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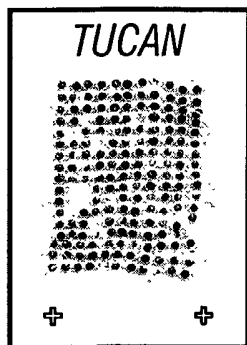
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(54) Title: METHODS FOR DETERMINING THE PROGNOSIS FOR CANCER PATIENTS USING TUCAN

(57) **Abstract:** This invention provides methods for determining a prognosis for survival for a cancer patient. One method involves (a) measuring a level of a TUCAN in a neoplastic cell-containing sample from the cancer patient, and (b) comparing the level of TUCAN in the sample to a reference level of TUCAN, wherein a low level of TUCAN in the sample correlates with increased survival of the patient. Another method involves (a) measuring a level of TUCAN in a neoplastic cell-containing sample from the cancer patient, and (b) classifying the patient as belonging to either a first or second group of patients, wherein the first group of patients having low levels of TUCAN is classified as having an increased likelihood of survival compared to the second group of patients having high level of TUCAN.

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**METHODS FOR DETERMINING THE PROGNOSIS
FOR CANCER PATIENTS USING TUCAN**

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5 by the National Institutes of Health. The United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention relates generally to cancer and, more specifically, to biomarkers that can be used to
10 diagnose or prognose cancer.

Cancer remains a major public health problem that profoundly affects the more than 1 million people diagnosed each year, as well as their families and
15 friends. As our Nation's population grows and ages, more people will get cancer. The use of screening tests to detect cancers early often leads to more effective treatment with fewer side effects. Patients whose cancers are found early also are less likely to die from
20 these cancers than are those whose cancers are not found until symptoms appear.

One type of cancer screening test involves the detection of a biomarker, such as a tumor marker, in a fluid or tissue obtained from a patient. Tumor markers
25 are substances produced by cancer cells that are not typically produced by normal cells. These substances generally can be detected in the body fluids or tissues of patients with cancer. Unfortunately, some tumor markers also can be detected in significant amounts in
30 the body fluids or tissues of people who do not have cancer, making certain markers less reliable for

diagnosis. Nevertheless, tumor markers remain an important tool for diagnosing cancer.

Another important use for tumor markers is for monitoring patients being treated for advanced cancer.

5 Measuring tumor markers for this purpose can be less invasive, less time-consuming, as well as less expensive, than repeating chest x-rays, computed tomography (CT) scans, bone scans, or other complicated tests, to determine if a therapy is reducing the cancer.

10 A further important use for tumor markers is for determining a prognosis of survival of a cancer patient. Such prognostic methods can be used to identify surgically treated patients likely to experience cancer recurrence so that they can be offered additional
15 therapeutic options. Biomarkers useful for prognosis of survival also can be especially effective for determining the risk of metastasis in patients who demonstrate no measurable metastasis at the time of examination or surgery. Knowledge of the likelihood of metastasis in a
20 cancer patient can be an important factor in selecting a treatment option. For example, a cancer patient likely to experience metastasis may be advantageously treated using a modality that is particularly aggressive.

Thus, there exists a need for identification of
25 biomarkers that can be used as diagnostic and prognostic indicators for cancer. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides methods for determining a prognosis for survival for a cancer patient. One method involves (a) measuring a level of a TUCAN in a
5 neoplastic cell-containing sample from the cancer patient, and (b) comparing the level of TUCAN in the sample to a reference level of TUCAN, wherein a lower level of TUCAN in the sample correlates with increased survival of the patient.

10 Another method for determining a prognosis for survival for a cancer patient involves (a) measuring levels of TUCAN and one or more biomarkers selected from the group consisting of cIAP2, Apaf1, Bcl-2 and Smac in a neoplastic cell-containing sample from the cancer
15 patient, and (b) comparing the level of TUCAN and the one or more selected biomarkers in the sample to a reference level of TUCAN and the one or more selected biomarkers, wherein a low level of TUCAN and a high level of any of Apaf1, Bcl-2 or Smac, or a low level of TUCAN and a low
20 level of cIAP2, in said sample correlate with increased survival of said patient.

A further method of determining a prognosis for survival for a cancer patient involves (a) measuring a
25 level of TUCAN in a neoplastic cell-containing sample from the cancer patient, and (b) classifying the patient as belonging to either a first or second group of patients, wherein the first group of patients having low levels of TUCAN is classified as having an increased
30 likelihood of survival than the second group of patients having high levels of TUCAN.

The invention also provides a method for monitoring the effectiveness of a course of treatment for a patient with cancer. The method involves (a) determining a level of a TUCAN in a neoplastic cell-
5 containing sample from the cancer patient prior to treatment, and (b) determining the level of TUCAN in a neoplastic cell-containing sample from the patient after treatment, whereby comparison of the TUCAN level prior to treatment with the TUCAN level after treatment indicates
10 the effectiveness of the treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows examples of immunohistochemical detection of IAP-family proteins, Apaf1 and TUCAN in normal and malignant colon tissues.

15 Figure 2 shows comparisons of immunoscores for normal and malignant colon tissues.

Figure 3 shows the specificity of antibodies and expression of IAPs, Apaf1 and TUCAN protein in colon carcinoma by immunoblotting.

20 Figure 4 shows correlations of biomarker immunostaining data with disease-free (Figure 4A) and overall survival (Figure 4B) for colon carcinoma patients.

Figure 5 shows correlations of biomarkers and
25 their combinations with disease-free (Figure 5A) and overall (Figure 5B) survival for colon carcinoma patients.

Figure 6 shows expression of TUCAN in several tumor cell lines.

Figure 7 shows immunohistochemical analysis of TUCAN expression in colorectal cancer.

5 Figure 8 shows that TUCAN binds selectively to pro-caspase-9 and to itself.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to the finding that expression of the CARD domain containing protein, TUCAN
10 (Tumor Up-regulated CARD-containing Antagonist of Caspase Nine), formerly known as CARD-X in PCT publication WO 01/16170, can be used to effectively predict clinical outcome for patients with cancer, either independently, or in combination with other biomarkers.

15 The prognostic methods of the invention are useful for determining if a patient is at risk for relapse. Cancer relapse is a concern relating to a variety of types of cancer. For example, of patients undergoing complete surgical removal of colon cancer,
20 25-40% of patients with stage II colon carcinoma and about 50% of patients with stage III colon carcinoma experience cancer recurrence. One explanation for cancer recurrence is that patients with relatively early stage disease (for example, stage II or stage III) already have
25 small amounts of cancer spread outside the affected organ that were not removed by surgery. These cancer cells, referred to as micrometastases, cannot typically be detected with currently available tests. The prognostic methods of the invention can be used to identify
30 surgically treated patients likely to experience cancer

recurrence so that they can be offered additional therapeutic options, including preoperative or postoperative adjuncts such as chemotherapy, radiation, biological modifiers and other suitable therapies. The methods are especially effective for determining the risk of metastasis in patients who demonstrate no measurable metastasis at the time of examination or surgery.

The prognostic methods of the invention also are useful for determining a proper course of treatment for a patient having cancer. A course of treatment refers to the therapeutic measures taken for a patient after diagnosis or after treatment for cancer. For example, a determination of the likelihood for cancer recurrence, spread, or patient survival, can assist in determining whether a more conservative or more radical approach to therapy should be taken, or whether treatment modalities should be combined. For example, when cancer recurrence is likely, it can be advantageous to precede or follow surgical treatment with chemotherapy, radiation, immunotherapy, biological modifier therapy, gene therapy, vaccines, and the like, or adjust the span of time during which the patient is treated.

As disclosed herein in Examples II and VIII, elevated levels of TUCAN were found in 49 and 64% of colon tumor specimens examined, respectively. Univariate analysis was used to determine significant correlations between longer disease-free survival (DFS) and low expression of TUCAN ($p = 0.0004$). As shown in Example IV, 78% (39/50) of patients whose tumors contained low levels of TUCAN remained alive and disease-free during the time covered by this study, compared to only 44% (21/48) of those with high expression of this protein. Example VIII also indicates that TUCAN immunostaining was

significantly higher among patients who died of colon cancer, as compared to patients who remained alive.

As shown in Example IV, at a median follow-up of 5 years, 49% of patients with high expression of TUCAN
5 had relapse or died of colon cancer, and only 19% had recurrence and 4% died of disease in a group of patients whose tumors expressed low levels of this protein. Multivariate analysis indicated that the presence of high TUCAN increased risk of death from colon cancer 17-fold
10 ($p = 0.00004$). Therefore, a high level of TUCAN in a sample from a patient with cancer correlates with increased likelihood of tumor metastasis and reduced survival. Similarly, a low level of TUCAN in a sample from a patient with cancer correlates with decreased
15 likelihood of tumor metastasis and increased likelihood of survival.

Also disclosed herein is the observation that the combination of low levels of cIAP2 and low levels of TUCAN identified a subgroup of early-stage colon cancer
20 patients with very favorable outcome. Approximately one-third of patients in a cohort of 92 patients had a combination of low cIAP2 and low TUCAN (33/92 [36%]). Among these 33 patients, 32 (97%) remained alive and 30 (91%) disease-free during the time covered by this study,
25 as opposed to 56% and 44% for other categories of patients. Similarly, in a cohort of 81 patients, 17 had a combination of high Apaf1 and low TUCAN. All (17) patients featuring high expression of Apaf1 and low TUCAN were alive and relapse-free at the end of the survey,
30 compared to only 65% (53/81) alive and 53% (43/81) recurrence-free for those who were not characterized by this feature. Therefore, a high level of TUCAN combined with a high level of cIAP2 or a low level of Apaf1 in a

sample from a patient with cancer correlates with increased likelihood of tumor metastasis and reduced likelihood of survival, whereas a low level of TUCAN combined with a low level of cIAP2 or a high level of Apaf1 in a sample from a patient with cancer correlates with reduced likelihood of tumor metastasis and increased likelihood of survival.

Based on these results, the invention provides methods for diagnosing neoplastic conditions, prognosing survival of patients suffering from cancer, and determining a stage of cancer using TUCAN as a biomarker. TUCAN can be used alone or in combination with other prognostic indicators as a specific biomarker for prognosing survival of patients suffering from cancer.

As disclosed herein, elevated levels of Apaf1, Survivin, XIAP, cIAP1, and cIAP2 were found in 38%, 54%, 74%, 61% and 35% of colon tumor specimens, respectively. Univariate analysis was used to determine significant correlations between longer disease-free survival (DFS) and low expression of cIAP2 ($p = 0.0002$), β -Catenin ($p = 0.04$), mutant p53 protein ($p = 0.03$), or high levels of Apaf1 ($p = 0.00008$), Bcl-2 ($p = 0.005$), and SMAC ($p = 0.03$) (see Figure 4a). Thus, 78% (39/50) of patients whose tumors contained low levels of TUCAN remained alive and disease-free during the time covered by this study, compared to only 44% (21/48) of those with high expression of this protein. Similarly, 74% (45/61) of low cIAP2 expressors were cancer-free at the time of last survey compared to only 36% (12/33) of those with high cIAP2 levels. At a median follow-up of 5 years, 60% of patients with high cIAP2 levels relapsed and 46% died of colon cancer, whereas in a low-cIAP2 group there were 20% relapses and 18% colon cancer-related deaths.

As further disclosed herein, high levels of Apaf1 were associated with longer survival, with 33/38 (87%) of colon cancer patients remaining disease-free compared to only 28/62 (45%) of those with low Apaf1 expression. In contrast, 43% of patients with low Apaf1 relapsed and 35% died of colon cancer, while only 14% had a cancer recurrence or died in a high-Apaf1 cohort. Low Bcl-2 levels also were associated with poor overall survival. Of 18 patients with low expression of this protein, 11 (61%) died of colon cancer, compared with 24% of patients who died in the high-Bcl-2 group (18/76). Similarly, patients whose tumors contained low Apaf1 staining had worse overall survival compared with those who overexpressed Bcl-2 (Figure 1N). Multivariate analysis indicated that high Apaf1 and Bcl-2 expression was associated with a decreased relative risk of dying of colon cancer by 75% ($p = 0.004$) and 82% ($p = 0.00006$). Therefore, a decreased level of Apaf1 or Bcl-2 in a sample from a patient with colon cancer correlates positively with increased chance of tumor metastasis and reduced survival.

Also disclosed herein is the observation that the combination of low levels of cIAP2 and high levels of Apaf1 identified a subgroup of early-stage colon cancer patients with very favorable outcome. Roughly one-quarter (25/94 [27%]) of the tumors analyzed contained both low cIAP2 and high Apaf1. Among these 25 patients, all 25 remained alive and free of disease after surgery at the time of last survey (median follow-up 5 years). Thus, the median 5 yr disease-free and overall survival rate for this group of patients was 100%, compared to only 50% and 64% for other categories of patients, respectively. Therefore, an increased level of cIAP2 and decreased level of Apaf1 in a sample from a

patient with colon cancer correlates with increased chance of tumor metastasis and reduced survival.

As used herein, the term "level" refers to mean the amount, accumulation or rate of a biomarker molecule, such as TUCAN. A level can be represented, for example, by the amount or synthesis rate of messenger RNA (mRNA) encoded by a gene, the amount or synthesis rate of polypeptide corresponding to a given amino acid sequence encoded by a gene, or the amount or synthesis rate of a biochemical form of a molecule accumulated in a cell, including, for example, the amount of particular post-synthetic modifications of a molecule such as a polypeptide, nucleic acid or small molecule. The term can be used to refer to an absolute amount of a molecule in a sample or to a relative amount of the molecule, including amounts determined under steady-state or non-steady-state conditions. The expression level of a molecule can be determined relative to a control molecule in a sample.

When used in reference to TUCAN mRNA or polypeptide, the term level refers to the extent, amount or rate of synthesis of the nucleic acid sequence shown as SEQ ID NO:1 or the TUCAN polypeptide shown as SEQ ID NO:2, or substantially the same nucleotide or amino acid sequences. The nucleic acid sequence and amino acid sequence of TUCAN are also described in PCT publication WO 01/16170, which is incorporated herein by reference. When used in reference to cIAP2 mRNA or polypeptide expression, the term level refers to the extent, amount or rate of synthesis of the nucleic acid sequence shown as SEQ ID NO:5 or the CIAP2 polypeptide shown as SEQ ID NO:6, or substantially the same nucleotide or amino acid sequences. When used in reference to β -catenin mRNA or

polypeptide, the term level refers to the extent, amount or rate of synthesis of the nucleic acid sequence shown as SEQ ID NO:7 or the β -catenin polypeptide shown as SEQ ID NO:8, or substantially the same nucleotide or amino acid sequences. When used in reference to Apaf1 mRNA or polypeptide, the term level refers to the extent, amount or rate of synthesis of the nucleic acid sequence shown as SEQ ID NO:9 or the Apaf1 polypeptide shown as SEQ ID NO:10, or substantially the same nucleotide or amino acid sequences. When used in reference to Bcl-2 mRNA or polypeptide, the term level refers to the extent, amount or rate of synthesis of the nucleic acid sequence shown as SEQ ID NO:11 or the Bcl-2 polypeptide shown as SEQ ID NO:12, or substantially the same nucleotide or amino acid sequences. When used in reference to Smac mRNA or polypeptide, the term level refers to the extent, amount or rate of synthesis of the nucleic acid sequence shown as SEQ ID NO:13 or the Smac polypeptide shown as SEQ ID NO:14, or substantially the same nucleotide or amino acid sequences. A level of these and other biomarkers of cancer, including XIAP, cIAP1, Survivin, Bcl-XL, Bax, BAG1, mutant p53, p53 and MIB-1, can be a gene expression level or a polypeptide expression level.

An amino acid sequence that has substantially the same amino acid sequence as a reference amino acid sequence contains a considerable degree of sequence identity or similarity, such as at least 70%, 80%, 90%, 95%, 98%, or 100% sequence identity or similarity, to a reference amino acid sequence. Such changes, gaps and insertions can be naturally occurring mutations, or can result from processing a sample containing the polypeptide. A nucleotide sequence that is substantially the same as a reference nucleotide sequences contains a considerable degree of sequence

identity or similarity, such as at least 70%, 80%, 90%, 95%, 98%, or 100% sequence identity or similarity, to the reference nucleotide sequence. Such differences can be due to genetic differences between individuals, such as mutations and polymorphisms of a gene. Differences between nucleotide and amino acid sequences can be determined using available algorithms and programs such as the Smith-Waterman algorithm and the BLAST homology search program (Altschul et al., J. Mol. Biol. 215:403-410 (1990)).

A gene expression level of a molecule is intended to mean the amount, accumulation or rate of synthesis of a biomarker gene. The gene expression level can be represented by, for example, the amount or transcription rate of hnRNA or mRNA encoded by a gene. A gene expression level similarly refers to an absolute or relative amount or a synthesis rate determined, for example, under steady-state or non-steady-state conditions.

A polypeptide expression level is intended to mean the amount, accumulation or rate of synthesis of a biomarker polypeptide. The polypeptide expression level can be represented by, for example, the amount or rate of synthesis of the polypeptide, a precursor form or a post-translationally modified form of the polypeptide. Various biochemical forms of a polypeptide resulting from post-synthetic modifications can be present in cell contained in a sample. Such modifications include post-translational modifications, proteolysis, and formation of macromolecular complexes. Post-translational modifications of polypeptides include, for example, phosphorylation, lipidation, prenylation, sulfation, hydroxylation, acetylation, addition of

carbohydrate, addition of prosthetic groups or cofactors, formation of disulfide bonds and the like. In addition, it is understood that fragments of a polypeptide are included within the definition of a polypeptide expression level. Fragments can include, for example, amino terminal, carboxyl terminal, or internal deletions of a full length polypeptide. Accumulation or synthesis rate with or without such modifications is included with in the meaning of the term. Similarly, a polypeptide expression level also refers to an absolute amount or a synthesis rate of the polypeptide determined, for example, under steady-state or non-steady-state conditions.

