine kinases and is genetically linked to the ERBB2 (HER2/neu) proto-oncogene. ERBB2 and GRB7 are commonly co-amplified in breast cancers. Interestingly, in an analysis that examined the expression of CD44 and TOX3, the inventor discovered that there was an inverse correlation between expression of CD44 and TOX3, which is consistent with poor expression of TOX3 in the basal subtype (CD44 has been suggested as one marker for cancer stem cells and expression of CD44 may be associated with basal-like disease (28)). Finally, the inventor found no association with c-fos, distinguishing the possible action of TOX3 in breast cancer from that observed in neurons (6). There is certainly cellular heterogeneity within all these samples and thus expression of these genes on a per cell basis is unknown.

TOX3 gene expression in breast cancer cell lines and tumors. The inventor examined directly whether TOX3 is expressed in breast cancer cells using quantitative RT-PCR. MOLT4, an oft-studied human acute lymphoblastic leukemia cell line highly expressed TOX but not TOX3 **(Figure 2)**. In contrast, three oft-studied breast cancer cell lines expressed TOX3 to various levels, but did not express TOX **(Figure 2)**. Interestingly, ZR75-1, the highest expresser,

is an ER+ luminal subtype of breast cancer cell (24). These results are consistent with the role of TOX in the immune system and the role of TOX3 in breast cancer. Moreover, since the tissue microenvironment can greatly influence cancer cells and microarray analysis of tumors includes a heterogeneous population of cells, this result confirms expression of TOX3 by the cancer cell itself.

EXAMPLES

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

EXAMPLE 1

TOX3 expression in subsets of breast cancer tumors

TOX3 expression was analyzed by qRT-PCR in RNA derived from 8 breast cancer tumors and 2 normal breast tissue samples; all samples were obtained from a commercial source. The breast cancer RNA samples were pre-selected by the following minimal criteria; the patients were (1) female, (2) White/Caucasian, and (3) had been diagnosed with estrogen receptor positive disease. All tumors were stage II or stage III infiltrating ducal carcinomas, from patients aged 41 to 78 years of age. For the two normal samples, one was from a 46-year-old patient who did not have cancer and one was derived from normal tissue from a 73-year-old patient diagnosed with stage II breast cancer. The inventor normalized the results to the sample from the non-cancer patient, arbitrarily assigning a value of 1 **(Figure 3)**. Surprisingly, the second "normal" sample had a 7-fold increase in TOX3 expression, similar to that seen in the ZR75-1 breast cancer cell line. According to certain embodiments, this result is related to the fact that this sample was derived from a cancer patient, and thus reflects an inherent variability in normal expression of this gene. Interestingly, there was great variability in expression of TOX3 among these tumor samples, ranging from well below that even detected in the normal tissue to greater than 100-fold upregulated in one tumor sample (MFT). This is reminiscent of the microarray data described above. According to certain embodiments, these expression differences correlate

with TOX3 locus allelic differences. Intriguingly, though, only two of the eight patients had reported that their mothers also had breast cancer, and these were among the top three expressers of TOX3 (samples MFT and SKBY).

EXAMPLE 2

Predominant TOX3 transcript in breast cancer cell lines and tumors

Two TOX3 transcripts have been reported that encode different N-terminal ends of the protein **(Figure 4A)**. The shorter variant 2 includes an alternative exon within the otherwise first intron of the TOX3 locus. A common 3' primer and distinct 5' primers that allowed the two transcripts to be distinguished were designed. These were used in end point RT-PCR on RNA derived from the ZR75.1 cell line and the MFT breast cancer tumor (see above). Results indicated that variant 1 is the predominant transcript, even in primary tumor cells **(Figure 4B)**. Thus, the *in vivo* work will focus on this form of the protein.

EXAMPLE 3

Transcriptome analysis of tumors with high TOX3 expression

Breast cancer tumors that highly express TOX3 were analyzed using transcriptome. Global expression analysis has enabled classification of molecularly-defined subsets of cancers. For breast cancer, five subtypes: luminal A, luminal B, Erbb2-enriched, basal, and normal-breast-like, were proposed based on gene expression clusters that were relatively stable over time and have some clinical correlations (26, 29). However, such classifications also belie the complexity and heterogeneity of the disease even within subtypes. Indeed, a subsequent large real-time RT-PCR analysis proposed twelve disease subtypes, based on expression of 47 genes (30). In addition, in terms of individual genes, this classification does not necessarily separate out important functional components of tumor formation or maintenance, which may be shared among subtypes, from useful but not necessarily causative biomarkers. As elaborated above, the inventor proposes to identify TOX3 as a disease susceptibility locus.

The data indicates that high level of expression of TOX3 may not fit neatly into otherwise defined subtypes. To address this issue, global gene expression analysis by microarray was performed to compare tumors with very high TOX3 expression and tumors with very low TOX3 expression **(Figure 3)**. While a small number of samples cannot be used to define a new

molecular subtype, the inventor can use this data to narrow the number of genes that may be proximal gene targets of TOX3. In addition, the data for expression of genes that have been previously used to define subtypes is examined to see how these samples fall within those groups. While much microarray analysis is dependent on calls of relatively modest changes in gene expression, the inventor will take a much more stringent approach. As TOX3 is a transcriptional regulator, the inventor has implemented a simplistic approach labeling genes that are highly expressed in the absence or presence of TOX3 as unlikely TOX3 gene targets and thus will not be investigated further.