As used herein, the term "reference level" refers to a control level of expression of a biomarker used to evaluate a test level of expression of a biomarker in a neoplastic cell-containing sample of a patient. For example, when the level of TUCAN in the neoplastic cells of a patient are higher than the reference level of TUCAN, the cells will be considered to have a high level of expression, or overproduction, of TUCAN. Conversely, when the level of TUCAN in the neoplastic cells of a patient are lower than the reference level, the cells will be considered to have a low level of expression, or underproduction, of TUCAN.

The reference level can be determined by a plurality of methods, provided that the resulting reference level accurately provides a level of a biomarker above which exists a first group of patients having a different probability of survival than that of a second group of patients having levels of the biomarker below the reference level. The reference level can be determined by, for example, measuring the level of

expression of a biomarker in non-tumorous cancer cells from the same tissue as the tissue of the neoplastic cells to be tested. The reference level can also be a level of a biomarker of *in vitro* cultured cells which can
5 be manipulated to simulate tumor cells, or can be manipulated in any other manner which yields expression levels which accurately determine the reference level.

The reference level can also be determined by comparison of the level of a biomarker, such as TUCAN, in
10 populations of patients having the same cancer. This can be accomplished, for example, by histogram analysis, in which an entire cohort of patients are graphically presented, wherein a first axis represents the level of the biomarker, and a second axis represents the number of
15 patients in the cohort whose neoplastic cells express the biomarker at a given level. Two or more separate groups of patients can be determined by identification of subsets populations of the cohort which have the same or similar levels of the biomarker. Determination of the
20 reference level can then be made based on a level which best distinguishes these separate groups. A reference level also can represent the levels of two or more markers. Two or more markers can be represented, for example, by a ratio of values for levels of each
25 biomarker.

The reference level can be a single number, equally applicable to every patient, or the reference level can vary, according to specific subpopulations of patients. For example, older men might have a different
30 reference level than younger men for the same cancer, and women might have a different reference level than men for the same cancer. Furthermore, the reference level can be some level determined for each patient individually. For

example, the reference level might be a certain ratio of a biomarker in the neoplastic cells of a patient relative to the biomarker levels in non-tumor cells within the same patient. Thus the reference level for each patient
5 can be proscribed by a reference ratio of one or more biomarkers, such as TUCAN, wherein the reference ratio can be determined by any of the methods for determining the reference levels described herein.

10 As used herein, the term "neoplastic cell" refers to any cell that is transformed such that it proliferates without normal homeostatic growth control. Such cells can result in a benign or malignant lesion of proliferating cells. Such a lesion can be located in a
15 variety of tissues and organs of the body. Table 1, below, provides a list of exemplary types of cancers from which a neoplastic cell can be derived.

 As used herein, the term "cancer" is intended to mean a class of diseases characterized by the
20 uncontrolled growth of aberrant cells, including all known cancers, and neoplastic conditions, whether characterized as malignant, benign, soft tissue or solid tumor. Specific cancers include digestive and
25 gastrointestinal cancers, such as anal cancer, bile duct cancer, gastrointestinal carcinoid tumor, colon cancer, esophageal cancer, gallbladder cancer, liver cancer, pancreatic cancer, rectal cancer, appendix cancer, small intestine cancer and stomach (gastric) cancer; breast
cancer; ovarian cancer; lung cancer; renal cancer; CNS
30 cancer; leukemia and melanoma. By exemplification, a list of known cancers is provided below in Table 1.

Table 1: Types of Cancer**HEMATOPORETTIC NEOPLASMS**

	Lymphoid Neoplasms	Adult T-cell leukemia/lymphoma
	Myeloid Neoplasms	Anaplastic large cell lymphoma
5	Histiocytoses	<i>Hodgkin Diseases (HD)</i>
	Precursor B lymphoblastic leukemia/lymphoma (ALL)	Acute myelogenous leukemia (AML)
	Precursor T lymphoblastic leukemia/lymphoma (ALL)	Myelodysplastic syndromes
10	Chronic lymphocytic leukemia/small lymphocytic lymphoma (SLL)	Chronic Myelofroliferative Disorders
	Lymphoplasmacytic lymphoma	Chronic Myelogenous Leukemia (CML)
	Mantle cell lymphoma	Polycythemia Vera
15	Follicular lymphoma	Essential Thrombocytosis
	Marginal zone lymphoma	Myelofibrosis with Myeloid Metaplasia
	Hairy cell leukemia	Hemangioma
	Plasmacytoma/plasma cell myeloma	Lymphangioma
20	Diffuse large B-cell lymphoma	Glomangioma
	Burkitt lymphoma	Kaposi Sarcoma
	T-cell chronic lymphocytic leukemia	Hemangioendothelioma
25	Large granular lymphocytic leukemia	Angiosarcoma
	Mycosis fungoids and sezary syndrome	Hemangiopericytoma
	Peripheral T-cell lymphoma, unspecified	
30	Angioimmunoblastic T-cell lymphoma	HEAD & NECK
	Angiocentric lymphoma (NK/T-cell lymphoma)	Basal Cell Carcinoma
35	Intestinal T-cell lymphoma	Squamous Cell Carcinoma
		Ceruminoma
		Osteoma
		Nonchromaffin Paraganglioma
		Acoustic Neurinoma
		Adenoid Cystic Carcinoma
		Mucoepidermoid Carcinoma
		Malignant Mixed Tumors
		Adenocarcinoma

	Lymphoma	Malignant Mesothelioma
	Fibrosarcoma	Sarcomatoid Type
	Osteosarcoma	Epithelial Type
	Chondrosarcoma	
5	Melanoma	THE GASTROINTESTINAL TRACT
	Olfactory Neuroblastoma	Squamous Cell Carcinoma
	Isolated Plasmocytoma	Adenocarcinoma
	Inverted Papillomas	Carcinoid
	Undifferentiated Carcinoma	Malignant Melanoma
10	Mucoepidermoid Carcinoma	Adenocarcinoma
	Acinic Cell Carcinoma	Gastric Carcinoma
	Malignant Mixed Tumor	Gastric Lymphoma
	Other Carcinomas	Gastric Stromal Cell Tumors
	Amenoblastoma	Lymphoma
15	Odontoma	Kaposi's Sarcoma
		Intestinal Stromal Cell Tumors
	THYMUS	Carcinids
	Malignant Thymoma	Malignant Mesethelioma
	Type I (Invasive thymoma)	Non-mucin producing
	Type II (Thymic carcinoma)	adenocarcinoma
20	Squamous cell carcinoma	
	Lymph epithelioma	
	THE LUNG	THE LIVER AND THE BILIARY TRACT
	Squamous Cell Carcinoma	Hepatocellular Carcinoma
	Adenocarcinoma	Cholangiocarcinoma
25	Bronchial derived	Hepatoblastoma
	Acinar; papillary; solid	Angiosarcoma
	Bronchioalveolar	Fibrolameller Carcinoma
	Small Cell Carcinoma	Carcinoma of the
	Oat Cell	Gallbladder
30	Intermediate Cell	Adenocarcinoma
	Large Cell Carcinoma	Squamous Cell Carcinoma
	Undifferentiated; giant cell; clear cell	Papillary, poorly differentiated

THE PANCREAS

Adenocarcinoma
 Cystadenocarcinoma
 Insulinoma
 5 Gastrinoma
 Glucagonamoia

THE KIDNEY

Renal Cell Carcinoma
 Nephroblastoma (Wilm's
 10 Tumor)

THE LOWER URINARY TRACT

Urothelial Tumors
 Squamous Cell Carcinoma
 Mixed Carcinoma
 15 Adenocarcinoma
 Small Cell Carcinoma
 Sarcoma

THE MALE GENITAL TRACT

Squamous Cell
 20 CarcinomaSarcinoma
 Speretocytic Sarcinoma
 Embyonal Carcinoma
 Choriocarcinoma
 Teratoma
 25 Leydig Cell Tumor
 Sertoli Cell Tumor
 Lymphoma
 Adenocarcinoma
 Undifferentiated Prostatic
 30 Carcinoma
 Ductal Transitional
 Carcinoma

THE FEMALE GENITAL TRACT

Squamous Cell Carcinoma
 Basal Cell Carcinoma
 Melanoma
 Fibrosarcoma
 Intaepithelial Carcinoma
 Adenocarcinoma Embryonal
 Rhabdomysarcoma
 Large Cell Carcinoma
 Neuroendocrine or Oat Cell
 Carcinoma
 Adenocarcinoma
 Adenosquamous Carcinoma
 Undifferentiated Carcinoma
 Carcinoma
 Adenoacanthoma
 Sarcoma
 Carcinosarcoma
 Leiomyosarcoma
 Endometrial Stromal Sarcoma
 Serous Cystadenocarcinoma
 Mucinous Cystadenocarcinoma
 Endometrioid Tumors
 Adenosarcoma
 Celioblastoma (Brenner
 Tumor)
 Clear Cell Carcinoma
 Unclassified Carcinoma
 Granulosa-Theca Cell Tumor
 Sertoli-Leydig Cell Tumor
 Disgerminoma
 Teratoma

THE BREAST

Phyllodes Tumor

Sarcoma
 Paget's Disease
 Carcinoma
 Insitu Carcinoma
 5 Invasive Carcinoma

THE ENDOCRINE SYSTEM

Adenoma
 Carcinoma
 Meningioma
 10 Craniopharyngioma
 Papillary Carcinoma
 Follicular Carcinoma
 Medullary Carcinoma
 Anaplastic Carcinoma
 15 Adenoma
 Carcinoma
 Pheochromocytoma
 Neuroblastoma
 Paraganglioma
 20 Pineal
 Pineoblastoma
 Pineocytoma

THE SKIN

Melanoma
 25 Squamous cell carcinoma
 Basal cell carcinoma
 Merkel cell carcinoma
 Extramammary Paget's Disease
 Paget's Disease of the
 30 nipple
 Kaposi's Sarcoma
 Cutaneous T-cell lymphoma

BONES, JOINTS, AND SOFT TISSUE TUMORS

Multiple Myeloma
 Malignant Lymphoma
 Chondrosarcoma
 Mesenchymal Chondrosarcoma
 Osteosarcoma
 Ewing Tumor (Ewing Sarcoma)
 Malignant Giant Cell Tumor
 Adamantinoma
 Malignant Fibrous
 Histiocytoma
 Desmoplastic Fibroma
 Fibrosarcoma
 Chordoma
 Hemangioendothelioma
 Kaposi's Sarcoma
 Liposarcoma
 Malignant Fibrous
 Histiocytoma
 Rhabdomyosarcoma
 Leiomyosarcoma
 Angiosarcoma

NERVOUS SYSTEM

Schwannoma
 Neurofibroma
 Malignant Peripheral Nerve
 Sheath Tumor
 Astrocytoma
 Fibrillary Astrocytoma
 Glioblastoma Multiforme
 Brain Stem Glioma
 Pilocytic Astrocytoma

- Pleomorphic
Xanthorastrocytoma
Oligodendroglioma
Ependymoma
5 Gangliocytoma
Cerebral Neuroblastoma
Central Neurocytoma
Dysembryoplastic
Neuroepithelial Tumor
10 Medulloblastoma
Malignant Meningioma
Primary Brain Lymphoma
Primary Brain Germ Cell
Tumor
- 15 **THE EYE**
Carcinoma
Squamous Cell Carcinoma
Mucoepidermoid Carcinoma
Melanoma
20 Retinoblastoma
Glioma
Meningioma
- THE HEART**
Myxoma
25 Fibroma
Lipoma
Papillary Fibroelastoma
Rhasdoyoma
Angiosarcoma
30 Other Sarcoma
- HISTIOCYTOSES**

As used herein, the term "specifically reactive" when used in reference to an antibody refers to the discriminatory binding of the antibody to the indicated target polypeptide. For such binding to be discriminating, the antibody will not substantially cross react with other polypeptides. Specific reactivity can include binding properties such as binding specificity, binding affinity and binding avidity. For example, an antibody can bind a target polypeptide with a binding affinity (Kd) of about 10^{-4} M or more, 10^{-6} M or more, 10^{-7} M or more, 10^{-8} M or more, 10^{-9} M or more, or 10^{-10} M or more. Several methods for detecting or measuring antibody binding are known in the art and disclosed herein.

As used herein, the term "sample" is intended to mean any biological fluid, cell, tissue, organ or portion thereof, that includes or potentially includes a neoplastic cell, such as a cell from the colon, rectum, breast, ovary, prostate, kidney, lung, blood, brain or other organ or tissue that contains or is suspected to contain a neoplastic cell. The term includes samples present in an individual as well as samples obtained or derived from the individual. For example, a sample can be a histologic section of a specimen obtained by biopsy, or cells that are placed in or adapted to tissue culture. A sample further can be a subcellular fraction or extract, or a crude or substantially pure nucleic acid molecule or protein preparation.

As used herein, the term "disease-free survival" refers to the lack of tumor recurrence and/or spread and the fate of a patient after diagnosis, for example, a patient who is alive without tumor recurrence. The phrase "overall survival" refers to the fate of the

patient after diagnosis, regardless of whether the patient has a recurrence of the tumor.

As used herein, the term "risk of recurrence" refers to the probability of tumor recurrence or spread in a patient subsequent to diagnosis of cancer, wherein the probability is determined according to the process of the invention.

Tumor recurrence refers to further growth of neoplastic or cancerous cells after diagnosis of cancer. Particularly, recurrence can occur when further cancerous cell growth occurs in the cancerous tissue. Tumor spread refers to dissemination of cancer cells into local or distant tissues and organs, for example during tumor metastasis. Tumor recurrence, in particular, metastasis, is a significant cause of mortality among patients who have undergone surgical treatment for cancer. Therefore, tumor recurrence or spread is correlated with disease-free and overall patient survival.

The invention relates to the use of TUCAN as a biomarker for prognosing survival and monitoring the effectiveness of a treatment for a cancer patient. TUCAN is a CARD domain-containing protein that has a role in regulating apoptosis. Apoptosis is a physiologic process that ensures homeostasis is maintained between cell production and cell turnover in essentially all self-renewing tissues. In addition to maintaining tissue homeostasis, apoptosis also occurs in response to a variety of external stimuli, including growth factor deprivation, alterations in calcium levels, free-radicals, cytotoxic lymphokines, infection by some viruses, radiation and most chemotherapeutic agents. Thus, apoptosis is an inducible event that likely is

subject to similar mechanisms of regulation as occur, for example, in a metabolic pathway. In this regard, dysregulation of apoptosis also can occur and is observed, for example, in some types of cancer cells, which survive for a longer time than corresponding normal cells, and in neurodegenerative diseases where neurons die prematurely. In viral infections, induction of apoptosis can figure prominently in the pathophysiology of the disease process, because immune-based eradication of viral infections depends on elimination of virus-producing host cells by immune cell attack resulting in apoptosis.

The principal effectors of apoptosis are a family of intracellular proteases known as Caspases, representing an abbreviation for Cysteine Aspartyl Proteases. Caspases are found as inactive zymogens in essentially all animal cells. During apoptosis, the caspases are activated by proteolytic processing at specific aspartic acid residues, resulting in the production of subunits that assemble into an active protease typically consisting of a heterotetramer containing two large and two small subunits (Thornberry and Lazebnik, Science 281:1312-1316 (1998)). The phenomenon of apoptosis is produced directly or indirectly by the activation of caspases in cells, resulting in the proteolytic cleavage of specific substrate proteins.

TUCAN contains at least two protein domains, one of which is a CARD (Caspase-Associated Recruitment Domain). CARDS are protein interaction motifs found in the N-terminal prodomains of several caspases and in apoptosis- regulatory proteins that either activate or suppress activation of CARD-containing pro-caspases. In

mammals, eight CARD-carrying caspases have been identified, including pro-caspases-1, 2, 4, 5, 9, 11, 12 and 13. To date, multiple non-caspase CARD-containing proteins have been discovered and functionally
5 characterized, including Apaf1, Nod1 (CARD4), NAC (DEPCAP), Raidd (CRADD), Cardiak (Rip2, RICK), Bcl10 (CIPER), ARC (Nop30), Asc, CARD9, CARD10, CARD11, CARD14, cIAP1, cIAP2, and CLAN. The CARD domains of many of these proteins are capable of binding the CARD-containing
10 prodomains of specific CARD-carrying caspases, either facilitating or inhibiting protease activation.

The CARD domain of TUCAN selectively binds to its own CARD and to pro-Caspase-9 (see Example IX). In addition, the binding of TUCAN to pro-caspase-9 has been
15 shown to interfere with the ability of pro-caspase-9 to interact with Apaf1. By inhibiting the interaction between pro-caspase-9 and Apaf1, TUCAN inhibits apoptosis signaling in the mitochondrial/cytochrome c pathway. Consistent with this observation is that finding that
20 over-expression of TUCAN reduces apoptosis induced by stimuli that are known to activate the mitochondrial pathway for caspase-activation, including Bax, DNA-damaging drugs, and staurosporine. In contrast, apoptosis induced via alternative pathways, including
25 GraB and Fas (TNF-family death receptor), is not inhibited by TUCAN. Further, over-expression of TUCAN in cells by stable or transient transfection inhibits apoptosis and caspase activation induced by Apaf1/caspase-9-dependent stimuli, including Bax, VP16,
30 and Staurosporine, but not by Apaf1/caspase-9-independent stimuli, Fas and Granzyme B. These cellular functions of TUCAN indicate that it has an important role in inhibiting mitochondrial signaling pathway-induced apoptosis.

TUCAN also contains an N-terminal domain that shares amino-acid similarity with a segment of the NAC protein, a CARD-carrying regulator of the Apaf1 apoptosome Chu et al. J Biol Chem 276:9239-9245 (2001) and Hlaing et al. J Biol Chem 276:9230-9238 (2001)). The TUCAN N-terminal domain contains several candidate phosphorylation sites, including PKC (S/T-x-R/K) sites at amino-acids 72, 286, 313, and 416, Casein kinase II (S/T-x-D/E) sites at 289, 376, 398, 414, and 416 and MAP kinase/CDK (S/T-P) sites at 187 and 289. The observed multiple forms of TUCAN identified by their different mobilities in SDS-PAGE experiments (see Figure 6B, for example) could be differently phosphorylated forms of TUCAN. TUCAN also contains a candidate caspase cleavage site (DEED) at residues 243-246.

These and other molecular characteristics and cellular functions of TUCAN are described, for example, in Pathan et al. J. Biol. Chem. 276:32220-32229 (2001), the entirety of which is incorporated herein by reference.

As disclosed herein in Example VII, relatively high levels of TUCAN are found in several human cancer cell lines. Moreover, as disclosed in Examples II and VIII, compared to normal colonic mucosa, TUCAN immunostaining was pathologically elevated in roughly two-thirds of early-stage colon cancers, indicating abnormal over-expression of this anti-apoptotic protein in association with malignant transformation. Studies of cells derived from pro-caspase-9 knock-out mice have indicated that pro-caspase-9 functions as a tumor suppressor in a p53-dependent pathway (Soengas et al. Science 284:156-159 (1999)). In view of the role of TUCAN in regulating pro-caspase-9, over-expression of

TUCAN can be functionally equivalent to loss of pro-caspase-9, indicating that elevated levels of TUCAN can promote tumor pathogenesis or progression. As shown herein in Examples IV and VIII, colon cancer patients
5 whose tumors contained higher levels of TUCAN indeed were more likely to die from their disease, based on retrospective analysis using archival specimens.

Therefore, the invention provides a method for determining a prognosis for survival for a cancer patient
10 using TUCAN. The method involves (a) measuring a level of a TUCAN in a neoplastic cell-containing sample from the cancer patient, and (b) comparing the level of TUCAN in the sample to a reference level of TUCAN, wherein low levels of TUCAN in the sample correlate with increased
15 survival of the patient.

A level of TUCAN in a neoplastic cell-containing sample that exceeds a determined basal level, or reference level, of TUCAN can be a significant factor in tumor recurrence or spread. When tumor cell
20 determined reference levels are exceeded, the level of TUCAN is characterized as high or overproduced. High or overproduced TUCAN can be indicative of increased risk of tumor recurrence or spread. Low or underproduced TUCAN can be indicative of decreased risk or tumor recurrence
25 or spread.

The methods of the invention for prognosing survival for a cancer patient involve obtaining a sample from a patient and measuring the level of one or more biomarkers, such as TUCAN. The level of the biomarker,
30 such as TUCAN, is used to determine the prognosis for disease-free or overall survival of a cancer patient based on the correlations provided herein. Such

prognosis is possible because the likelihood of tumor recurrence or spread correlates with the level of TUCAN in a tumor cell. For example, as shown in Examples VI and VIII, it has been found that when the levels of TUCAN expression are low, the likelihood of cancer recurrence is low. The level of TUCAN in a neoplastic-cell containing sample from a patient can be used as the sole factor in assessing disease status or can be used in addition to other predictive methods.

10 TUCAN can be used to prognose survival or monitor the effectiveness of a course of treatment for patients suffering from a variety of types of cancer. As described in Example VII, TUCAN is present in multiple different cancer cell types, including leukemia, melanoma
15 and breast, ovarian, lung, CNS, prostate and renal cancers. Also as described above, a cellular function of TUCAN is suppression of mitochondrial signaling pathway-induced apoptosis. Mitochondrial signaling pathway-induced apoptosis is an apoptotic mechanism that can
20 occur in any cell type, and that can become dysregulated or suppressed in any type cell, resulting in transformation of a cell such that it proliferates without normal homeostatic growth control. Therefore, a level of TUCAN can be correlated with tumor recurrence or
25 survival of a patient having any type of cancer. Using the guidance provided herein and other well-known methods, those skilled in the art will be able to determine if a level of TUCAN in a particular tumor cell type correlates with patient survival. Having determined
30 a correlation between a reference level of TUCAN and survival of a cancer patient, those skilled in the art can practice the methods for determining the prognosis for survival for a cancer patient and the method for

monitoring the effectiveness of a course of treatment for a patient with cancer described herein.

In the methods of the invention, a sample can
5 be, for example, a cell or tissue obtained using a biopsy procedure or can be a fluid sample containing cells, such as blood, serum, semen, urine, or stool. Those skilled in the art will be able to determine an appropriate sample, which will depend on cancer type, and an
10 appropriate method for obtaining a biopsy sample, if necessary. When possible, it can be preferable to obtain a sample from a patient using the least invasive collection means. For example, obtaining a fluid sample from a patient, such as blood, saliva, serum, semen,
15 urine or stool, is less invasive than collecting a tissue sample.

In one embodiment, a level of TUCAN can be determined by measuring the amount of a TUCAN using a selective binding agent, such as an antibody specifically
20 reactive with a TUCAN polypeptide. Other selective binding agents include polypeptides that bind to a TUCAN polypeptide, such as a TUCAN polypeptide that contains the TUCAN CARD domain (amino acids 345-431 (SEQ ID NO:3)), and a caspase 9 polypeptide, the amino acid
25 sequence of which (SEQ ID NO:4) is referenced as P55211 in the Prosite database, or modifications thereof that bind to a TUCAN polypeptide. Selective binding of TUCAN to pro-caspase and to itself is described in Example IX.

Essentially all modes of affinity binding
30 assays are applicable for use in determining a level of TUCAN, or another biomarker polypeptide, such as cIAP2, Apaf1, Smac, β -catenin, Bcl-2 or p53, in a sample. Such methods are rapid, efficient and sensitive. Moreover,

affinity binding methods are simple and can be modified to be performed under a variety of clinical settings and conditions to suit a variety of particular needs.

Affinity binding assays that are known and can be used in the methods of the invention include both soluble and solid phase formats. A specific example of a soluble phase affinity binding assay is immunoprecipitation using a biomarker selective antibody or other binding agent. Solid phase formats are advantageous for the methods of the invention since they are rapid and can be performed more easily on multiple different samples simultaneously without losing sensitivity or accuracy. Moreover, solid phase affinity binding assays are further amenable to high throughput screening and automation.

Specific examples of solid phase affinity binding assays include immunohistochemical binding assays, immunoaffinity binding assays such as an ELISA and radioimmune assay (RIA). Other solid phase affinity binding assays are known to those skilled in the art and are applicable to the methods of the invention. Although affinity binding assays are generally formatted for use with an antibody binding molecules that is selective for the analyte or ligand of interest, essentially any binding agent can be alternatively substituted for the selectively binding antibody. Such binding agents include, for example, macromolecules such as polypeptides, peptides, nucleic acid molecules, lipids and sugars as well as small molecule compounds. Methods are known in the art for identifying such molecules which bind selectively to a particular analyte or ligand and include, for example, surface display libraries and combinatorial libraries. Thus, for a molecule other than an antibody to be used in an affinity binding assay, all

that is necessary is for the binding agent to exhibit selective binding activity for a biomarker.

The various modes of affinity binding assays, such as immunoaffinity binding assays, include, for example, immunohistochemistry methods, solid phase ELISA and RIA as well as modifications thereof. Such modifications thereof include, for example, capture assays and sandwich assays as well as the use of either mode in combination with a competition assay format. The choice of which mode or format of immunoaffinity binding assay to use will depend on the intent of the user. Such methods can be found described in common laboratory manuals such as Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1999).

An antibody useful in the methods of the invention includes a polyclonal and monoclonal antibody, as well as an antigen binding fragment of such antibodies. Methods of preparing polyclonal or monoclonal antibodies are well known to those skilled in the art and are described in Example I and in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988).

An antibody useful in the methods of the invention also includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial

libraries consisting of variable heavy chains and variable light chains as described by Huse et al. (Science 246:1275-1281 (1989)). These and other methods of making, for example, chimeric, humanized, CDR-grafted, 5 single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989) ; Harlow and Lane, *supra*, 1988); Hilyard et al., Protein Engineering: A practical approach (IRL Press 10 1992); Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995)).

Formats employing affinity binding can be used in conjunction with a variety of detection labels and systems known in the art to quantitate amounts of 15 biomarkers in the analyzed sample. Detection systems include the detection of bound biomarker by both direct and indirect means. Direct detection methods include labeling of the biomarker-specifically reactive antibody or binding agent. Indirect detection systems include, 20 for example, the use of labeled secondary antibodies and binding agents.

Secondary antibodies, labels and detection systems are well known in the art and can be obtained commercially or by techniques well known in the art. The 25 detectable labels and systems employed with the biomarker-selective binding agent should not impair binding of the agent to the biomarker. Moreover, multiple antibody and label systems can be employed for detecting the bound biomarker-specifically reactive 30 antibody to enhance the sensitivity of the binding assay if desired.

Detectable labels can be essentially any label that can be quantitated or measured by analytical methods. Such labels include, for example, enzymes, radioisotopes, fluorochromes as well as chemi- and
5 bioluminescent compounds. Specific examples of enzyme labels include horseradish peroxidase (HRP), alkaline phosphatase (AP), β -galactosidase, urease and luciferase.

A horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate
10 tetramethylbenzidine (TMB), which yields a soluble product in the presence of hydrogen peroxide that is detectable by measuring absorbance at 450 nm. An alkaline phosphatase detection system can be used with the chromogenic substrate p-nitrophenyl phosphate, for
15 example, which yields a soluble product readily detectable by measuring absorbance at 405 nm. Similarly, a β -galactosidase detection system can be used with the chromogenic substrate o-nitrophenyl- β -D-galactopyranoside (ONPG), which yields a soluble product
20 detectable by measuring absorbance at 410 nm, or a urease detection system can be used with a substrate such as urea-bromocresol purple (Sigma Immunochemicals, St. Louis, MO). Luciferin is the substrate compound for luciferase which emits light following ATP-dependent
25 oxidation.

Fluorochrome detection labels are rendered detectable through the emission of light of ultraviolet or visible wavelength after excitation by light or another energy source. DAPI, fluorescein, Hoechst 33258,
30 R-phycoerythrin, B-phycoerythrin, R-phycoerythrin, rhodamine, Texas red and lissamine are specific examples of fluorochrome detection labels that can be utilized in the affinity binding formats of the invention. A

particularly useful fluorochrome is fluorescein or rhodamine.

Chemiluminescent as well as bioluminescent detection labels are convenient for sensitive,
5 non-radioactive detection of a biomarker and can be obtained commercially from various sources such as Amersham Lifesciences, Inc. (Arlington Heights, IL).

Alternatively, radioisotopes can be used as detectable labels in the methods of the invention.
10 Iodine-125 is a specific example of a radioisotope useful as a detectable label.

Signals from detectable labels can be analyzed, for example, using a spectrophotometer to detect color from a chromogenic substrate; a fluorometer to detect
15 fluorescence in the presence of light of a certain wavelength; or a radiation counter to detect radiation, such as a gamma counter for detection of iodine-125. For detection of an enzyme-linked secondary antibody, for example, a quantitative analysis of the amount of bound
20 agent can be made using a spectrophotometer such as an EMAX Microplate Reader (Molecular Devices, Menlo Park, CA) in accordance with the manufacturer's instructions. If desired, the assays of the invention can be automated or performed robotically, and the signal from multiple
25 samples can be detected simultaneously.

The prognostic formats of the present invention can be forward, reverse or simultaneous as described in U.S. Patent No. 4,376,110 and No. 4,778,751. Separation steps for the various assay formats described herein,
30 including the removal of unbound secondary antibody, can be performed by methods known in the art (Harlow and

Lane, *supra*). For example, washing with a suitable buffer can be followed by filtration, aspiration, vacuum or magnetic separation as well as by centrifugation.

A binding agent selective for a biomarker also
5 can be utilized in imaging methods that are targeted at biomarker- expressing neoplastic cells. These imaging techniques will have utility in identification of residual neoplastic cells at the primary site following standard treatments including, for example, surgical
10 resection of an organ of the gastrointestinal system, such as the colon, and radiation therapy. In addition, imaging techniques that detect neoplastic cells have utility in detecting secondary sites of metastasis. The biomarker specific binding agent can be radiolabeled
15 with, for example, ¹¹¹indium and infused intravenously as described by Kahn et al., Journal of Urology 152:1952-1955 (1994). The binding agent selective for a biomarker can be, for example, a monoclonal antibody specifically reactive with TUCAN or another biomarker, such as cIAP2,
20 Apaf1, Smac, β -catenin, Bcl-2 or p53. Imaging can be accomplished by, for example, radioimmunosciintigraphy as described by Kahn et al., *supra*.

The level of TUCAN, or another biomarker, such as cIAP2, Apaf1, Smac, β -catenin, Bcl-2 or p53, also can
25 be determined by measuring the amount of a biomarker mRNA or DNA using a binding agent selective for the biomarker, such as a nucleic acid probe. The methods used to detect mRNA levels include detection of hybridization or amplification of mRNA encoding the biomarker. This
30 detection can be carried out by analysis of mRNA either *in vitro* or *in situ* using one of the methods known to one of ordinary skill in the art as exemplified in the Current Protocols in Molecular Biology (John Wiley &

Sons, 1999); in U.S. Patent No. 5,882,864; and the like. A TUCAN mRNA, or other biomarker mRNA, detected will be any RNA transcript of a TUCAN gene, or fragment thereof, or cIAP2, Bcl-2, p53, b- catenin, survivin or Apaf1 gene,
5 or fragment thereof.

There are numerous methods well known in the art for detecting nucleic acid molecules by specific or selective hybridization with a complementary probe. Briefly, for detection by hybridization, a TUCAN nucleic
10 acid probe complementary to a TUCAN gene, having a detectable label is added to a neoplastic cell-containing sample obtained from the individual having, or suspected of having cancer under conditions which allow annealing of the probe to TUCAN RNA. Methods for detecting TUCAN
15 RNA in a sample can include the use of, for example, RT-PCR. Conditions are well known in the art for both solution and solid phase hybridization procedures. Moreover, optimization of hybridization conditions can be performed, if desired, by hybridization of an aliquot of
20 the sample at different temperatures, durations and in different buffer conditions. Such procedures are routine and well known to those skilled. Following annealing, the sample is washed and the signal is measured and compared with a suitable control or standard value. The
25 magnitude of the hybridization signal is directly proportional to the mRNA level of TUCAN. A level of TUCAN mRNA in a neoplastic cell-containing sample is compared to a suitable reference level for TUCAN mRNA. The levels of other biomarker mRNA, such as cIAP2, Apaf1,
30 Smac, β -catenin, Bcl-2 or p53, can be similarly determined and compared to a suitable reference level for the particular biomarker.

Other examples of methods include PCR and other amplification methods such as RT-PCR, 5' or 3' RACE, RNase protection, RNA blot, dot blot or other membrane-based technologies, dip stick, pin, ELISA or two-dimensional arrays immobilized onto a solid support. These methods can be performed using either qualitative or quantitative measurements, all of which are well known to those skilled in the art.

PCR or RT-PCR can be used with isolated RNA or crude cell lysate preparations. PCR is advantageous when there is limiting amounts of starting material. A further description of PCR methods can be found in, for example, Dieffenbach, C.W., and Dveksler, G.S., PCR Primer: A Laboratory Manual, Cold Spring Harbor Press, Plainview, New York (1995). Multisample formats such as microarrays offer the advantage of analyzing numerous, different samples in a single assay. In contrast, solid-phase dip stick-based methods offer the advantage of being able to rapidly analyze a patient's fluid sample for an immediate result.

Nucleic acid probes useful for measuring the expression level of a biomarker, such as cIAP2, TUCAN, Apaf1, β -catenin, Bcl-2, or Smac by hybridization include, for example, probes prepared using the nucleotide sequences provided herein. Nucleic acid molecules corresponding to the entire cDNA sequences and fragments thereof, including oligonucleotides corresponding to cIAP2, TUCAN, Apaf1, β -catenin, Bcl-2, or Smac nucleotide sequences and which are capable of specifically or selectively hybridizing to cIAP2, TUCAN, Apaf1, β -catenin, Bcl-2, or Smac RNA, are useful for hybridization methods.

A reference level is a level a biomarker, such as cIAP2, TUCAN, Apaf1, Smac, β -catenin, of Bcl-2, used to evaluate the level of the biomarker in cancerous cells of a patient. Specifically, when the level of a
5 biomarker in the cancerous cells of a patient are higher than the reference level, the cells will be considered to have a high level of, or overproduction, of the biomarker. Conversely, when the level of biomarker in the cancerous cells of a patient are lower than the
10 reference level, the cells will be considered to have a low level of, or underproduction, of the biomarker.