Those genes whose expression is shared in TOX3 high cells (or show correlation with levels of TOX3 expression) and whose expression is absent or low in tumors that express little TOX3 are first examined. For that reason, quantitative data on TOX3 from the inventor's qRT-PCR are used initially. The high degree of genome annotation makes it likely that sorting through even a larger number of genes looking for known regulators of cell growth, survival, differentiation, or gene regulation that are good candidates for follow-up as potential TOX3 gene targets in the context of breast cancer.

EXAMPLE 4

Molecular and cellular effects of TOX3 expression

The molecular and cellular effects of manipulating TOX3 expression in breast cancer are examined using the ZR75-1 cell line that expresses TOX3 in a complementary approach. ZR75-1 cells previously have been reported to be transfectable and susceptible to siRNA-mediated knockdown (31). Thus using siRNA-mediated TOX3 knockdown the inventor can determine directly whether expression of candidate genes is modulated by expression of TOX3. Therefore, this analysis is accomplished on a global level via microarray, and is used as comparison with the data set obtained from primary tumor samples as above. Importantly, the inventor determined if knockdown of TOX3 alters the growth, adhesion, or morphologic characteristics of this cell line, including the migration and invasion properties of the cells as assessed *in vitro* (31). Similarly, TOX3 is over-expressed in these cells to test for complementary changes in cellular behavior or gene expression. If differences are detected upon loss of TOX3 in these cells, then the cells can be used to test if TOX might act as a dominant negative of TOX3 function in this cellular context. This provides the basis for thinking of ways to manipulate

TOX3 activity, rather than expression. Together, these studies represent a powerful approach to identify gene targets of TOX3 and correlate that with cell behavior, as well as expression in primary tumors. This result indicates that TOX3 activation can be manipulated by overexpressing TOX in breast cancer tumors.

EXAMPLE 5

Production of anti-TOX3 antibodies

A rabbit polyclonal antibody against TOX was produced utilizing recent advances in production of rabbit monoclonal antibodies (rabbit antibodies are often of much higher affinity than those produced in other species).

An anti-TOX3 antibody is invaluable in characterizing expression of TOX3 in breast cancer tumors. There are large numbers of well-characterized tissue arrays available for breast cancer (*i.e.* of known histological appearance and grade, metastatic properties, hormone receptor expression, and Her2 expression), some including adjacent normal tissue. Thus, analogous to the molecular subtyping approach, the inventor determines the expression pattern of TOX in tumors at the protein level. Since these are fixed samples, initially an anti-peptide antibody that is likely to recognize denatured protein (and thus will also be useful for immunoblotting) is used. In addition, the inventor used the N-terminal regions of the protein as a peptide source, which allows for discrimination from other family members, avoiding the highly conserved DNA binding domain and the Q-rich C-terminal domain. In addition, the inventor utilized native TOX3 peptide to produce an antibody that binds to the native form of TOX3 and thus is useful as an *in vivo* reagent.

EXAMPLE 6

Analysis of TOX3 protein expression

TOX3 protein levels in breast cancer tumors were analyzed using anti-TOX3 antibodies. These studies complement those performed above at the level of gene expression, in order to understand the distinction between tumors that express or do not express TOX3. Indeed, protein expression profiling has also been undertaken as a method to subtype breast cancers (32). Analysis of tissue arrays that include normal breast tissue as well as normal tumor-adjacent breast tissue is also of great interest. As shown above, the inventor found variability in TOX3

expression between two ostensibly normal breast tissue samples, with higher expression from tissue derived from a breast cancer patient. Thus, up regulation of TOX3 is examined to show that this up regulation leads to tumorigenesis. Thus, expression of TOX3 in normal tissue adjacent to a TOX3 high-expressing tumor is also examined.

EXAMPLE 7

Animal model for TOX3 expression

The role of TOX3 in initiating tumorigenesis was examined using a novel *in vivo* animal model for expression of TOX3 in breast tissue. The above Examples focus on a continuing role for TOX3 in tumor maintenance or progression. However, the most significant role for TOX3 may be as an initiator of disease rather than maintenance of the tumor phenotype. Indeed, TOX itself plays a transient role during development of the immune system. Thus, creation of an *in vivo* model system to allow mechanistic dissection of the role of TOX3 in breast cancer, including tumor induction is necessary. Data disclosed herein suggest that overexpression rather than mutation or loss of TOX3 likely is involved in disease; thus a conditional deletion or mutation of the protein *in vivo* is generated.

To determine whether alterations in TOX3 expression can directly induce cancerous changes in breast tissue or increase susceptibility to cancer, transgenic mice were produced that highly express this nuclear factor specifically in the breast. In addition, mice were generated with reversible transgene expression, such as with a tetracycline-inducible system, which gives finer control of timing of expression and allow experiments to distinguish a role for the protein in induction versus maintenance of tumors. To accomplish this, human TOX3 has been cloned from a highly-expressing tumor sample **(Figure 3)**, by high fidelity RT-PCR. For the reasons presented above **(Figure 4)**, primers were designed to clone TOX3 variant 1 for this purpose. From preliminary sequence analysis (based on independent PCR reactions) a single silent polymorphism in the coding region from this patient's tumor, when compared to the public database sequence, is found. This is consistent with the inventor's belief that mutations in the coding sequence of TOX3 are not associated with breast cancer, while level of expression is. This most basic issue has not been addressed in the context of breast cancer.