A high level of a biomarker, such as cIAP2, TUCAN, Apaf1, Smac, β -catenin, Bcl-2 or p53, or overproduction of a biomarker gene is related to a level
15 of the biomarker above a determined basal level. Thus, a reference or basal level of a biomarker, such as cIAP2, TUCAN, Apaf1, Smac, β -catenin, Bcl-2 or p53, in a cancer cell is identified as a "cutoff" value, above which there is a significant correlation between the presence of the
20 biomarker and increased or decreased tumor recurrence or spread. Those of skill in the art will recognize that some "cutoff" values are not sharp in that clinical correlations are still significant over a range of values on either side of the cutoff; however, it is possible to
25 select an optimal cutoff value (for example varying H-scores, and the like) of a level of a biomarker for a cancer cell type. It is understood that improvements in optimal cutoff values could be determined, depending on the sophistication of statistical methods used and on the
30 number and source of samples used to determine reference or basal values.

Such overproduction is not typically calculated in terms of absolute biomarker levels, but is determined

using relative measurements. These relative measurements are illustrated for quantitation purposes with an internal standard; however, it will be appreciated that other standards or methods of determination can be used, such as comparison with external standards, biomarker polypeptide measurements, biomarker mRNA measurements, absolute values of protein, mRNA or DNA levels, and the like.

A reference level can also be determined by comparison of biomarker levels in populations of patients having cancer, such as patients having cancer of the same stage. This can be accomplished by histogram analysis, in which the entire cohort of patients tested are graphically presented, wherein a first axis represents the level of a biomarker, and a second axis represents the number of patients in the cohort whose tumor cells contain the biomarker at a given level. Two or more separate groups of patients can be determined by identification of subsets populations of the cohort which have the same or similar levels of the biomarker. Determination of the reference level can then be made based on a biomarker level that best distinguishes these separate groups.

Verification that the reference level distinguishes the likelihood of tumor recurrence or spread in cancer patients expressing below-reference biomarker levels versus cancer patients expressing above-reference biomarker levels can be carried out using single variable or multi-variable analysis. These methods determine the likelihood of a correlation between one or more variables and a given outcome. In the specific case, the methods will determine the likelihood of a correlation between a biomarker levels (or biomarker

level coupled with another variable) and disease-free or overall survival of cancer patients. Any one of a plurality of methods well known to those of ordinary skill in the art for carrying out these analyses can be
5 used. Examples of single variable analysis is the Kaplan-Meir method or the log-rank test. An example of multi-variable analysis is the Cox proportional-hazards regression model (see, for example, Example VI).

Population-based determination of reference
10 levels, for example, by histogram analysis can be carried out using a cohort of patients sufficient in size in order to determine two or more separate groups of patients having different biomarker levels. Typically, such a cohort comprises at least 25 patients, such as at
15 least 50 patients, including at least 75 patients, and at least 100 patients. Similarly, verification of determined reference levels can also comprise at least 25 patients, such as at least 50 patients, including at least 75 patients, and at least 100 patients.

20 The reference level can be a single number, equally applicable to every patient, or the reference level can vary according to specific subpopulations of patients. For example, men might have a different reference level than women for the same cancer.
25 Furthermore, the reference level can be a level determined for each patient individually. For example, the reference level might be a certain ratio of a biomarker level in the tumor cells of a patient relative to the biomarker level in non-tumor cells within the same
30 patient. Thus the reference level for each patient can be proscribed by a reference ratio of biomarker levels, wherein the reference ratio can be determined by any of

the methods for determining the reference levels described above.

Further, while a reference level can separate two groups of patients, it is within the scope of the invention that numerous reference values might exist which separate a plurality of populations. For example, two reference values can separate a first group of patients with high levels of a biomarker from a second group of patients with intermediate levels the biomarker, and from a third group of patients with low levels of the biomarker. The number of different reference levels can be sufficient to proscribe a curve, such as a continuous line, which describes the likelihood of disease-free or overall survival in a patient as a function of the biomarker level in that patient. Such a curve will constitute a "continuous" biomarker level, where the likelihood of disease free or overall survival in a patient is proportional to the biomarker level in that patient. Two or more biomarker levels also can be represented by such a curve.

The reference level can also represent the level of a biomarker protein, such as cIAP2, TUCAN, Apaf1, Smac, β -catenin, Bcl-2 or p53, in one or more compartments of the cell. Typically, the reference level will represent the level of biomarker protein in (a) the whole cell, (b) the nucleus, or (c) the cytosol. This level will be useful when cell compartmentalization of the protein correlates with the risk of tumor recurrence or spread of a certain cancer. Similarly, the reference level can be a ratio of levels of biomarker protein in the different compartments (for example, the ratio of nuclear biomarker protein to whole cell biomarker

protein, or the ratio of nuclear to cytosolic biomarker protein).

The reference level of a biomarker, such as cIAP2, TUCAN, Apaf1, or Smac, can further be used in conjunction with another variable found to be a statistically significant indicator of the likelihood of disease-free or overall survival for cancer. Such indicators include the presence or levels of known cancer markers (for example, colon cancer markers include sialosyl-TnCEA, CA19-9, and LASA), or can be clinical or pathological indicators (for example, age, tumor size, tumor histology, clinical stage, family history and the like). For example, clinical stage of the cancer is also a statistically significant indicator of disease-free or overall survival, wherein the reference level of a biomarker can vary according to the clinical stage of the cancer. For example, the level of a biomarker, such as a low level of TUCAN, in conjunction with clinical stage II of a cancer for a given patient, together are indicators for increased likelihood of disease free or overall survival. Hence, the reference level of a biomarker can vary as a function of another statistically significant indicator of disease-free or overall survival for cancer.

The levels of biomarkers, such as cIAP2, Apaf1, TUCAN, Bcl-2 and Smac, in a cancer cell can correlate with each other and with other molecules because these molecules participate in common dysregulated molecular pathways that contribute to the hyperproliferative state of a cancer cell. Therefore a combination of TUCAN with one or more additional biomarkers can be used in the methods of the invention for determining a prognosis for survival for a cancer patient. A second or additional biomarker can be, for example, Apaf1, cIAP1, cIAP2,

survivin, AIF, Bcl-2, Bcl-XL, Bax, Bid, BAG1, p53, mutant p53, β -catenin, MIB-1 or another well-known tumor marker, such as the exemplary commercially available tumor markers described below. Furthermore, the use of a
5 combination of TUCAN with one or more biomarkers can provide increased prognostic significance or confidence in a prognostic determination.

Therefore, the invention provides a method for determining a prognosis for survival for a cancer patient
10 that involves the use of two or more biomarkers. The method is practiced by (a) measuring the levels of TUCAN and one or more biomarkers selected from the group consisting of cIAP2, Apaf1, Bcl-2 and Smac in a neoplastic cell-containing sample from the cancer
15 patient, and (b) comparing the level of TUCAN and the one or more selected biomarkers in the sample to a reference level of TUCAN and the biomarkers, wherein a low level of TUCAN and a high level of any of Apaf1, Bcl-2 or Smac, or a low level of TUCAN and a low level of cIAP2, in said
20 sample correlate with increased survival of said patient.

The methods of the invention can be practiced, for example, by selecting a combination of TUCAN and one or more biomarkers for which increased or decreased
25 expression correlates with improved survival, such as any of cIAP2, Apaf1, Bcl-2, Smac, or another known or standard biomarker for cancer. The selected biomarker can be a general diagnostic or prognostic marker useful for multiple types of cancer, such as CA 125, CEA or LDH,
30 or can be a cancer-specific diagnostic or prognostic marker, such as a colon cancer marker (for example, sialosyl-TnCEA, CA19-9, or LASA), breast cancer marker (for example, CA 15-2, Her-2/neu and CA 27.29), ovarian cancer marker (for example, CA72-4), lung cancer (for

example, neuron-specific enolase (NSE) and tissue polypeptide antigen (TPA)), prostate cancer (for example, PSA, prostate-specific membrane antigen and prostatic acid phosphatase), melanoma (for example, S-100 and TA-
5 90), as well as other biomarkers specific for other types of cancer. Those skilled in the art will be able to select useful diagnostic or prognostic markers for detection in combination with TUCAN. Similarly, three or more, four or more or five or more or a multitude of
10 biomarkers can be used together for determining a prognosis for survival for a cancer patient.

The use of two or more biomarkers can provide increased confidence in prognostic outcome. For example, as disclosed herein, combinations of low cIAP and low
15 TUCAN, and high Apaf1 and low TUCAN were correlated with increased disease-free survival (see Example V). In particular, among 33 patients examined for levels of cIAP and TUCAN in a neoplastic cell-containing sample, 97% of patients having low cIAP and low TUCAN remained alive
20 (91% disease-free), as opposed to 56% alive and 44% disease-free for other categories of patients. In addition, among 17 patients examined for levels of Apaf1 and TUCAN in a neoplastic cell-containing sample, 100% of patients having high Apaf1 and low TUCAN remained alive
25 and disease-free, as opposed to 65% alive and 53% disease-free for other categories of patients. Those skilled in the art will recognize that such correlations can be observed using other combinations of biomarkers using methods described herein.

30 Combinations of biomarkers useful in the prognostic methods of the invention include, for example, cIAP2 and TUCAN, Apaf1 and TUCAN, and a multiplicity of other combination of TUCAN with biomarkers such as cIAP2,

Apaf1, Bcl-2 and Smac and other molecules, including AIF, Bcl-2, Bcl-XL, Bax, Bid, BAG1, p53, mutant p53, β -catenin, MIB-1 and a variety of other general and tumor-specific biomarkers, such as commercially available
5 diagnostic markers described herein above. Such combinations can be useful indicators of the metastatic state of a cancer cell because elevated levels of these biomarkers was observed in a portion of all cancer specimens evaluated (see Example II). Further, elevated
10 levels of various biomarkers correlated with another colon cancer marker, Ki-67, and positive correlations between the expression of biomarkers was observed, for example, between cIAP2 and TUCAN ($p=0.003$) in patient populations.

15

The invention also provides a method for monitoring the effectiveness of a course of treatment for a patient with cancer. The method involves (a) determining the level of a TUCAN in a neoplastic cell-
20 containing sample from the cancer patient, and (b) determining the level of TUCAN in a neoplastic cell-containing sample from the patient after treatment, whereby comparison of the TUCAN level prior to treatment with the biomarker level after treatment indicates the
25 effectiveness of the treatment.

As used in the context of a course of treatment, "effectiveness" refers to the ability of the course of treatment to decrease the risk of tumor recurrence or spread and therefore to increase the
30 likelihood of disease-free or overall survival of the patient. This method will have particular utility when the level a biomarker, such as cIAP, TUCAN, Apaf1 or Smac, in the tumor cells of a patient is abnormal compared to the level of cIAP, TUCAN, Apaf1 and Smac in

the non-tumor cells of the patient. Comparison of biomarker levels in a neoplastic cell-containing sample from a patient before and after treatment will thereby serve to indicate whether a biomarker level is returning
5 to that of non-tumor cells, implying a more effective course of treatment, or whether a biomarker level is remaining abnormal or increasing in abnormality, implying a less effective course of treatment. For example, an increase in the level of Apaf1, Bcl-2 or Smac in a
10 patient sample after treatment indicates that treatment is effective because high levels of Apaf1 or Smac correlate with a lower incidence of colon cancer recurrence. Further, a low in the level of β -catenin, cIAP2 or TUCAN in a patient sample after treatment
15 indicates that treatment is effective because low levels of β -catenin, cIAP2 or TUCAN correlate with a lower incidence of colon cancer recurrence.

Patients having cancer can be classified according to whether a high level of a particular
20 biomarker, or a low level of the biomarker, is measured in a neoplastic cell-containing sample obtained from the patient. Determination of the prognosis for the patient can be made by determining whether the group to which the patient has been assigned correlates with a higher or
25 lower likelihood of disease-free or overall survival with respect to the group to which the patient was not assigned.

Therefore, the invention also provides a method of determining a prognosis for survival for a cancer
30 patient that involves patient classification. The method is practiced by (a) measuring a level of TUCAN in a neoplastic cell-containing sample from the cancer patient, and (b) classifying the patient as belonging to

either a first or second group of patients, wherein the first group of patients having low levels of TUCAN is classified as having an increased likelihood of survival compared to the second group of patients having high
5 levels of TUCAN.

A high level of TUCAN, or overproduction of TUCAN, correlates with patients having an increased risk of tumor recurrence or spread. Thus, patients belonging to a first group having high levels of TUCAN are
10 classified as having an increased risk of tumor recurrence or spread compared to a second group of patients having low levels TUCAN. Patients belonging to a first group having low levels of TUCAN are classified as having increased likelihood of survival compared to a
15 second group of patients having high levels of TUCAN.

The method of determining a prognosis for survival for a cancer patient can be practiced using one or more additional biomarkers. A variety of biomarkers,
20 including known cancer markers and the prognostic biomarkers disclosed herein, can be used in combination with TUCAN to determine a prognosis for survival for a cancer patient. In one embodiment, the method involves (a) determining a level of cIAP2 the neoplastic cell-
25 containing sample from the cancer patient, and (b) classifying the patient as belonging to either a first or second group of patient, wherein the first group of patients having low levels of TUCAN and low levels of cIAP2 is classified as having increased likelihood of
30 survival compared to the second group of patients having high levels of TUCAN and high levels of cIAP2.

In another embodiment, the method involves (a) determining a level of a biomarker selected from the

group consisting of Apaf1, Smac and Bcl-2 in the neoplastic cell-containing sample from the cancer patient, and (b) classifying the patient as belonging to either a first or second group of patient, wherein the
5 first group of patients having low levels of TUCAN and high levels of any of Apaf1, Smac or Bcl-2 is classified as having increased likelihood of survival compared to the second group of patients having high levels of TUCAN and low levels of any of Apaf1, Smac or Bcl-2.

10 After the levels of one or more biomarker in patient sample have been determined and compared to a reference level, the patient is then classified into a group having a certain likelihood of disease free or overall survival. Then the likelihood of disease-free or
15 overall survival for the patient is assessed based on the likelihood of disease-free or overall survival for patients in that group.

For example, a neoplastic cell containing sample from a cancer patient can be determined to have
20 high levels of Apaf1, Bcl-2 or Smac relative to a reference level. This patient would then be classified into a group of patients having high levels of Apaf1, Bcl-2 or Smac. Because it has been discovered that there is an increased likelihood of disease-free or overall
25 survival for the group of patients expressing high levels of Apaf1, Bcl-2 or Smac in cancer cells (relative to those expressing low levels of Apaf1, Bcl-2 or Smac in cancer cells), the specific cancer patient would be considered to have an increased likelihood of disease
30 free or overall survival.

Conversely, a neoplastic cell containing sample from a cancer patient can be determined to have high

levels of cIAP2, β -catenin or TUCAN relative to a reference level. This patient would then be classified into a group of patients having high levels of cIAP2, β -catenin or TUCAN. Because it has been discovered that
5 there is a decreased likelihood of disease-free or overall survival for the group of patients expressing high levels of cIAP2, β -catenin or TUCAN in cancer cells (relative to those expressing low levels of cIAP2, β -catenin or TUCAN in cancer cells), the specific cancer
10 patient would be considered to have an decreased likelihood of disease free or overall survival.

The methods of the invention are applicable to determining the susceptibility of an individual for developing cancer. The methods are applicable to a
15 variety of cancers, including gastrointestinal, lung, colon, prostate, breast, ovarian, skin, blood and kidney cancers. In particular, colon cancers develop from premalignant precursor lesions known as adenomatous colon polyps. Multiple epidemiological studies have
20 demonstrated that once one member of a family has developed an adenomatous colon polyp, his or her siblings are at markedly elevated risk for developing both colon adenomas and colon cancers. Those skilled in the art understand that the method of the invention can be
25 practiced as described herein for neoplastic conditions, including colon neoplastic conditions, such as adenomatous colon polyps, for example, by collecting an appropriate biopsy sample.

30 The methods of the invention for determining a prognosis for survival for a cancer patient are applicable to patients at any stage of tumor progression, and further can be used to determine a stage of tumor progress. A stage of a tumor refers to the degree of

progression of a tumor. Various stages of tumor development are well known to those of skill in the art, as exemplified in Markman, "Basic Cancer Medicine," Saunders, (ed. Zorab, R.) (1997). For example, cancers
5 can be staged into three general stages -- localized, regional spread, and distant spread. Cancers also can be staged using the TNM system, which considers the extent of direct spread within affected and nearby tissues, the extent of spread to nearby lymph nodes, and the extent of
10 spread to distant organs. Based on these features, spread of cancers can be summarized by assigning Roman numerals from 0 through IV. Those skilled in the art can select an appropriate staging system for a particular type of cancer.

15 In particular, colon cancer can be staged using the Dukes, Astler-Coller and AJCC/TNM systems, which describe the spread of the cancer in relation to the layers of the wall of the colon or rectum, organs next to the colon and rectum, and other organs farther away.
20 Dukes stage A is equivalent to AJCC/TNM stage I and Astler-Coller stage A, B1; Duke's stage B is equivalent to AJCC/TNM stage II and Astler-Coller stage B2, B3. Dukes stage C is equivalent to AJCC/TNM stage III and Astler-Coller stage C1, C2, C3. AJCC/TNM stages of
25 colorectal cancer are as follow: Stage 0: the cancer has not grown beyond the inner layer (mucosa) of the colon or rectum. This stage is also known as carcinoma in situ or intramucosal carcinoma; Stage I: the cancer has grown through the mucosa into the submucosa, or can also have
30 grown into the muscularis propria, but it has not spread outside the wall itself into nearby tissue such as lymph nodes; Stage II: the cancer has grown through the wall of the colon or rectum, into the outermost layers and may have invaded other nearby tissues, but has not yet spread

to the nearby lymph nodes; Stage III: the cancer can be of any size, but has spread to 3 or fewer nearby lymph nodes, or has spread to 4 or more nodes but it has not spread to other parts of the body; Stage IV: the cancer
5 has spread to distant organs such as the liver, lung, peritoneum or ovary.