Transgenic mice were produced using a mouse mammary tumor virus (MMTV) promoter-based expression vector obtained from Dr. Windle (Virginia Commonwealth

University). The human TOX3 cDNA was inserted into exon 3 of the rabbit beta globin gene in this vector. There are no translation start sites in the globin sequences upstream of the cDNA, but there is an upstream exon/intron to allow splicing, necessary to obtain expression in transgenic mice. This vector gives high-level expression in breast tissue *in vivo* (33). Genetic background can play an important role in rodent tumor models, as it does in human disease. These transgenic mice were produced in the FVB/N strain, an easy strain for production of transgenic mice, but most importantly, also found to be susceptible to mammary tumor formation by expression of various genes under control of the MMTV promoter, including Erbb2 (Her2/neu) (as described herein), Hras1 (34), and Wnt1 (35).

First generation progeny of transgenic founder mice (MMTV-huTOX3 Tg) are screened for expression of TOX3 in breast tissue by RT-PCR, and via Western blot using the antibody produced as described herein. The rabbit β-globin untranslated sequence allows specific detection of the transgene, both at the level of RNA and DNA by PCR. Strains with high level of expression are bred for additional characterization. Two types of analyses are conducted. First, spontaneous tumor formation in these mice is examined. Second, the ability of TOX3 expression to modulate oncogene-driven tumor formation as described below is analyzed. These experiments are conducted simultaneously, as the former also acts as a control for the latter.

Mammary glands from MMTV-huTOX3 Tg and wildtype littermate virgin mice were examined at 5 weeks, 2 months, and 4 months postpartum to look for structural differences, and of course tumor formation. Since, TOX3 appears to be associated with ER+ disease it is also possible that there may be effects induced by hormone responsiveness. To test this, MMTV-huTOX3 Tg and wild type littermate mice during pregnancy are compared. The mouse mammary gland undergoes well-characterized differentiation changes during pregnancy and lactation that might affect TOX3 activity (36).

The ability of TOX3 to modulate the timing, incidence, phenotype, or progression of disease induced by Erbb2 (Her2/neu) is examined. Although the Erbb2 subtype is more associated with ER- disease, preliminary data has indicted that there can be overlap in expression of Erbb2 and TOX3 in breast cancer tumors **(Figure 1)**. There are two relevant transgenic mouse models, both on a FVB/N background, and both commercially available that express Erbb2 under the MMTV promoter and lead to disease. In one (37), expression of unactivated rat Erbb2 in mice leads to focal mammary tumors that first appear at 4 months. There is also a high

frequency of secondary metastatic disease in the lung. In the other model (38), expression of a transforming mutated version of rat Erbb2 results in multifocal disease involving the whole epithelium.

These mice are utilized to determine the level of up regulation of endogenous TOX3 in Erbb2-induced tumors. This foundational experiment was of great interest, particularly because the comparison between the incidence of expression of TOX3 in focal and the incidence of expression of TOX3 to multi-focal disease. In addition, these Tg lines are bred to MMTV-huTOX3 Tg produced as described herein, to determine if disease induction or progression is affected. Given the relatively long lag time for disease induction in Erbb2 Tg mice, TOX3 expression supplies a "second hit" to promote disease, it is reasonable to expect that this is detectable by a significant shift in kinetics. Other differences in disease due to expression of TOX3 may be detected.

EXAMPLE 8

TOX3 sequences

TOX high mobility group box family member 3 [Homo sapiens] (SEQ ID NO:1):

MDVRFYPAAAGDPASLDFAQCLGYYGYSKFGNNNNYMNMAEANNAFFAASEQTFHTP
SLGDEEFEIPPITPPPESDPALGMPDVLLPFQALSDPLPSQGSEFTPQFPPQSLDLPSITISRN
LVEQDGVLHSSGLHMDQSHTQVSQYRQDPSLIMRSIVHMTDAARSGVMPPAQLTTINQ
SQLSAQLGLNLGGASMPHTSPSPPASKSATPSPSSSINEEDADEANRAIGEKRAAPDSGK
KPKTPKKKKKKDPNEPQKPVSAYALFFRDTQAAIKGQNPNATFGEVSKIVASMWDSLG
EEQKQVYKRKTEAAKKEYLKALAAYRASLVSKAAAESAEAQTIRSVQQTLASTNLTSSL
LLNTPLSQHGTVSASPQTLQQSLPRSIAPKPLTMRLPMNQIVTSVTIAANMPSNIGAPLISS
MGTTMVGSAPSTQVSPSVQTQQHQMQLQQQQQQQQQMQQMQQQQLQQHQMHQQI
QQQMQQQHFQHHMQQHLQQQQQHLQQQINQQQLQQQLQQRLQLQQLQHMQHQSQP
SPRQHSPVASQITSPIPAIGSPQPASQQHQSQIQSQTQTQVLSQAIPTICESNCLMNPGTY

N-terminus of TOX high mobility group box family member 3 [Homo sapiens] (SEQ ID NO:2):

MDVRFYPAAAGDPASLDFAQCLGYYGYSKFGNNNNYMNMAEANNAFFAASEQTFHTP
SLGDEEFEIPPITPPPESDPALGMPDVLLPFQALSDPLPSQGSEFTPQFPPQSLDLPSITISRN
LVEQDGVLHSSGLHMDQSHTQVSQYRQDPSLIMRSIVHMTDAARSGVMPPAQLTTINQ
SQLSAQLGLNLGGASMPHTSPSPPASKSATPSPSSSINEEDADEANRAIGEKRAAPDSG

TOX high mobility group box family member 3 DNA [Rattus norvegicus] (SEQ ID NO:3):

atggatgtga ggttctaccc cgcggcggcc ggggatcccg ccggcctgga cttcgcgcag
tgcctggggt actacggcta cagcaagttg ggaaataata actacatgaa catggctgag
gcaaacaacg ccttctttgc tgccagtgag cagacattcc acacgccaag ccttggggat
gaagagtttg aaattccgcc gatcacgcct cctccagagt cagaccccac cctgggcatg

cccgatgtac tgctaccctt tcagacactc agcgatccgt tgccttccca gggaaatgag
ttcacacccc agtttccccc tcagagcctg gatcttcctt ccatcacaat ctcaaggaat
ctggtggagc aagatggtgt gcttcatagc aacgggctgc atatggatca gagccacaca
caagtgtcgc agtaccgcca ggatccttct ttggtcatga ggtcaattgt ccacatgaca
gatgctgctc gctctgggat catgcctcct gcccaactga ccaccatcaa ccagtctcag
ctcagtgcac agttgggctt gaatctggga ggagccagtg tgccccacac gtctccttca
cctccagcaa gcaaatcagc cactccctcc ccttccagct ctatcaatga agaggatgct
gatgaaacaa acagagccgt tggagagaaa agaacagccc cagattctgg caagaagccc
aagactccaa agaaaaagaa aaagaaagat cccaacgagc ctcagaagcc agtgtcagca
tacgccctgt ttttcagaga tacacaggct gcaattaaag gtcaaaaccc caatgcgacc
tttggagaag tctcaaaaat tgtagcatct atgtgggaca gccttggaga ggagcaaaag
caggtatata aaaggaaaac agaagctgcc aagaaagaat atttgaaggc cctggctgcc
taccgggcca gcctcgtttc taaggctgct gctgagtccg cagaagccca gactatccgc
tctgtccagc agactctggc atcaaccaac ctgacatcct ctctccttct gaacacgtca
ctgtctcaac atgggacagt cccggcttca cctcagactc tcccgcagtc actccctagg
tcgattgccc ccaaaccctt aaccatgaga ctacccatga gccagattgt cacatcagtc
accattgcag ccaacatgcc ctcgaacatt ggggctccac ttatcagctc catgggaacg
accatggttg gctcagtatc ctccacgcag gtgagccctt cggtacaaac ccagcaacat
cagctgcagc tgcagcagca gcaacaacag cagcagcagc agatgcagca gatgcaacat
cagcagctgc agcagcacca gatgcatcag cagattcagc agcagatgca gcagcagcat
ttccagcacc acatgcaaca gcacctgcag cagcagcaac agcagcacct ccagcagcag
atcagccaac agcagctgca gcagcagctg cagcagcatc tccagctgca gcagcagctg
cagcacatgc agcaccagtc tcagccttct ccccggcagc actcgcccgt cacctcacag
atcacgtccc ccatccccgc cattggcagc ccccagccag cctctcagca gcaccagcct
caaatccagt cgcagacaca gactcaagtg ttaccgcagg tcagtatttt ttaa

TOX high mobility group box family member 3 DNA (trinucleotide repeat containing 9, transcript variant 1 (TNRC9)) [Homo sapiens] (SEQ ID NO:4):

gcggccgcgg ctcccgagct cctcgggctc tgggtcccgg cgcccctccg gccgcgagtc
ccacgcgcca cccccgggcg ccctcgacgg tggatctagc ggcggcgagg aggcgggtcc
cggccccggc gaaccccagt cccggccccc ggccccgggc ccagcttcgg catggatgtg
aggttctacc ccgcggcggc cggggaccct gccagcctgg acttcgcgca gtgcctgggg
tactacggct acagcaagtt tggaaataat aataactata tgaatatggc tgaggcgaac
aatgcgttct tcgctgccag tgagcagaca ttccacacac caagccttgg ggacgaggaa
ttcgaaattc caccaatcac gcctcctcca gagtcagacc ctgccctagg catgccggat
gtactgctac cctttcaagc cctcagcgat ccattgcctt cccagggaag tgaattcaca
ccccagtttc cccctcaaag cctggacctc ccttccatta caatctcaag aaatctcgtg
gaacaagatg gcgtgcttca tagcagtggg ttgcatatgg atcagagcca cacacaagtg
tcccagtacc ggcaggatcc ctccctgatc atgcggtcca tcgtccacat gaccgatgct
gcgcgttctg gggtcatgcc tcctgcccag ctcaccacca tcaaccagtc tcagctcagc
gcccagttgg ggttgaattt gggaggtgcc agtatgcctc acacatctcc ttcacctcca
gcaagcaaat cagccactcc ctccccttcc agctccatca atgaagagga tgctgatgaa
gccaacagag ccattggaga gaaaagagct gctccagact ctggcaagaa gcccaagact
ccaaagaaaa agaaaaagaa agatcccaat gagccacaga agccagtgtc agcatatgcc
ctgtttttca gagacacaca ggctgcaatt aaaggtcaaa accccaatgc aacctttgga
gaggtctcaa aaattgtagc atctatgtgg gacagccttg gagaagaaca aaagcaggta
tataaaagga aaacagaagc tgccaaaaaa gaatacctga aggccctggc ggcatacagg
gccagcctcg tttctaaggc tgctgctgag tcagcagaag cccagaccat ccgttctgtt
cagcagaccc tggcgtcgac caatctaaca tcctctctcc ttctcaacac tccactgtct
caacatggaa cagtgtcagc atcacctcag actctccagc aatccctccc taggtcaatc
gctcccaaac ccttaaccat gagactcccc atgaaccaga ttgtcacatc agtcaccatt