Early stages of tumor development shall be understood to refer to stages in tumor development in which the tumor has detectably spread no further than the
10 lymph nodes local to the organ of the primary tumor. Typically, early stages will be considered to be stages I and II.

The predictive value of the method of the invention will be particularly effective in the case of
15 patients in the early stages of cancer. This is because the method of the invention is advantageously effective in determining the risk of metastasis in patients who demonstrate no measurable metastasis at the time of examination. One of ordinary skill in the art would
20 appreciate that the prognostic indicators of survival for cancer patients suffering from stage I cancer may be different from those for cancer patients suffering from stage IV cancer. For example, prognosis for stage I cancer patients may be oriented toward the likelihood of
25 continued growth and/or metastasis of the cancer, whereas prognosis for stage IV cancer patients may be oriented toward the likely effectiveness of therapeutic methods for treating the cancer.

A stage of cancer progression can be correlated
30 with a level of one or more biomarkers, such as a level of TUCAN, Apaf1 or an IAP, such as cIAP2 or Smac. Therefore, a determination of a level of a biomarker in a

sample from a cancer patient can be used to determine a stage of the tumor from which the sample was derived by comparing the sample with a reference level of the biomarker indicative of a particular stage of cancer.

5 The methods of the invention are applicable for use with a variety of different types of samples isolated or obtained from an individual having, or suspected of having a cancer or neoplastic condition. For example, samples applicable for use in one or more prognostic
10 formats of the invention, include tissue and cell samples. A tissue or cell sample can be obtained, for example, from a fluid sample obtained from the patient, by biopsy or surgery. For example, in the case of solid tumors which have not metastasized, a tissue sample from
15 the surgically removed tumor can be obtained and prepared for testing by conventional techniques. In addition, a sample can be removed from a patient, for example, using well-known biopsy procedures. For example, in the case of colon cancer, to obtain a sample of very small, raised
20 polyps, a colonoscope can be fitted with a snare to remove a polyp without damage to the wall of the colon (polypectomy); or to obtain small, flatter polyps, a biopsy forceps can be attached to a colonoscope to collect a small sample of tissue.

25 As described below, and depending on the format of the method, the tissue can be used whole or subjected to various methods known in the art to disassociate the sample into smaller pieces, cell aggregates or individual cells. Additionally, when combined with amplification
30 methods such as polymerase chain reaction (PCR), a single cell sample is sufficient for use in prognostic assays of the invention which employ hybridization detection methods. Similarly, when measuring biomarker polypeptide

levels, amplification of the signal with enzymatic coupling or photometric enhancement can be employed using only a few or a small number of cells.

Whole tissue obtained from a biopsy or surgery
5 is one example of a neoplastic cell-containing sample. Tumor tissue cell samples can be assayed employing any of the formats described below. For example, the tumor tissue sample can be mounted and hybridized *in situ* with biomarker nucleic acid probes. Similar histological
10 formats employing protein detection methods and *in situ* activity assays also can be used to detect a biomarker polypeptide in whole tissue tumor cell samples. Protein detection methods include, for example, staining with a biomarker specific antibody, as described herein, in
15 Example II. Such histological methods as well as others well known to those skilled in the art are applicable for use in the prognostic methods of the invention using whole tissue as the source of a neoplastic cell-containing sample. Methods for preparing and mounting
20 the samples are similarly well known in the art.

Individual cells and cell aggregates from an individual having, or suspected of having a neoplastic condition or cancer is another example of a neoplastic cell-containing sample that can be analyzed for increased
25 or decreased expression of biomarker RNA or polypeptide. The cells can be grown in culture and analyzed using procedures such as those described above. Whole cell samples expressing cell surface markers associated with biomarker expression can be rapidly tested using
30 fluorescent or magnetic activated cell sorting (FACS or MACS) with labeled binding agents selective for the surface marker or using binding agents selective for specific cell populations, for example, and then

determining a level of a biomarker within this population. A level of a biomarker can be determined using, for example, binding specifically reacting agents for a biomarker or by hybridization to a biomarker
5 specific probe. Other methods for measuring the level of a biomarker in whole cell samples are known in the art and are similarly applicable in any of the prognostic formats described below.

The tissue or whole cell tumor cell sample
10 obtained from an individual also can be analyzed for increased or decreased biomarker levels by lysing the cell and measuring the level of a biomarker in the lysate, a fractionated portion thereof or a purified component thereof using any of formats described herein.
15 For example, if a hybridization format is used, biomarker RNA can be amplified directly from the lysate using PCR, or other amplification procedures well known in the art such as RT-PCR, 5' or 3' RACE to directly measure the level of a biomarker nucleic acid molecules. RNA also
20 can be isolated and probed directly such as by solution hybridization or indirectly by hybridization to immobilized RNA. Similarly, when determining a level of a biomarker using polypeptide detection formats, lysates can be assayed directly, or they can be further
25 fractionated to enrich for a biomarker. For example, an immunochemical method, such as immunoblot analysis (see Example III) can be performed using a neoplastic cell-containing sample. Numerous other methods applicable for use with whole tumor cell samples are well known to those
30 skilled in the art and can accordingly be used in the methods of the invention.

The tumor tissue or cell sample can be obtained directly from the individual or, alternatively, it can be obtained from other sources for testing. Similarly, a cell sample can be tested when it is freshly isolated or
5 it can be tested following short or prolonged periods of cryopreservation without substantial loss in accuracy or sensitivity. If the sample is to be tested following an indeterminate period of time, it can be obtained and then cryopreserved, or stored at 4°C for short periods of time,
10 for example. An advantage of the prognostic methods of the invention is that they do not require histological analysis of the sample. As such, the sample can be initially disaggregated, lysed, fractionated or purified and the active component stored for later diagnosis.

15 The prognostic methods of the invention are applicable for use with a variety of different types of samples other than tumor cell samples. For example, a biomarker polypeptide or fragment thereof that is released into the extracellular space, including
20 circulatory fluids as well as other bodily fluids, can be used in prognostic methods to detect a secreted polypeptide or fragment related to a biomarker polypeptide. In such a case, the methods of the invention are applicable with fluid samples collected
25 from an individual having, or suspected of having a neoplastic condition or cancer.

Fluid samples, which can be measured for biomarker levels, include, for example, blood, serum, lymph, urine and stool. Other bodily fluids are known to
30 those skilled in the art and are similarly applicable for use as a sample in the prognostic methods of the invention. One advantage of analyzing fluid samples is that they are readily obtainable, in sufficient quantity,

without invasive procedures as required by biopsy and surgery. Analysis of fluid samples such as blood, serum and urine will generally be in the prognostic formats described herein which measure biomarker polypeptide
5 levels. As the biomarker related polypeptide is circulating in a soluble form, the methods will be similar to those which measure expression levels from cell lysates, fractionated portions thereof or purified components.

10 Neoplastic conditions and cancer can be diagnosed, predicted or prognosed by measuring a level of a biomarker in a neoplastic cell-containing sample, circulating fluid or other bodily fluid obtained from the individual. As described herein, levels of a biomarker
15 can be measured by a variety methods known in the art.

One skilled in the art can readily determine an appropriate assay system given the teachings and guidance provided herein and choose a method based on measuring RNA or polypeptide. Considerations such as the sample
20 type, availability and amount will also influence selection of a particular prognostic format. For example, if the sample is a tumor cell sample and there is only a small amount available, then prognostic formats which measure the amount of biomarker RNA by, for
25 example, PCR amplification, or which measure biomarker polypeptide by, for example, FACS analysis can be appropriate choices for determining the level of a biomarker. Alternatively, if the sample is a blood sample and the user is analyzing numerous different
30 samples simultaneous, such as in a clinical setting, then a multisample format, such as an Enzyme Linked Immunoabsorbant Assay (ELISA), which measures the amount of a biomarker polypeptide can be an appropriate choice

for determining the level of a biomarker. Additionally, biomarker nucleic acid molecules released into bodily fluids from the neoplastic or pathological cells can also be analyzed by, for example, PCR or RT-PCR. Those
5 skilled in the art will know, or can determine which format is amenable for a particular application and which methods or modifications known within the art are compatible with a particular type of format.

Nucleic acid probes can be produced
10 recombinantly or chemically synthesized using methods well known in the art. Additionally, hybridization probes can be labeled with a variety of detectable labels including, for example, radioisotopes, fluorescent tags, reporter enzymes, biotin and other ligands. Such
15 detectable labels can additionally be coupled with, for example, colorimetric or photometric indicator substrate for spectrophotometric detection. Methods for labeling and detecting such probes are well known in the art and can be found described in, for example, Sambrook et al.,
20 Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainview, New York (1989), and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999).

Nucleic acid probes useful for detecting a
25 biomarker in a sample can be hybridized under various stringency conditions readily determined by one skilled in the art. Depending on the particular assay, one skilled in the art can readily vary the stringency conditions to optimize detection of a particular
30 biomarker in a particular sample type.

In general, the stability of a hybrid is a function of the ion concentration and temperature.

Typically, a hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Moderately stringent hybridization refers to conditions that permit a nucleic acid molecule such as a probe to bind a complementary nucleic acid molecule. The hybridized nucleic acid molecules generally have at least 60% identity, at least 75% identity, at least 85% identity; or at least 90% identity. Moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 42°C. High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C.

Low stringency hybridization refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 22°C, followed by washing in 1X SSPE, 0.2% SDS, at 37°C. Denhart's solution contains 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin (BSA). 20X SSPE (sodium chloride, sodium phosphate, ethylene diamide tetraacetic acid (EDTA)) contains 3M sodium chloride, 0.2M sodium phosphate, and 0.025 M (EDTA). Other suitable moderate stringency and high stringency hybridization buffers and conditions are well known to those of skill in the art and are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainview, New York (1989); and Ausubel et al., *supra*, 1999). Nucleic acid molecules encoding polypeptides hybridize under moderately stringent or high stringency conditions to substantially the entire sequence, or substantial

portions, for example, typically at least 15-30 nucleotides of the nucleic acid sequences of cIAP2, TUCAN, Apaf1, Bcl-2, Smac, β -catenin or another biomarker.

5

The invention relates to the discovery that high or low amounts of particular biomarkers, including cIAP2, TUCAN, Apaf1, Bcl-2, β -catenin and Smac are predictive of survival of patients having cancer. The over-expression or under-expression of these biomarkers can contribute to the genetic malfunction of cancer cells that leads to uncontrolled proliferation. Therefore, modulation of the level of a biomarker in a cancer cell to a level consistent with a normal cell can be used to return a cancer cell to a more normal proliferation state. In the case of over-expressed biomarker genes, such as cIAP2, TUCAN and b-catenin a variety of strategies can be employed to reduce gene expression. For example, inhibition of transcription or translation of cIAP2, TUCAN and b-catenin, or reduction in the amount of active cIAP2, TUCAN and b-catenin polypeptide, can be used to reduce the levels of these biomarkers to a level representative of a normal cell. In the case of under-expressed biomarker genes, such as Apaf1, Bcl-2 and Smac, a variety of strategies can be employed to increase gene expression. For example, introduction of Apaf1, Bcl-2 and Smac from an exogenous nucleic acid molecule, promotion of transcription or translation of Apaf1, Bcl-2 or Smac, or promotion in the amount of active Apaf1, Bcl-2 or Smac polypeptide, can be used to increase the levels of these biomarkers to a level representative of a normal cell.

Therefore, the invention additionally provides a method for treating or reducing the progression of a

neoplastic condition such as cancer by reducing neoplastic cell proliferation. In one embodiment, the method involves administering a nucleic acid encoding Apaf1, Bcl-2 or Smac into a neoplastic cell and
5 expressing the Apaf1, Bcl-2 or Smac polypeptide in an amount effective to reduce neoplastic cell proliferation. In another embodiment, the method of reducing neoplastic cell proliferation involves contacting a neoplastic cell with an effective amount of an agent that, under
10 sufficient conditions, increases the amount of Apaf1, Bcl-2 or Smac in the cell.

Such an agent can increase the amount of a biomarker directly or indirectly, for example, by
15 increasing the amount of a biomarker polypeptide in a cell, such as by stimulating increased mRNA expression. Apaf1, Bcl-2 or Smac mRNA expression can be increased, for example, by inducing or derepressing transcription of Apaf1, Bcl-2 or Smac genes and by regulating the
20 expression of a cellular protein that acts as a transcription factor to regulate gene expression. An agent can act to increase the amount of Apaf1, Bcl-2 or Smac by increasing the stability of a Apaf1, Bcl-2 or Smac mRNA or polypeptide, for example, by decreasing a
25 cellular degradation activity, such as a protease activity. Molecules that mediate the regulation of Apaf1, Bcl-2 or Smac expression, such as receptors and corresponding signal transduction molecules, can also be targets of agents that increase the amount of Apaf1,
30 Bcl-2 or Smac in a cell. For example, a signal transduction pathway that stimulates the expression of Apaf1, Bcl-2 or Smac can be modulated to increase the level of Apaf1, Bcl-2 or Smac expression, for example, by increasing the rate of Apaf1, Bcl-2 or Smac synthesis or
35 the length of time that gene expression remains active.

Conversely, a decrease in the amount of a biomarker in a cell can be affected by inducing changes in biomarker transcription, translation or protein stability opposite to those described above. As such, in a further embodiment, the method of reducing neoplastic cell proliferation involves contacting a neoplastic cell with an effective amount of an agent that, under sufficient conditions, decreases the amount of cIAP2, b-catenin or TUCAN in the cell.

10 The amount of a biomarker in a cell, such as cIAP2, TUCAN, b-catenin, Bcl-2, Apaf1 or Smac, can be modulated, for example, by increasing expression of the biomarker from an exogenous nucleic acid molecule, by introducing a biomarker polypeptide or functional analog thereof into a cell, by introducing inhibitor of a biomarker polypeptide into a cell, and by modulating the expression or activity of a gene or protein product that regulates the level of a biomarker in a cell. The amount of a biomarker in a cell also can be modulated using an antisense molecule to block transcription or translation of the biomarker mRNA. Specifically, cells can be transformed with sequences complementary to cIAP2, β -catenin or TUCAN nucleic acid molecules. Such methods are well known in the art, and sense or antisense oligonucleotides or larger fragments, can be designed from various locations along the coding or control regions of sequences encoding biomarkers. Thus, antisense molecules can be used to modulate biomarker activity, or to achieve regulation of gene function.

30 Ribozymes, enzymatic RNA molecules, can also be used to catalyze the specific cleavage of a biomarker mRNA, such as cIAP2, β -catenin or TUCAN. The mechanism of ribozyme action involves sequence-specific

hybridization of the ribozyme molecule to complementary target biomarker RNA, followed by endonucleolytic cleavage. Specific ribozyme cleavage sites within any potential RNA target are identified by scanning the biomarker RNA for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for secondary structural features which can render the oligonucleotide inoperable. The suitability of candidate targets can also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. Antisense molecules and ribozymes of the invention can be prepared by any method known in the art for the synthesis of nucleic acid molecules.

RNA interference (RNAi) can also be used to modulate the amount of a biomarker mRNA, such as cIAP2, β -catenin or TUCAN. RNAi is a process of sequence-specific gene silencing by post-transcriptional RNA degradation, which is initiated by double-stranded RNA (dsRNA) homologous in sequence to the silenced gene. A suitable double-stranded RNA (dsRNA) for RNAi contains sense and antisense strands of about 21 contiguous nucleotides corresponding to the gene to be targeted that form 19 RNA base pairs, leaving overhangs of two nucleotides at each 3' end (Elbashir et al., Nature 411:494-498 (2001); Bass, Nature 411:428-429 (2001); Zamore, Nat. Struct. Biol. 8:746-750 (2001)). dsRNAs of about 25-30 nucleotides have also been used successfully for RNAi (Karabinos et al., Proc. Natl. Acad. Sci. 98:7863-7868 (2001). dsRNA can be synthesized in vitro and introduced into a cell by methods known in the art.

A variety of methods are known in the art for introducing a nucleic acid molecule into a cell, including a cancer cell. Such methods include microinjection, electroporation, lipofection, calcium-phosphate mediated transfection, DEAE-Dextran-mediated transfection, polybrene- or polylysine-mediated transfection, and conjugation to an antibody, gramicidinS, artificial viral envelopes or other intracellular carriers such as TAT. For example, cells can be transformed by microinjection as described in Cibelli et al., Nat. Biotech. 16:642-646 (1998) or Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995); by lipofection as described in Choi (U.S. Pat # 6,069,010) or Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995); by electroporation as described in Current Protocols in Molecular Biology, John Wiley and Sons, pp 9.16.4-9.16.11 (2000) or Cibelli et al., Nat. Biotech. 16:642-646 (1998); or by fusion with yeast spheroplasts Lamb and Gearhart, Cur. Opin. Gen. Dev. 5:342-348 (1995).

A nucleic acid encoding a biomarker polypeptide, such as Apaf1, Bcl-2 or Smac, or other molecule useful for reducing proliferation of a cancer cell, can be delivered into a mammalian cell, either *in vivo* or *in vitro* using suitable vectors well-known in the art. Suitable vectors for delivering a nucleic acid encoding a biomarker polypeptide to a mammalian cell, include viral vectors and non-viral vectors such as plasmid vectors. Such vectors are useful for providing therapeutic amounts of a biomarker polypeptide, such as Apaf1, Bcl-2 or Smac, as well as for delivering antisense nucleic acid molecules and ribozymes.