gcagccaaca tgccctcgaa cattggggct ccactgataa gctccatggg aacgaccatg
gttggctcag caccctccac ccaagtgagt ccttcggtgc aaacccagca gcatcagatg
caattgcagc agcagcagca gcagcaacaa caacagatgc aacagatgca gcagcagcaa
ctccagcagc accaaatgca tcagcaaatc cagcagcaga tgcagcagca gcatttccag
caccacatgc agcagcacct gcagcagcag cagcagcatc tccagcagca aattaatcaa
cagcagctgc agcagcagct gcagcagcgc ctccagctgc agcagctgca acacatgcag
caccagtctc agccttctcc tcggcagcac tcccctgtcg cctctcagat aacatccccc
atccctgcca tcgggagccc ccagccagcc tctcagcagc accagtcgca aatacagtct
cagacacaga ctcaagtatt atcgcaggct atacctacaa tatgtgaatc aaactgttta
atgaatcctg ggacatactg atgactataa actggcctct ctgagtcata gaaaaatggc
cttatttctc cagaagtgag taaaccacac ttccaggcta tctgaactcc tgaagcccta
aaaataaaaa gcacagttgt aactacctga aatatgaaga tccagtttca tacaaacatt
tgtatgacgt gaatagttga tggcattttt ttgtcatgaa aaaaataatg taaatcacag
acttttgcca aagctcttat ttttttttcct aaatctctcc agaaaaaaaa tgcaagtgac
taaattcaat tattgactaa tttccacttt ttatccatga cttctccaaa tcaaaccaca
gtatatgttg taacaatatc tatgaccact gttagcccat tatattcatt ccaattagaa
gaaatgtgaa tactatattc cgtgttttga gtgacaagtt tcgaaaaata aaaacactgt
atttttaaaa gggaaatgca cttaaatgaa aacagttatt acaaaagtta agatttaaaa
agaaaaagca agagttttta ttatgatgta ataccagtag aatatttaaa aggcacacca
catctgaata atcaatgtaa atattttctt tcaaagttgt aagttttcat atcatgtgct
gtaaagtttt cctaaatgag gctttaacgt aaacactggt gacataaacc attcattgct
acgttgctta ttgtgttttt atgctgtttt atacttttt atgagttatg atagcagcaa
ttaagttgtt tgtattttgc ttaactaaaa caaaaatgct tttatcttgc tatagaataa
acacatttca gtaaaaactg tggactgtat tttgatgcaa caacaaagaa actgttcact
tttcaaataa aatgatatgt cagatttca

While particular embodiments of the present invention have been shown and described, it will be obvious to those skilled in the art that, based upon the teachings herein, changes and modifications may be made without departing from this invention and its broader aspects and, therefore, the appended claims are to encompass within their scope all such changes and modifications as are within the true spirit and scope of this invention.

Many modifications and variations of the invention as hereinbefore set forth can be made without departing from the spirit and scope thereof and therefore only such limitations should be imposed as are indicated by the appended claims.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

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WHAT IS CLAIMED IS:

1. A method of determining an increased susceptibility of a subject to cancer, comprising:

obtaining a sample from the subject;

determining the expression level of a biomarker in the sample;

comparing the expression level of the biomarker with the expression level of the biomarker from a healthy individual; and

determining that the subject has an increased susceptibility to cancer if the level of expression of the biomarker in the sample is greater than the level of expression of the biomarker in the healthy individual.

2. The method of claim 1, wherein the sample comprises a tissue or a cell.

3. The method of claim 1, wherein the biomarker is TOX3.

4. The method of claim 1, wherein determining the expression level of the biomarker comprises analyzing the transcription level of the biomarker or analyzing the protein level of the biomarker.

5. The method of claim 1, wherein the cancer is breast cancer.

6. The method of claim 1, wherein the cancer is a subset of breast cancer.

7. The method of claim 6, wherein the subset of breast cancer is luminal A, luminal B, Erbb2-enriched, basal, or normal-breast-like cancer.

8. A method of treating cancer in a subject, comprising:

providing a composition comprising a TOX3 modulator; and

administering the composition to the subject in an amount sufficient to reduce or inhibit TOX 3 expression in the subject's cancer cells, whereby the cancer is treated.

9. The method of claim 8, wherein the TOX3 modulator is an antisense molecule, antibody, antibody fragment, polypeptide, or a small molecule.

10. The method of claim 8, wherein the cancer is breast cancer.

11. The method of claim 8, wherein the cancer is a subset of breast cancer.

12. The method of claim 11, wherein the subset of breast cancer is luminal A, luminal B, Erbb2-enriched, basal, or normal-breast-like cancer.

13. A method of reducing the likelihood of the development of cancer in a subject comprising:

providing a composition comprising a TOX3 modulator; and

administering a TOX3 modulator to the subject in an amount that is sufficient to reduce or inhibit TOX 3 expression in the cancer cells and thereby inhibit the development of cancer.

14. The method of claim 13, wherein the TOX3 modulator is an antisense molecule, antibody, antibody fragment, polypeptide, or a small molecule.