Viral based systems provide the advantage of being able to introduce relatively high levels of the heterologous nucleic acid into a variety of cells. Suitable viral vectors for introducing a nucleic acid encoding a biomarker polypeptide, such as Bcl-2, Smac or Apaf1, into a mammalian cell are well known in the art. These viral vectors include, for example, Herpes simplex virus vectors (Geller et al., Science, 241:1667-1669 (1988)); vaccinia virus vectors (Piccini et al., Meth. Enzymology, 153:545-563 (1987)); cytomegalovirus vectors (Mocarski et al., in Viral Vectors, Y. Gluzman and S.H. Hughes, Eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988, pp. 78-84)); Moloney murine leukemia virus vectors (Danos et al., Proc. Natl. Acad. Sci. USA, 85:6460-6464 (1988); Blaese et al., Science, 270:475-479 (1995); Onodera et al., J. Virol., 72:1769-1774 (1998)); adenovirus vectors (Berkner, Biotechniques, 6:616-626 (1988); Cotten et al., Proc. Natl. Acad. Sci. USA, 89:6094-6098 (1992); Graham et al., Meth. Mol. Biol., 7:109-127 (1991); Li et al., Human Gene Therapy, 4:403-409 (1993); Zabner et al., Nature Genetics, 6:75-83 (1994)); adeno-associated virus vectors (Goldman et al., Human Gene Therapy, 10:2261-2268 (1997); Greelish et al., Nature Med., 5:439-443 (1999); Wang et al., Proc. Natl. Acad. Sci. USA, 96:3906-3910 (1999); Snyder et al., Nature Med., 5:64-70 (1999); Herzog et al., Nature Med., 5:56-63 (1999)); retrovirus vectors (Donahue et al., Nature Med., 4:181-186 (1998); Shackelford et al., Proc. Natl. Acad. Sci. USA, 85:9655-9659 (1988); U.S. Patent Nos. 4,405,712, 4,650,764 and 5,252,479, and WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829; and lentivirus vectors (Kafri et al., Nature Genetics, 17:314-317 (1997)). It is understood that both permanent and transient expression can be useful in a method of the invention.

An Apaf1, Bcl-2 or Smac polypeptide-encoding recombinant nucleic acid can be directed into a particular tissue or organ system, for example, by vector targeting or tissue-restricted gene expression.

5 Therefore, a vector useful for therapeutic administration of a nucleic acid encoding an Apaf1, Bcl-2 or Smac polypeptide can contain a regulatory element that provides tissue specific expression of the polypeptide. For example, a nucleic acid sequence encoding a Apaf1,
10 Bcl-2 or Smac polypeptide can be operatively linked to a cell specific promoter.

Any of a variety of inducible promoters or enhancers can also be included in a nucleic acid or vector of the invention to allow control of expression of
15 a Apaf1, Bcl-2 or Smac polypeptide, or another molecule useful for modulating cell proliferation, such as an antisense nucleic acid molecule or ribozyme, by added stimuli or molecules. Such inducible systems, include, for example, tetracycline inducible system (Gossen &
20 Bizard, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992); Gossen et al., Science, 268:1766-1769 (1995); Clontech, Palo Alto, CA); metallothionein promoter induced by heavy metals; insect steroid hormone responsive to ecdysone or related steroids such as muristerone (No et al., Proc.
25 Natl. Acad. Sci. USA, 93:3346-3351 (1996); Yao et al., Nature, 366:476-479 (1993); Invitrogen, Carlsbad, CA); mouse mammary tumor virus (MMTV) induced by steroids such as glucocorticoid and estrogen (Lee et al., Nature, 294:228-232 (1981); and heat shock promoters inducible by
30 temperature changes.

An inducible system particularly useful for therapeutic administration utilizes an inducible promoter that can be regulated to deliver a level of therapeutic

product in response to a given level of drug administered to an individual and to have little or no expression of the therapeutic product in the absence of the drug. One such system utilizes a Gal4 fusion that is inducible by
5 an antiprogesterin such as mifepristone in a modified adenovirus vector (Burien et al., Proc. Natl. Acad. Sci. USA, 96:355-360 (1999)). The GENE SWITCH inducible expression system (US Patent Nos. 5,935,934 and 5,874,534) is an example of such a system. Other
10 inducible systems use the drug rapamycin to induce reconstitution of a transcriptional activator containing rapamycin binding domains of FKBP12 and FRAP in an adeno-associated virus vector (Ye et al., Science, 283:88-91 (1999)), use tetracycline to control
15 transcription (Baron Nucleic Acids Res. 25:2723-2729 (1997)) and use synthetic dimerizers to regulate gene expression (Pollock et al., Methods Enzymol. 306:263-281 (1999)). Such a regulatable inducible system is advantageous because the level of expression of the
20 therapeutic product can be controlled by the amount of drug administered to the individual or, if desired, expression of the therapeutic product can be terminated by stopping administration of the drug.

It is understood that modifications which do
25 not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Generation of Antibodies for Immunodetection of
IAPs and Apaf1

5 This example shows preparation and
characterization of antibodies useful for detecting IAPs
and Apaf1.

 Antisera were raised against recombinant
proteins and synthetic peptides for immunodetection of
10 Survivin, XIAP, Apaf1, AIF and Smac. Prior to employing
these antibodies for analysis of cancers, the specificity
of these antibodies for their intended protein targets
was confirmed by SDS-PAGE/immunoblot analysis. Examples
of data are provided in Figure 3. Figure 3A shows in
15 vitro translated Survivin, XIAP, cIAP1, cIAP2, NAIP,
BRUCE, and baculovirus Cp-IAP proteins were subjected to
SDS-PAGE/immunoblot analysis, using polyclonal XIAP
antiserum (AR-27A). Incubation with XIAP antiserum
detected only XIAP in vitro translated protein.
20 Detergent lysates were prepared from various normal human
tissues, normalized for total protein content (50 ug),
and subjected to SDS-PAGE/immunoblot assay using antisera
specific for Survivin (B), Apaf1 (C), SMAC (D) or AIF
(E); molecular weight markers are indicated in
25 kilo-Daltons (F). In addition, lysates from 5 matched
pairs of colon carcinoma (T) and normal colonic mucosa
(N) specimens were analyzed for total protein content
(100 mg per lane) and subjected to SDS-PAGE/immunoblot
analysis, using the antisera specific for c-IAP1, c-IAP2,
30 XIAP, Survivin, Apaf-1, and TUCAN (G). Antibody
detection was accomplished by an ECL method. Immunoblot
data were quantified by scanning densitometry using
Pro-Image software system.

The anti-XIAP antiserum reacted specifically with the expected ~ 57 kDa XIAP protein, but not with other IAP-family members including Survivin, cIAP1, cIAP2, NAIP, BRUCE, and baculovirus Cp-IAP - which were all produced by in vitro transcription and translation from cDNAs (Figure 3A). Similarly, monospecificity of the anti-Survivin antiserum was demonstrated by SDS-PAGE/immunoblot analysis of recombinant IAP-family proteins and lysates from normal tissues which lack Survivin mRNA and protein versus tumor cell lines which express Survivin protein (Figure 3B). The anti-Smac antiserum displayed specific reactivity against GST-Smac recombinant protein (Figure 3C). The antibody detected abundant amounts of Smac protein in RS11 and Jurkat cells, and several human tissues, such as epidermis, brain and testis. Barely detectable Smac levels in normal colon contrasted with relatively high amount of this protein in a colon cancer lysate.

Polyclonal antisera for Survivin, Apaf1, XIAP and Smac were generated in rabbits using recombinant protein immunogens. Survivin (full-length protein) and Apaf1 (residues 264 -282) were produced as GST-fusion proteins from pGEX vectors using *Escherichia coli* BL21 (DE3) as the host strain. The protein purification method has been described previously. An additional anti-Apaf-1 serum was generated in rabbits using a synthetic peptide as the immunogen. A peptide (NH₂ -CGPKYVVPVESSLGKEKGL -amide (SEQ ID NO:15)) corresponding to residues 264-282 of human (hu) Apaf-1, was synthesized with an N-terminal cysteine appended to permit conjugation to maleimide-activated carrier proteins KLH and OVA (Pierce, Inc.). This peptide conjugate was used to generate a polyclonal antiserum (AR-23) in rabbits. Affinity-purified His 6 -tagged - XIAP BIR2 recombinant

protein was produced using published methods and was used as an immunogen to produce XIAP-specific antiserum (AR-27A). An anti-AIF serum was produced in rabbits using a synthetic peptide corresponding to residues 151-170 of human AIF. New Zealand white female rabbits were injected subcutaneously with a mixture of 0.25 ml KLH-peptide (1 mg/ml), 0.25 ml OVA-peptide (1 mg/ml), or recombinant protein (0.1 - 0.25 µg protein per immunization) and 0.5 ml Freund's complete adjuvant (dose divided over 10 injections sites) and then boosted 3 times at weekly intervals, followed by another 3 - 20 boostings at monthly intervals with 0.25 mg each of KLH-peptide, OVA-peptide, or recombinant protein immunogens in Freund's incomplete adjuvant, collecting blood at 1-3 weeks after each boosting to obtain immune serum. The generation of Bcl-2, Bcl-XL, Bax, and TUCAN-specific antisera has been described. Anti-c-IAP1 and c-IAP2 antibodies were obtained from Santa Cruz Biotechnology Inc., CA and R&D Systems, Inc., β-Catenin antiserum from BD Transduction Laboratories, and p53 clone DO-7, MIB-1, and BAG1 clone KS-6C8 from DAKO.

EXAMPLE II

Immunohistochemical Analysis of IAPs and Other Biomarkers in Normal Colonic Mucosa and Colon Cancer

This example shows immunohistochemical analysis of IAPs and other biomarkers in a tissue microarray representing tissue samples obtained from 102 individuals.

A tissue microarray was constructed using primary tumor specimens derived from a relatively homogenous cohort of 102 patients presenting with stage II disease (Dukes' B stage) to a single institution, and

who were treated by surgical resection with curative intent. Colon carcinoma specimens were obtained from Department of Pathology, Yonsei University, College of Medicine, Seoul, Korea. Samples were taken from primary
5 tumors derived from patients who presented between 1986 and 1996 with Dukes' B stage [stage II disease, as defined by American Joint Committee on Cancer and Union Internationale Contre le Cancer (AJCC/UICC) criteria]. Patients with Dukes' stage B2 (T3N0M0) constituted 91% of
10 the cohort, whereas 9% suffered from a Dukes' B3 (T4N0M0) cancer. All patients were treated by surgical resection of the involved segment of colon. No postoperative adjuvant chemotherapy was performed initially in all cases. However, chemotherapy was administered for some
15 patients after relapse. Clinical data represent a median follow up of 60 months.

To construct colon cancer microarrays, 2-5 cylinders of 1 mm diameter tissue were taken from representative areas of archival paraffin blocks
20 containing 8% formalin-fixed tumor and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). Serial sections (4 μ m) were applied to 3-aminopropyl-triethoxysilane (APES)-coated slides (Sigma).

25 Microarrays were immunostained using antisera specific for the IAP family members Survivin, XIAP, cIAP1, and cIAP2 (Figure 1A), and other markers such as Apaf1, Smac, AIF, Bcl-2, Bcl-XL, Bax, BAG1, β -Catenin, MIB-1 and p53. Dewaxed tissue sections were
30 immunostained using a diaminobenzidine (DAB)-based detection method as described in detail, employing the Envision-Plus-Horse Radish Peroxidase (HRP) system (DAKO) using an automated immunostainer (Dako Universal Staining

System). Antisera specific for Survivin, XIAP, Apaf1, TUCAN, AIF, Smac, Bax, and Bid were applied at 1:3000 to 10000 (v/v), for Bcl-2 and Bcl-XL at 1:2000 (v/v). The dilutions of c-IAP1, c-IAP2 and β -Catenin antibodies were 1:600 (v/v), BAG1 and MIB-1 1:100, and p53 1:50. For all tissues examined, the immunostaining procedure was performed in parallel using preimmune serum to verify specificity of the results. Initial confirmations of antibody specificity also included experiments in which antiserum was preabsorbed with 5 - 10 μ g/ml of either synthetic peptide immunogen or recombinant protein immunogen. The scoring of tumor immunostaining was based on the percentage of immunopositive cells (0-100) multiplied by staining intensity score (0/1/2/3), yielding scores of 0-300. All immunostaining results were quantified according the approximate percentage of immunopositive cells (0-100%) and immunointensity on a 0-3 scale, and then an immunoscore was calculated from the product of the percentage immunopositivity and immunointensity (0-300).

Tissue sections were immunostained using various antisera, as described above, followed by detection using a HRPase-based method with diaminobenzidine colorimetric substrate (brown). Nuclei were counterstained with hematoxylin (blue). Representative data are shown in Figure 1. Figure 1A shows a colon cancer microarray slide stained for cIAP2 (x5 magnification). Examples of normal colonic epithelium immunostaining are presented for cIAP1 (B; x100), Survivin (D; x150), Smac (E; x150), AIF (G; x150), and TUCAN (K; x20). Immunostaining results in regions of invasive cancer are shown for Smac (F; x400), AIF (H; x250), Apaf1 (I, J; x200), TUCAN (L x20; M x400), and Bcl-2 (N; x150). Examples of malignant and the adjacent

normal colonic epithelium are presented for cIAP2 (C; x40), p53 (O; x150) and MIB-1 (P; x400).

Several of the 102 tumor specimens on the array (~ 65%) contained adjacent normal colonic mucosa (59-70), depending on the particular slide), permitting side-by-side comparisons of immunostaining results for normal versus malignant epithelium. In addition, 4 specimens of normal colon derived from individuals who were not diagnosed with colon cancer were stained separately. Immunoreactivity for the cIAP1 and cIAP2 proteins was detected in 62/62 (100%) and 34/65 (52%) of normal colonic mucosa specimens examined, respectively. The intensity of cIAP1 staining in non-malignant epithelium progressively increased from the base of the crypts to the luminal surface (Figure 1B). In contrast, low cIAP2 immunoreactivity was more evenly distributed along the crypt-villus axis, though a slight increase in immunointensity in the upper regions of the villi was sometimes evident. XIAP was also expressed in non-malignant colonic epithelium (63/63 [100%]) and was distributed in a gradient similar to cIAP1, with XIAP immunoreactivity highest in the upper portions of the villi. Low intensity Survivin immunostaining was present in 60/62 (97%) of specimens containing normal colonic epithelium. Survivin immunoreactivity was predominantly nuclear in the crypt epithelial cells, and became progressively stronger in intensity and predominantly cytoplasmic towards the luminal surface along the crypt-villus axis (Figure 1D). Immunohistochemical analysis of Apaf1 in normal colonic mucosa revealed the presence of immunoreactivity in 58/60 (97%) of specimens. Apaf1 immunoreactivity was present predominantly in peri-nuclear and cytosolic locations in normal colonic epithelial cells, with the intensity slightly increasing

as the cells migrated from the crypt bases to the upper regions of the villi. Along with Apaf1, the intracellular concentration of Smac protein increased towards the luminal surface in 58/62 (94%) of normal colonic mucosa specimens (Figure 1E). A relatively high mostly granular cytosolic expression of AIF was uniformly distributed along the colonic crypts in 100% of specimens (60/60) (Figure 1G). The specificity of these immunostaining results was confirmed by control stainings performed using either preimmune serum or immune antisera which had been preabsorbed with the relevant immunogens.

Immunohistochemical analysis of tumor tissues on the microarray revealed several examples of cancer-specific alterations in the expression of these apoptosis-regulatory proteins. Figure 1 shows some examples of the immunostaining results for tumor specimens. The mean intensity of immunostaining was significantly higher in the invasive cancer compared to normal colonic epithelium for all investigated proteins (Figure 1C, E-F, K-M, O, P) with the exception of Bcl-2, Bax, and AIF (Figure 1G, H). Moreover, while immunostaining results varied widely among specimens examined, the immunoscores for a portion of the cancer specimens clustered into groups displaying clear elevations in immunoreactivity when compared to normal specimens (Figure 2). For example, while all normal colonic specimens had cIAP1 immunoscores of <200, 35 of 94 (37%) invasive cancer specimens had immunoscores of = 200 ($p < .0001$), thus suggesting that a subgroup of colon cancers develops pathological elevations in the levels of this anti-apoptotic protein. Similarly, cIAP2 immunoscores were < 100 for normal colonic epithelium, in contrast to invasive cancers where 25 of 94 (27%) had immunoscores of >100 ($p < .0001$). Likewise, all normal

colonic epithelium samples possessed immunoscores of <190 for XIAP, while 34 of 97 invasive cancers (35%) had XIAP immunoscores of > 190 ($p < .0001$). Survivin immunoscores for non-malignant epithelium were < 190, compared to
5 invasive cancers where scores > 190 were found for 33 of 100 (33%) of specimens ($p < .0001$). For Apaf1, two clusters of immunoscores emerged for both normal and malignant epithelium. Most normal colonic specimens (50/60; 83%) had immunoscores < 100. In tumors, a group
10 of specimens with similarly low immunoscores (< 100) was observed (64/102; 63%) (Figure 1J) but an additional group of cancers (38/102; 37%) was identified in which immunoscores clustered above 100, ranging from 140-280 (Figure 1I). These results show that for all biomarkers
15 examined, evidence of tumor-associated upregulation of expression was observed in a portion of the cancer specimens evaluated.