15. The method of claim 13, wherein the cancer is breast cancer.

16. The method of claim 13, wherein the cancer is a subset of breast cancer.

17. The method of claim 13, wherein the subset of breast cancer is luminal A, luminal B, Erbb2-enriched, basal, or normal-breast-like cancer.

18. An isolated peptide, comprising SEQ ID NO: 1, wherein the peptide binds to TOX3 and modulates TOX3 expression.

19. A pharmaceutical composition comprising the peptide of claim 18.

20. An isolated peptide, comprising SEQ ID NO: 2, wherein the peptide binds to TOX3 and modulates TOX3 expression.

21. A pharmaceutical composition comprising the peptide of claim 20.

22. An isolated antibody that binds to TOX3 and modulates TOX3 expression, wherein the antibody is derived from the peptide of claim 18.

23. A pharmaceutical composition comprising the antibody of claim 22.

24. An isolated antibody that binds to TOX3 and modulates TOX3 expression, wherein the antibody is derived from the peptide of claim 20.

25. A pharmaceutical composition comprising the antibody of claim 24.

26. A kit, comprising:

a composition comprising a TOX3 modulator; and

instructions for the use of said composition for treating cancer in a subject.

27. The kit according to claim 26, wherein the TOX3 modulator is an antisense molecule, antibody, antibody fragment, polypeptide, or a small molecule.

28. A kit, comprising:

a composition comprising a TOX3 modulator; and

instructions for the use of said composition for determining an increase in the susceptibility of cancer in a subject.

29. The kit according to claim 28, wherein the TOX3 modulator is an antisense molecule, antibody, antibody fragment, polypeptide, or a small molecule.

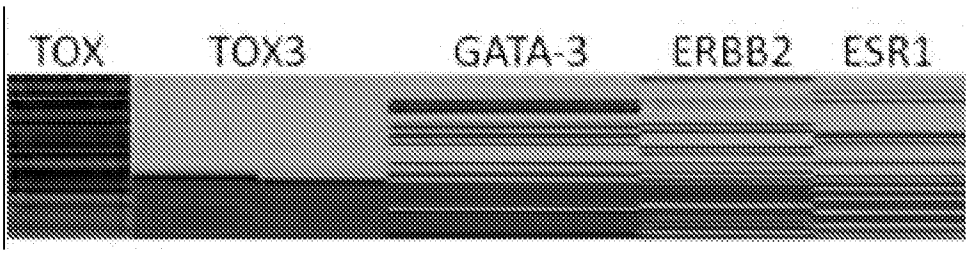
30. A kit, comprising:

a composition comprising a TOX3 modulator; and

instructions for the use of said composition for reducing the likelihood of the development of cancer in a subject.

31. The kit according to claim 30, wherein the TOX3 modulator is an antisense molecule, antibody, antibody fragment, polypeptide, or a small molecule.