Elevated levels of cIAP2, Survivin, and β -Catenin correlated with high Ki-67 labeling index ($p =$
20 0.006 , $p = 0.005$, and $p = < .0001$, respectively). Statistical analysis revealed a significant correlation between the levels of Survivin and those of XIAP and cIAP1 ($p = 0.01$), or cIAP2 ($p = 0.008$). Elevated levels of survivin were associated with high expression of
25 Bcl-2. A positive correlation between the expression of cIAP2 and TUCAN ($p = 0.003$) agrees with an observed positive impact that a combination of low levels of these proteins has on survival in our cohort of colon carcinoma patients. However, an inverse correlation between TUCAN
30 and Bcl-2 or AIF, did not reach a statistical significance. No significant association between cIAP2 and Apaf1 or Bcl-2 was found. Bcl-2, which has implications of a good prognostic marker in our cohort, correlates significantly with some pro-apoptotic

proteins, such as Apaf1 ($p < .0001$), AIF ($p = 0.002$) and Smac ($p = 0.008$), but also with an anti-apoptotic BAG1 protein which was found to predict long-term survival in early-stage breast cancer (#7874). An increased nuclear
5 concentration of p53, which in 80% is related to p53 point missense mutation correlated with increased expression of Bcl-XL ($p < .0001$).

EXAMPLE III

10 Immunoblot Analysis of IAPs and Apaf1 in Colon Carcinoma

This example shows immunoblot analysis of IAPs, Apaf1 and other apoptosis-regulators in five frozen colon cancer specimens.

To corroborate the immunohistochemistry data,
15 five frozen colon cancer specimens were identified that had sufficient amounts of both adjacent normal (N) and tumor (T) tissue for immunoblot analysis using antibodies specific for IAPs, Apaf1, and other proteins. Detergent-lysates of these tissues specimens were
20 prepared and normalized for total protein content prior SDS-PAGE/immunoblot analysis (Figure 3E). Densitometry analysis was also performed to quantify band intensities, and the results from the loading control blot were used to normalize all data (Figure 3F).

25 Colon cancer specimens ($n = 10$) with high ratios of cancer cells relative to stroma ($>70\%$) were selected for immunoblotting analysis. The protein lysates were prepared without additional microdissection or fractionation. The tumor lysates and the samples of the
30 normal mucosa from the same patients were prepared using modified RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.25% Na-deoxycholate, 1% NP40, 1 mM EDTA, 1 mM Na₃VO₄, 1

mM NaF, 1 mM PMSF) containing complete protease inhibitor cocktail (SIGMA), Pan-Caspase inhibitor z-Asp-2.6-dichlorobenzoyloxy-methylketone and ZVAD-fmk, normalized for total protein content (100 ug) and
5 resolved by SDS-PAGE (12% and 15% gels). Protein quantification was performed using the Bio-Rad Protein Assay Kit (Bio-Rad). Proteins were transferred (overnight 150 mA, 4°C) to PVDF membranes (Amersham Pharmacia). After blocking with 5% skim milk in TBST (50
10 mM Tris [pH 7.6], 150 mM NaCl, 0.05% Tween 20) at room temperature for 2 hours, blots were incubated overnight with antisera specific for particular IAP family members, Apaf1, and TUCAN, using 1:1,000-1:10,000 (v/v) dilutions at 4°C. After incubation with HRPase-conjugated secondary
15 goat anti-rabbit (either Bio-Rad or Santa Cruz) antibody at room temperature for 1hr, immunodetection was accomplished by an enhanced chemoluminescence (ECL) method (Amersham), with exposure to x-ray film (Kodak/XAR). Densitometry was performed to quantify the
20 intensity of bands, using Image-pro Plus software.

Higher levels of cIAP2, XIAP, Survivin, and Apaf1 were detected in every specimen evaluated, compared to case-matched normal tissue. Levels of cIAP1 protein, as well as the anti-apoptotic protein TUCAN, were
25 elevated in some tumor specimens compared to normal, but not others. A nonspecific band obtained during preblocking procedure with a secondary ECL antibody (Biorad), served as a loading control.

The immunoblotting results confirmed the
30 immunohistochemistry observations described in Example II (Figure 1 E, F). Higher levels of cIAP2, XIAP, Survivin, and Apaf1 were detected in every specimen evaluated as compared to case-matched normal tissue. Levels of cIAP1

and TUCAN were elevated in some tumor specimens compared to normal, but not others.

EXAMPLE IV

Correlation of Protein Expression with Clinical Outcome

5 To analyze the relation of biomarkers with patient survival, the comparisons of the immunoscores obtained for normal colonic epithelium and colon cancers shown in Figure 3 were used to set logical cut-offs for dichotomization of data.

10 Clinical data were available for all patient specimens included on the tissue microarray with respect to relapse and overall survival, with a median follow-up of 5 years. Patients were categorized as: (i) Alive without disease (A); (ii) Alive with recurrent disease
15 (R); or (iii) Dead (D). As shown in Table 2, no significant differences in the immunoscores for cIAP1, XIAP, or Survivin were observed when comparing the A, R, and D groups of patients. cIAP2 immunostaining was significantly higher in colon cancer patients who had
20 either died of disease (D) or who had relapsed after surgery (R) ($p < 0.0001$). In contrast, immunoscores for Apaf1 were significantly lower in the groups of patients who had relapsed (R) or died (D), compared to patients who were alive without disease ($p < 0.0001$) (Table 2).
25 An unpaired t-test method was used for comparisons of XIAP, Survivin, cIAP1, cIAP2, and Apaf1 immunoscores in the A, R, and D groups of patients. P-values refer to a comparison of group A with the combined groups R and D.

Table 2: Summary of immunostaining results for colon cancer patients

Patient Status	XIAP	Survivin	cIAP1	cIAP2	Apaf1
	Mean ±SE Median	Mean ±SE Median	Mean ±SE Median	Mean ±SE Median	Mean ±SE Median
A	168±9 160	152±10 160	166±9 190	81±9 70	132±8 140
R	176±22 170	125±25 95	169±23 180	162±22 160	46±20 50
D	177±13 180	135±13 120	174±13 180	146±13 130	77±12 70
p-values A vs R+D	0.5	0.2	0.7	<.0001	<.0001

10 To analyze the relation of biomarkers to
 patient survival by another method, immunostaining data
 for these proteins were dichotomized into high- versus
 low-expression groups. For this purpose, the comparisons
 of the immunoscores obtained for normal colonic
 15 epithelium and colon cancers shown in Figure 2 were used
 to set logical cut-offs for dichotomization of data.
 Immunoscores for normal and malignant colon epithelium
 were depicted in a graphic form in Figure 2. Based on
 comparisons with normal colonic epithelium, cutoffs for
 20 dichotomizing immunostaining data were selected. The
 range of immunoscore for 95% of normal specimens defined

a group of tumors with low immunoscore for cIAP1, cIAP2, XIAP, Survivin, Bcl-XL, and BAG1. Bimodal distribution of proteins helped to identify cut-offs for Bax, Apaf1 and TUCAN. The application of median immunoscores as
5 cut-offs for Bcl-2, Bid, AIF, Smac, and β -catenin, increased accuracy in the subcategorisation of tumors into low and high expressors. The histograms for p53 and MIB-1 present the immunopercentage, classifying cases > 0% as high p53 expressors and = 20% as those expressing
10 high levels of MIB.

Based on this method, high levels of Apaf1, TUCAN, Survivin, XIAP, cIAP1, and cIAP2 were found in 38%, 49%, 54%, 74%, 61% and 35% tumor specimens, respectively. In univariate analysis, significant
15 correlations were observed in this cohort between longer disease-free survival (DFS) and low expression of cIAP2 ($p = 0.0002$), TUCAN ($p = 0.0004$), β -Catenin ($p = 0.04$), mutant p53 protein ($p = 0.03$), or high levels of Apaf1 ($p = 0.00008$), Bcl-2 ($p = 0.005$), and SMAC ($p = 0.03$)
20 (Figure 4a). Thus, 78% (39/50) of patients whose tumors contained low levels of TUCAN remained alive and disease-free during the time covered by this study, compared to only 44% (21/48) of those with high expression of this protein. Similarly, 74% (45/61) of
25 low cIAP2 expressors enjoyed colon cancer-free life at the time of last survey compared to only 36% (12/33) of those with high cIAP2 levels. In contrast, high levels of Apaf1 were associated with longer survival in this cohort of colon cancer patients, with 33/38 (87%) of
30 patients remaining disease-free compared to only 28/62 (45%) of those with low Apaf1 expression.

The most significant improvement of overall survival was noticed in a group of patients whose colon carcinoma specimens contained low levels of TUCAN ($p < .0001$) (Figure 4b). Among 50 patients expressing low
5 TUCAN, only 4% (2/50) died, as opposed to 54% (26/48) of those presenting high levels of this protein. Significant correlations were also observed between longer overall survival and low cIAP2 ($p = 0.01$) or low mutant p53 protein ($p = 0.03$). Low Bcl-2 levels were associated
10 with poor overall survival. Of 18 patients with low expression of this protein, 11 (61%) died of colon cancer, compared with 24% of patients who died in the high-Bcl-2 group (18/76). Similarly, patients whose tumors contained low Apaf1 staining had worse overall
15 survival compared with those who overexpressed Bcl-2 (Figure 1N).

Elevated levels of Bcl-2 conferred a significant advantage for both overall ($p = 0.0008$) and disease-free survival ($p = 0.005$). Of 76 patients whose
20 tumors revealed high Bcl-2, 58 (76%) remained alive and 50 (66%) relapse-free, compared to 39% and 33% of those with low Bcl-2 levels. Independent of its anti-apoptotic function, Bcl-2 can delay entry into the cell cycle and promote exit of cells from the cycle. Thus, a positive
25 effect of Bcl-2 on clinical outcome may be linked to its cell cycle-inhibitory role.

Figure 4 shows correlations of biomarkers immunostaining data with disease-free (A) survival and overall survival (B) for colon carcinoma patients. All
30 biomarkers data and outcome measures were entered into a database using STATISTICA software system (StatSoft). The log rank test was used to for correlation of immunoscore data with the patient survival. The

Kaplan-Meier curves illustrate correlations of the investigated biomarkers with survival for this cohort of patients.

In summary, at a median follow-up of 5 years, 5 60% of patients with high cIAP2 levels relapsed and 46% died of colon cancer, whereas in a low-cIAP2 group there were 20% relapses and 18% colon cancer-related deaths. At the same time point, 49% of patients with high expression of TUCAN had relapse or died of colon cancer, 10 and only 19% had recurrence and 4% died of disease in a group of patients whose tumors expressed low levels of this protein. In contrast, 43% of patients with low Apaf1 relapsed and 35% died of colon cancer, while only 14% had a cancer recurrence or died in a high-Apaf1 15 cohort. Thus, these findings indicate that higher levels of the anti-apoptotic protein cIAP2 and lower levels of the pro-apoptotic protein Apaf1 are associated with adverse outcome in patients with early-stage colon cancer. No significant differences were noted in the 20 age, or gender of the patients in the high- versus low-expression groups for cIAP2, Apaf1, or TUCAN.

EXAMPLE V

Combined Analysis of cIAP2 and Apaf1 Expression data

This example shows combined analysis of cIAP2 25 and Apaf1 expression data.

Since certain proteins had significant prognostic value, it was determined whether combining two biomarkers could identify a subgroup of patients with distinct survival characteristics. Patients with two 30 favorable variables (low cIAP2 and high Apaf1) were compared with all other patients in this cohort.

Figure 5 shows correlations of biomarkers and their combinations with disease-free (A, B) survival and overall survival (C, D) for colon carcinoma patients. Using the Kaplan-Meier curves, panels A and B illustrate a combination of biomarkers [low cIAP2 and high Apaf1 (A); low cIAP2 and low TUCAN (B)] with positive impact on disease-free survival. The two combinations of markers with an adverse effect on survival are presented in panel C (low Apaf1 and high TUCAN), and panel D (low Bcl-2 and high cIAP2).

Of the 94 patient samples successfully analyzed for cIAP2 and Apaf1, 25 (27%) had both a low cIAP2 and a high Apaf1 immunoscore. All (100%) of these patients with the combination of low cIAP2 and high Apaf1 were alive and disease-free at 5 years after diagnosis, compared to 52% disease-free or 64% alive of others ($p = .00007$ for OS; $p < .0001$ for DFS) (Figure 5A; DFS). At the same time point, among patients with a combination of low cIAP2 and low TUCAN 97% were alive and 94% disease-free, compared to 59% alive and 50% disease-free of others ($p = .00001$) (Figure 5B; DFS). Thus, the combinations of cIAP2 and Apaf1 or cIAP2 and TUCAN immunostaining data identify a subgroup of colon cancer patients with distinct survival characteristics.

However, when patients with two adverse biomarkers (low Apaf1 and high TUCAN) were compared with other patients, 34% of patients with this combination of proteins and 90% of others were alive at 5 years after diagnosis ($p < .0001$) (Figure 4C). The discrepancy was even larger at the end of the survey, with 0% and 90% of those who remained alive, respectively. When combination data were examined for another pair of adverse biomarkers (cIAP2 high and Bcl-2 low), none of the patients was alive in this group 5 years after surgery, but 75% of others

survived ($p = 0.04$) (Figure 4D). These results are in agreement with an outcome of the LERS data analysis.

EXAMPLE VI

5 Multivariate Analysis Identifies cIAP2, Apaf1, TUCAN and Bcl-2 as Independent Prognostic Indicators of Survival in Early-stage Colon Cancer

This example shows that multivariate analysis confirms that cIAP2, Apaf1, TUCAN and Bcl-2 are independent prognostic indicators of survival in
10 early-stage colon cancer.

Multivariate Cox proportional hazards models were fitted to assess whether elevated levels of biomarkers were associated with disease-free survival (DFS) and overall survival (OS). The variables were not
15 stratified into T3N0M0 and T4N0M0 subgroups due to a small number of patients involved in this study. In addition, the data mining system LERS (Learning from Examples based on Rough Sets) was employed to perform a multivariate analysis of immunohistochemical staining
20 data.

In this project, the algorithms LEM2 was determined to be the most applicable to the data and therefore was employed for multivariate analysis. The presence of high cIAP2 and high TUCAN increased risk of
25 death from colon cancer within this cohort of patients 2.7-fold ($p = 0.01$) and 17-fold ($p = 0.00004$), respectively. High Apaf1 and Bcl-2 expression was associated with a decreased relative risk of dying of colon cancer by 75% ($p = 0.004$) and 82% ($p = 0.00006$).

When an association of protein levels with disease-free survival was assessed by multivariate analysis, cIAP2 and TUCAN maintained prognostic significance ($p = 0.000005$, $p = 0.0005$), with high levels of these proteins increasing risk of recurrence 6-fold and 3.4-fold, respectively. Also Apaf1 and Bcl-2 retained their significant prognostic role ($p = 0.006$, $p = 0.0004$), decreasing the hazard rate of colon cancer recurrence by approximately 75%. Additionally, high levels of Smac decreased the risk of recurrence by 63%. No role of Smac was evident for overall survival of patients in this cohort. Taken together, these findings indicate that immunostaining data for cIAP2, Apaf1, TUCAN, Bcl-2 and their combination can have prognostic significance for patients with early-stage colon cancer. Table 3 A-B shows multivariate analysis of DFS (A) and OS (B) in stage II colon carcinoma patients using backward stepwise Cox proportional hazards regression analysis to assess whether elevated levels of biomarkers were associated with disease-free survival or overall survival.

Table 3: Multivariate analysis of DFS (A) and OS (B) in stage II colon carcinoma patients

A. DFS

	BIOMARKER	coeffifcient	HR (95% CI)	p
5	cIAP2	1.79	5.96 (2.78 - 12.8)	0.000005
	Apaf1	-1.27	0.28 (0.11 - 0.68)	0.006
	TUCAN	1.23	3.43 (1.6 - 6.55)	0.0005
	Bcl-2	-1.37	0.25 (0.13 - 0.60)	0.007
	Smac	-1.00	0.37 (0.19 - 0.81)	0.007

10 B. OS

	BIOMARKER	coeffifcient	HR (95% CI)	p
	cIAP2	0.98	2.66 (1.04 - 5.42)	0.01
	Apaf1	-1.36	0.26 (0.10 - 0.65)	0.004
	TUCAN	2.84	17.19 (5.12 - 57.48)	0.000004
15	Bcl-2	-1.69	0.18 (0.08 - 0.43)	0.00006

EXAMPLE VII

Expression of TUCAN in Multiple Cancer Cell Lines

This example shows that TUCAN is expressed in several tumor cell lines.