FIGURE 1A-B



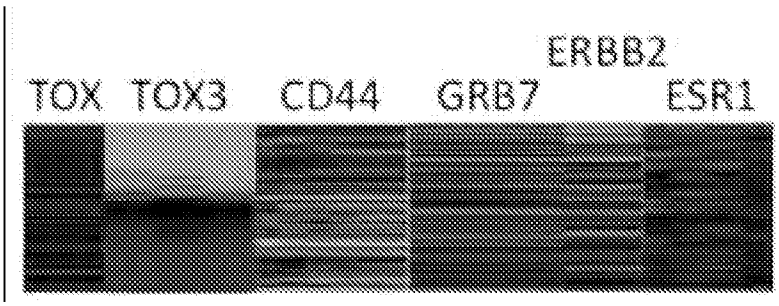


FIGURE 2

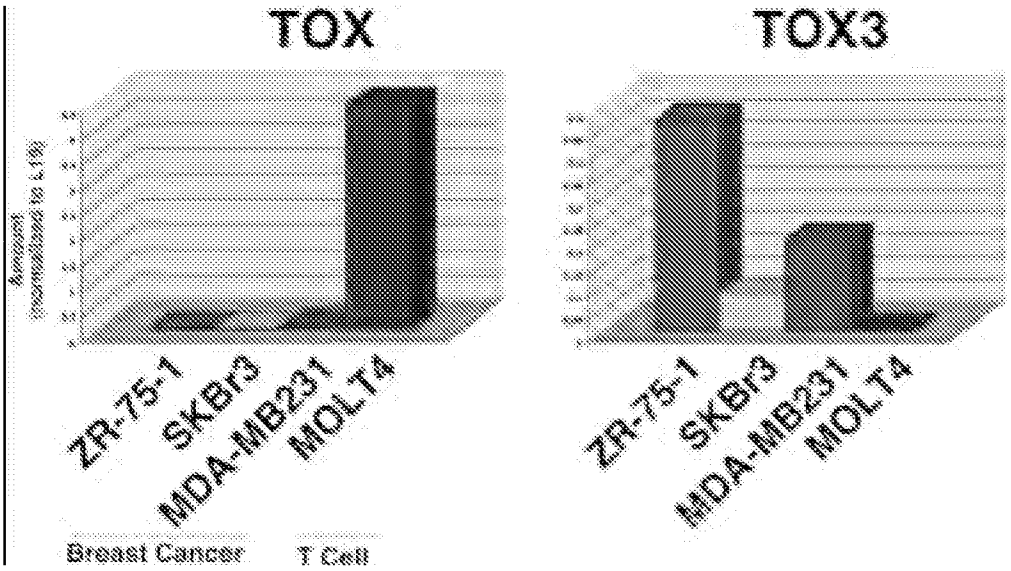


FIGURE 3A-B

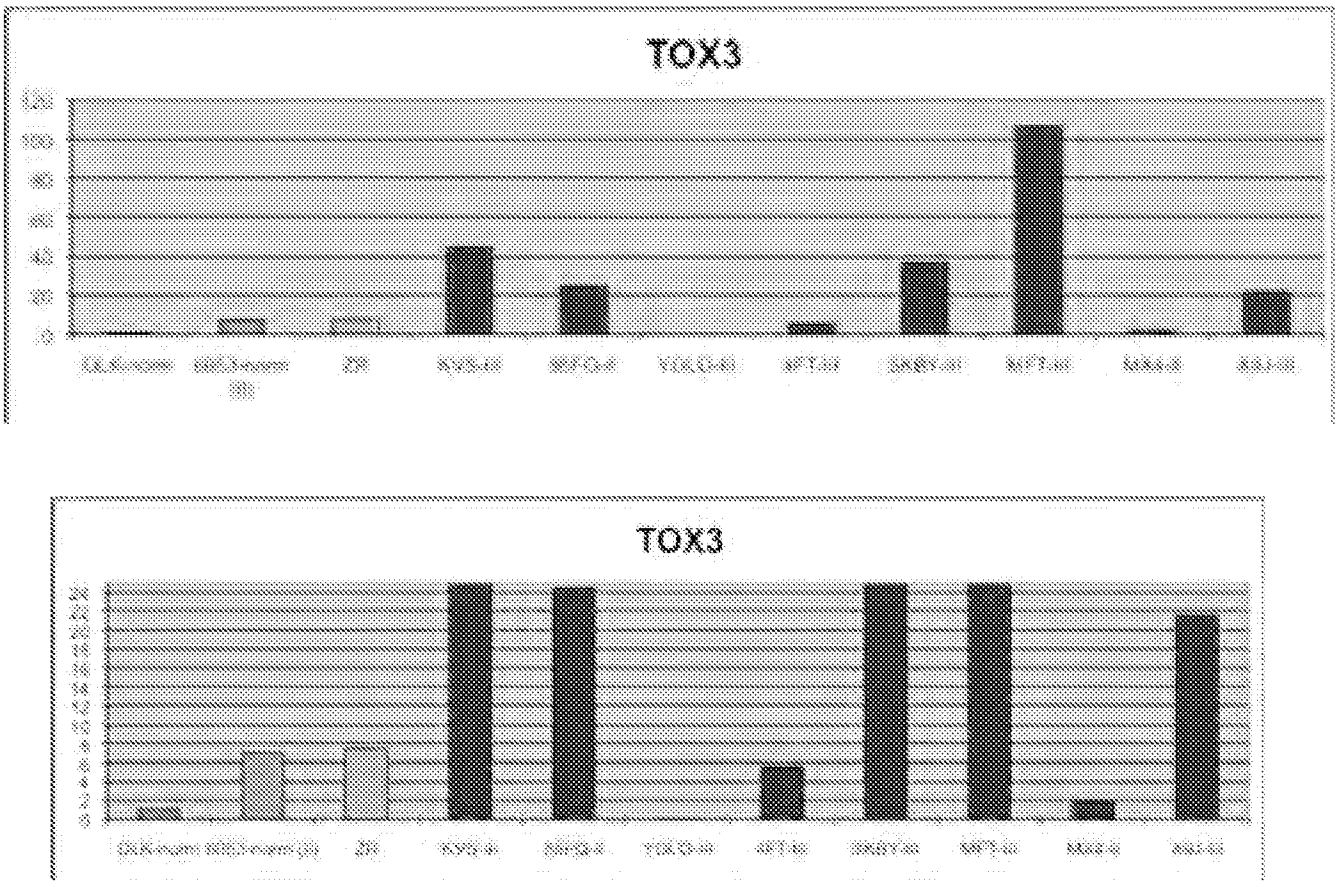
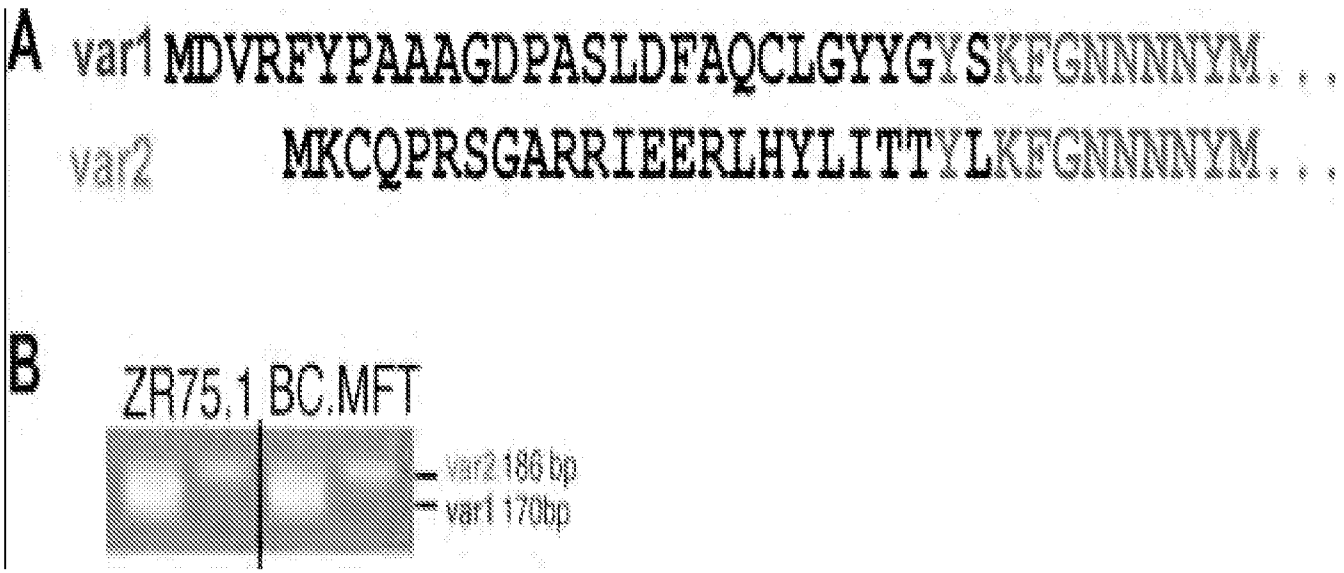


FIGURE 4A-B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var2 MKCQPRSGARRIEERLHYLITTYLKFGNNNNYM. . .
B
ZR75.1 BC.MFT
var2 186 bp
var1 170bp

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/58795

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/48, 33/53 (2011.01)
USPC - 436/64; 436/86; 436/94
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC 436/64; 436/86; 436/94

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 436/501; 435/6

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ), Google Scholar(TOX3 breast cancer, TNRC9 breast cancer, CAGF9 breast cancer), Google(TOX3)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2009/0175844 A1 (NAKAMURA et al.), 9 July 2009 (09.07.2009); para [0017]-[0019], [0046]-[0048]; Table 4	1-4 ----- 5-7
Y	NORDGARD et al., Genes harbouring susceptibility SNPs are differentially expressed in the breast cancer subtypes. Breast Cancer Research, 2007, vol 9, no 113; pp 1-2; pg 1, left col, para 1, right col, para 1.	5-7
A	US 2008/0292546 A1 (CLARKE et al.) 27 November 2008 (27.11.2008)	1-7

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2011 (06.04.2011)	25 APR 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/58795

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-7, directed to a method of determining increased susceptibility of a subject to cancer, comprising: obtaining a sample from the subject, determining the expression level of a biomarker in the sample, comparing the expression level of the biomarker to the level in a healthy individual and determining that the subject has an increased susceptibility to cancer if the level of expression of the biomarker is greater than the level in a healthy individual.

- Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/58795

Continuation of Box III: Lack of Unity of Invention

Group II: claims 8-17, 26, 27, 30 and 31, directed to a method of treating or reducing the likelihood of cancer in a subject, comprising administering a composition comprising a TOX3 modulator in an effective amount to the subject's cancer cells.

Group III: Claims 18-25, directed to isolated peptides that bind to TOX3 and modulate TOX3 expression.

Group IV: claims 28 and 29, directed to a kit comprising a TOX3 modulator and instructions for the use of said composition for determining an increase in the susceptibility of cancer in a subject.

The inventions listed as Groups I - IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the Group I claims is a method of determining increased susceptibility of a subject to cancer, comprising: obtaining a sample from the subject, determining the expression level of a biomarker in the sample, comparing the expression level of the biomarker to the level in a healthy individual and determining that the subject has an increased susceptibility to cancer if the level of expression of the biomarker is greater than the level in a healthy individual. The special technical feature of the Group II claims is a method of treating or reducing the likelihood of cancer in a subject, comprising administering a composition comprising a TOX3 modulator in an effective amount to the subject's cancer cells. The special technical feature of the Group III claims is isolated peptides that bind to TOX3 and modulate TOX3 expression. The special technical feature of the Group IV claims is a kit comprising a TOX3 modulator and instructions for the use of said composition for determining an increase in the susceptibility of cancer in a subject.

There is no common technical element shared by all of the above Groups, because, although at least one dependent claim in Group I is directed to TOX3, the Group is not limited to TOX3 overexpression in association with cancer. This one element of Group I, being related to TOX is shared in common with the other Groups, and in particular to Group IV. However, while Group I is specific to the assessment of overexpression of a marker in association with susceptibility to cancer, Group IV is not. Groups II and IV share a common technical element of being related to a modulator of TOX3. However, these common technical elements do not represent an improvement over the prior art of US 2008/0292546 A1 to Clarke et al., which discloses assessing expression levels of markers in cancer cells (para [0008]), wherein detection may include exposing a cancer cell marker mRNA to a nucleic acid probe complementary to the marker mRNA (para [0009]), further wherein a treatment for cancer targeted at the marker may comprise antisense or SiRNA to the marker mRNA (para [0014]), and wherein marker expression may be increased (para [0059]). Clarke et al. also includes TOX3 (Table 5) amongst the genes potentially upregulated, wherein TOX3 is one of a large list of genes. Further, in a related disclosure, US 2009/0208962 A1 to Cox discloses polymorphisms associated with breast cancer (abstract), wherein TOX3 (TNRC9) (para [0010]) is amongst a short list of target markers. It would have been obvious to a person skilled in the art to closely examine the expression of TOX3 in association with cancer based on the combination of the inclusion of TOX3 in a longer list by Clarke, and the more significant association of altered forms of TOX3 being associated with breast cancer disclosed by Cox. Therefore, the inventions of Groups I-IV lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.