5 To determine the expression of TUCAN in cancers, the NCI panel of 60 human tumor cell lines (Weinstein, et al. Science 17:343-349 (1997)) was analyzed by immuno-blotting using an antiserum specific for TUCAN (Figure 6A). Cell lines included in the panel
10 are shown in Table 4, below:

Table 4: NCI panel of 60 human tumor cell lines

	<u>Cell Line Name</u>	<u>Cell Type</u>
	CCRF-CEM	Leukemia
	HL-60 (TB)	Leukemia
15	K-562	Leukemia
	MOLT-4	Leukemia
	RPMI-8226	Leukemia
	SR	Leukemia
	A549/ATCC	Non-Small Cell Lung
20	EKVX	Non-Small Cell Lung
	HOP-62	Non-Small Cell Lung
	HOP-92	Non-Small Cell Lung
	NCI-H226	Non-Small Cell Lung
	NCI-H23	Non-Small Cell Lung
25	NCI-H322M	Non-Small Cell Lung
	NCI-H460	Non-Small Cell Lung
	NCI-H522	Non-Small Cell Lung
	COLO 205	Colon
	HCC-2998	Colon
30	HCT-116	Colon
	HCT-15	Colon
	HT29	Colon

Table 4, continued

	<u>Cell Line Name</u>	<u>Cell Type</u>
	KM12	Colon
	SW-620	Colon
5	SF-268	CNS
	SF-295	CNS
	SF-539	CNS
	SNB-19	CNS
	SNB-75	CNS
10	U251	CNS
	LOX IMVI	Melanoma
	MALME-3M	Melanoma
	M14	Melanoma
	SK-MEL-2	Melanoma
15	SK-MEL-28	Melanoma
	SK-MEL-5	Melanoma
	UACC-257	Melanoma
	UACC-62	Melanoma
	IGR-OV1	Ovarian
20	OVCAR-3	Ovarian
	OVCAR-4	Ovarian
	OVCAR-5	Ovarian
	OVCAR-8	Ovarian
	SK-OV-3	Ovarian
25	786-0	Renal
	A498	Renal
	ACHN	Renal
	CAKI-1	Renal
	RXF 393	Renal
30	SN12C	Renal
	TK-10	Renal
	UO-31	Renal
	PC-3	Prostate
	DU-145	Prostate
35	MCF7	Breast

Table 4, continued

	<u>Cell Line Name</u>	<u>Cell Type</u>
	NCI/ADR-RES	Breast
	MDA-MB-231/ATCC	Breast
5	HS 578T	Breast
	MDA-MB-435	Breast
	MDA-N	Breast
	BT-549	Breast
	T-47D	Breast

10 Lysates were normalized for total protein
content prior to analysis. Relative levels of TUCAN
protein varied widely among the tumor lines tested, with
some cell lines containing especially abundant levels of
this protein (for example, MCF7 breast cancer cells,
15 OVCAR5 ovarian cancer cells, and NCI-H322M lung cancer
cells). TUCAN protein also was present in HL-60 leukemia
cells, SNB-19 CNS cancer cells, MDA-MB-231 breast cancer
cells, IGROV1 ovarian cancer cells, NCI-H226 non-small
cell lung cancer cells, NCI-H23 non small cell lung
20 cancer cells, M14 melanoma cells, Du-145 prostate cancer
cells, UO-31 renal cancer cells, and K562 leukemia cells.
In some of these tumor lines, TUCAN migrated in SDS-PAGE
as a broad band or as an apparent doublet, indicating
that multiple forms of TUCAN protein can be present in
25 cancer cells (Figure 6A).

The levels of endogenous TUCAN protein in some
of these cancer cell lines were compared with the
transfected HEK293T and Jurkat cells. The levels of
plasmid-derived TUCAN produced in transiently transfected
30 HEK293T cells were comparable to the endogenous levels of
TUCAN found in MCF7 breast cancer cells (Figure 6B).
Levels of plasmid-derived TUCAN produced in the stably
transfected Jurkat cells were comparable in amount to

endogenous TUCAN measured in OVCAR5 ovarian and NCI-H322M lung cancer cell lines.

In summary, TUCAN is expressed in a variety of tumor cell lines, including cancer cells obtained from
5 human breast, ovarian, lung, CNS, leukemia, kidney, prostate, skin and colon tumors.

EXAMPLE VIII

Elevated TUCAN Expression in Colon Cancers Correlates 10 with Reduced Patient Survival

This example shows that TUCAN expression is elevated in colon cancers and that TUCAN elevation correlates with reduced colon cancer patient survival.

15 Using anti-TUCAN antibodies, the expression of TUCAN protein was analyzed by immunohistochemical methods in a collection of 102 archival paraffin-embedded colon cancer specimens derived from patients with uniform clinical stage (Duke's B; Stage II) and treatment
20 (surgery without adjuvant chemotherapy). A tissue microarray was constructed so that all 102 tumor specimens could be analyzed on a single glass slide, thus minimizing differences in immuno-intensity due to technical artifacts (Figure 7).

25 Normal human tissues for immunohistochemistry analysis were obtained from biopsy and autopsy specimens, fixed in Bouin's solution (Sigma), and embedded in paraffin. Colon carcinoma specimens were obtained from Department of Pathology, Yonsei University, College of
30 Medicine, Seoul, Korea. Tissue samples included 102 primary tumors derived from patients who presented

between 1986 and 1996 with stage II disease (Duke's B-stage), as defined by American Joint Committee on Cancer and Union Internationale Contre le Cancer (AJCC/UICC) criteria. All patients were treated by surgical resection of the involved segment of colon. No postoperative adjuvant chemotherapy was performed initially in all cases. However, chemotherapy was administered for some patients after relapse. Clinical data represent a median follow up of 60 months.

To construct colon cancer microarrays, 2-5 cylinders of 1 mm diameter tissue were taken from representative areas of archival paraffin blocks containing 8% formalin-fixed tumor and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). Serial sections (4 um) were applied to 3-aminopropyltriethoxysilane (APES)-coated slides (Sigma), as described in Rentrop et al. Histochem J. 18:271-276 (1986).

For immunohistochemistry, dewaxed tissue sections were immunostained using a diaminobenzidine (DAB)-based detection method, employing the Envision-Plus-Horse Radish Peroxidase (HRP) system (DAKO) using an automated immunostainer (Dako Universal Staining System) (Krajewski et al. Proc. Natl. Acad. Sci. USA 96:5752-5757 (1999)). Anti-TUCAN antibody was applied at 1:5000 (v/v). Incubation with antiserum preabsorbed with 5 ug/ml of either synthetic peptide (BUR215) or recombinant GST-CARD/TUCAN protein (BUR206) immunogen was used to verify specificity of the results. The scoring of tumor immunostaining was based on the percentage of immunopositive cells (0-100) multiplied by staining intensity score (0/1/2/3), yielding scores of 0-300.

Data were analyzed using the JMP Statistics software package (SAS Institute). An unpaired t-test method and Pearson ChiSquare test were used for correlation of TUCAN immunostaining data with the patient survival.

Of the 102 tumor specimens arrayed, 66 contained adjacent normal colonic epithelium in the same section, permitting comparisons of the intensity of TUCAN immunostaining in tumor versus normal cells. TUCAN immuno-intensity was stronger in the invasive cancer cells compared to normal colonic epithelial cells in 42 of 66 (64%) of these specimens, indicating that roughly two-thirds of colon cancers have up-regulated levels of TUCAN protein. TUCAN immunoreactivity was present diffusely through the cytosol of these cells (Figure 7). Control staining performed with either preimmune serum or with anti-TUCAN antiserum that had been preabsorbed with TUCAN immunogen confirmed that these results were specific for anti-TUCAN.

Tumor immunostaining results were scored with respect to immuno-intensity (ranked on a scale of 0-3), percentage immunopositivity (0-100%), and immunoscore (which is the product of immuno-intensity and immuno-percentage), and these data were correlated with patient survival information. TUCAN immunostaining was significantly higher among patients who died of their cancer (n = 31), compared to patients who remained alive without disease (n = 61) or alive with recurrent disease (n = 10). A summary of TUCAN immunostaining results is shown below in Table 5.

Table 5: Summary of TUCAN Immunostaining Results

Patient Status	n	%Immunopositivity	Immunointensity	Immunoscore
		Mean Median ± SE	Mean Median ± SE	Mean Median ± SE
5 Alive without disease	61	58 ± 3 60	1.4 ± 0.1 1	92 ± 9 80
Alive with disease	10	54 ± 7 55	1.3 ± 0.2 1	73 ± 21 65
10 Dead from disease	31	90 ± 4 95	2.5 ± 0.1 3	224 ± 12 240
p-values	10 2	p < .0001	p < .0001	p < .0001

In summary, TUCAN expression is abnormally elevated in a substantial proportion of early-stage colon cancers. Furthermore, elevated TUCAN expression correlates with reduced patient survival.

EXAMPLE IX

TUCAN Binds Selectively to Pro-Caspase-9 and to Itself

This example shows that TUCAN binds selectively to Pro-Caspase-9 and to itself. Since CARDS are known for their ability to bind each other, TUCAN was tested for interactions with the CARD-containing proteins pro-Caspase-1, pro-Caspase-2, pro-Caspase-9, Apaf1, Nod1 (CARD4), CED4, NAC (DEFCAP), Cardiak (RIP2), Raidd (CRADD), Bcl10 (CIPER; huE10), cIAP1, cIAP2, CLAN, CARD9, and itself. Among these, TUCAN associated only with pro-Caspase-9 and itself.

Figure 8 shows representative results from co-immunoprecipitation experiments performed using TUCAN containing either Flag or Myc epitope tags. The TUCAN polypeptides were expressed by transient transfection in HEK293T cells together with epitope-tagged pro-caspase-9 or other proteins. An inactive mutant of pro-caspase-9 in which the catalytic cysteine was substituted with alanine (Cys287/Ala) was employed for these experiments to avoid induction of apoptosis (Cardone et al. Science 282:1318-1321 (1998)). Cell lysates were prepared from transfected cells and immunoprecipitations were performed using anti-Flag or anti-Myc antibodies, followed by SDS-PAGE/immunoblot analysis. Representative results are presented in Figure 8A, which shows that TUCAN co-immunoprecipitated with pro-Caspase-9 but not the CARD-containing protein Apaf1. TUCAN also did not co-immunoprecipitate with the CARD-containing proteins pro-Caspase-1, pro-Caspase-2, Nod1, CED4, NAC, Cardiak, Raidd, Bcl10, CLAN, CARD9, cIAP1 and cIAP2. Moreover, TUCAN did not associate non-specifically with caspases, as co-immunoprecipitation experiments did not reveal interactions with the DED-containing caspases, pro-caspase-8 and-10 (Figure 8A).

To determine the role of the CARD domain within TUCAN for interactions with pro-Caspase-9, fragments of TUCAN were expressed. The TUCAN fragments contained essentially only the CARD (residues 345-431) or lacked the CARD (residues 1-337) (Δ CARD). Pro-Caspase-9 co-immunoprecipitated with full-length TUCAN and the CARD only fragment but not the Δ CARD fragment of TUCAN (Figure 8B). Thus, the CARD domain of TUCAN is necessary and sufficient for association with pro-Caspase-9. Self-association of TUCAN was also confirmed by co-immunoprecipitation experiments, using HA and

Myc-tagged proteins and contrasting the full-length, CARD-only, and Δ CARD proteins. Full-length TUCAN interacted with full-length TUCAN and the CARD-only fragment but not the Δ CARD fragment (Figure 8C). Thus, 5 the CARD domain of TUCAN is necessary and sufficient for self-association.

Throughout this application various publications have been referenced within parentheses. 10 The disclosures of these publications in their entirety are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with 15 reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the 20 spirit of the invention.

What is claimed is:

1. A method for determining a prognosis for survival for a cancer patient, comprising:

5 (a) measuring a level of a TUCAN in a neoplastic cell-containing sample from said cancer patient, and

(b) comparing the level of TUCAN in said sample to a reference level of TUCAN, wherein a low level of
10 TUCAN in said sample correlates with increased survival of said patient.

2. The method of claim 1, wherein said survival is overall survival.

3. The method of claim 1, wherein said
15 survival is disease-free survival.

4. The method of claim 1, wherein said cancer patient has a cancer selected from the group consisting of: colon cancer, gastrointestinal cancer, breast cancer,
20 ovarian cancer, lung cancer, leukemia, CNS cancer, melanoma, prostate cancer, and renal cancer.

5. The method of claim 1, wherein said sample is colon tumor tissue.

25 6. The method of claim 1, wherein said sample is a fluid selected from the group consisting of blood, serum, urine, semen and stool.

7. The method of claim 1, wherein a level of
30 a TUCAN polypeptide is measured.

8. The method of claim 7, wherein a level of said TUCAN polypeptide is measured using an antibody specifically reactive with TUCAN.

9. The method of claim 1, wherein a level of
5 a TUCAN nucleic acid is measured.

10. The method of claim 1, wherein said patient has an early stage of cancer.

11. The method of claim 1, wherein said level of TUCAN is used to determine if said patient is at risk
10 for relapse.

12. The method of claim 1, wherein said level of TUCAN is used to determine a proper course of treatment for said patient.

13. A method of determining a prognosis for
15 survival for a cancer patient, comprising:

(a) measuring levels of TUCAN and one or more biomarkers selected from the group consisting of cIAP2, Apaf1, Bcl-2 and Smac in a neoplastic cell-containing sample from said cancer patient, and

20 (b) comparing the level of TUCAN and the one or more selected biomarkers in said sample to a reference level of TUCAN and said one more selected biomarkers, wherein a low level of TUCAN and a high level of any of Apaf1, Bcl-2 or Smac, or a low level of TUCAN and a low
25 level of cIAP2, in said sample correlate with increased survival of said patient.

14. The method of claim 13, wherein said survival is overall survival.

15. The method of claim 13, wherein said survival is disease-free survival.

16. The method of claim 13, wherein cIAP2 is a
5 selected biomarker.

17. The method of claim 13, wherein Apaf1 is a selected biomarker.

18. The method of claim 13, wherein Bcl-2 is a selected biomarker.

19. The method of claim 13 wherein Smac is a
10 selected biomarker.

20. The method of claim 13, wherein said cancer patient has a cancer selected from the group consisting of: colon cancer, gastrointestinal cancer,
15 breast cancer, ovarian cancer, lung cancer, leukemia, CNS cancer, melanoma, prostate cancer, and renal cancer.

21. The method of claim 13, wherein said sample is colon tumor tissue.

22. The method of claim 13, wherein said
20 sample is a fluid selected from the group consisting of blood, serum, semen, urine, and stool.

23. The method of claim 13, wherein a level of TUCAN or a biomarker polypeptide is measured.

24. The method of claim 23, wherein a level of
25 TUCAN or a biomarker polypeptide is measured using an antibody specifically reactive with TUCAN or the biomarker polypeptide.

25. The method of claim 13, wherein a level of TUCAN or a biomarker nucleic acid is measured.

26. The method of claim 13, wherein said patient has an early stage of cancer.

5 27. The method of claim 13, wherein the levels of TUCAN and one or more biomarkers are used to determine if said patient is at risk for relapse.

28. The method of claim 13, wherein the levels of TUCAN and one or more biomarkers are used to determine
10 a proper course of treatment for said patient.

29. The method of claim 13, further comprising selecting two or more biomarkers from the group consisting of cIAP2, Apaf1, Bcl-2 and Smac.

15 30. A method for monitoring the effectiveness of a course of treatment for a patient with cancer, comprising:

(a) determining a level of a TUCAN in a neoplastic cell-containing sample from a cancer patient
20 prior to treatment, and

(b) determining the level of TUCAN in a neoplastic cell-containing sample from said patient after treatment, whereby comparison of said TUCAN level prior to treatment with the TUCAN level after treatment
25 indicates the effectiveness of said treatment.

31. The method of claim 30, wherein said cancer patient has a cancer selected from the group consisting of: colon cancer, gastrointestinal cancer,
30 breast cancer, ovarian cancer, lung cancer, leukemia, CNS cancer, melanoma, prostate cancer, and renal cancer.

32. The method of claim 30, wherein said sample is colon tumor tissue.

33. The method of claim 30, wherein said sample is a fluid selected from the group consisting of
5 blood, serum, urine, semen and stool.

34. The method of claim 30, wherein a level of a TUCAN polypeptide is measured.

35. The method of claim 34, wherein a level of said TUCAN polypeptide is measured using an antibody
10 specifically reactive with TUCAN.

36. The method of claim 30, wherein a level of a TUCAN nucleic acid is measured.

37. The method of claim 30, wherein said patient has an early stage of cancer.

15 38. A method of determining a prognosis for survival for a cancer patient, comprising:
(a) measuring a level of TUCAN in a neoplastic cell-containing sample from said cancer patient, and
(b) classifying said patient as belonging to
20 either a first or second group of patients, wherein said first group of patients having low levels of TUCAN is classified as having an increased likelihood of survival compared to said second group of patients having high levels of TUCAN.

25 39. The method of claim 38, wherein said survival is overall survival.

40. The method of claim 38, wherein said survival is disease-free survival.

41. The method of claim 38, wherein said cancer patient has a cancer selected from the group
5 consisting of: colon cancer, gastrointestinal cancer, breast cancer, ovarian cancer, lung cancer, leukemia, brain cancer, melanoma, prostate cancer, and renal cancer.

42. The method of claim 38, wherein said
10 sample is colon tumor tissue.

43. The method of claim 38, wherein said sample is a fluid selected from the group consisting of blood, serum, urine, semen and stool.

44. The method of claim 38, wherein a level of
15 a TUCAN polypeptide is measured.

45. The method of claim 44, wherein a level of a TUCAN polypeptide is measured using an antibody specifically reactive with TUCAN polypeptide.

46. The method of claim 38, wherein a level of
20 TUCAN nucleic acid is measured.

47. The method of claim 38, wherein said patient has an early stage of cancer.

48. The method of claim 38, further
25 comprising:

(a) determining a level of cIAP2 in said neoplastic cell-containing sample from said cancer patient, and

(b) classifying said patient as belonging to either a first or second group of patient, wherein said first group of patients having low levels of TUCAN and low levels of cIAP2 is classified as having increased
5 likelihood of survival compared to said second group of patients having high levels of TUCAN and high levels of cIAP2.

49. The method of claim 38, further comprising:

10 (a) determining a level of a biomarker selected from the group consisting of Apaf1, Smac and Bcl-2 in said neoplastic cell-containing sample from said cancer patient, and

(b) classifying said patient as belonging to
15 either a first or second group of patient, wherein said first group of patients having low levels of TUCAN and high levels of any of Apaf1, Smac or Bcl-2 is classified as having increased likelihood of survival compared to said second group of patients having high levels of TUCAN
20 and low levels of any of Apaf1, Smac or Bcl-2.

50. The method of claim 30, further comprising determining a level of a biomarker selected from the group consisting of cIAP2, Apaf1, Smac and Bcl-2 in said
25 neoplastic cell-containing sample from said cancer patient prior to and after treatment, wherein the levels of the selected biomarker and TUCAN prior to treatment are compared with the levels of the selected biomarker and TUCAN after treatment to indicate the effectiveness
30 of said treatment.

1/19

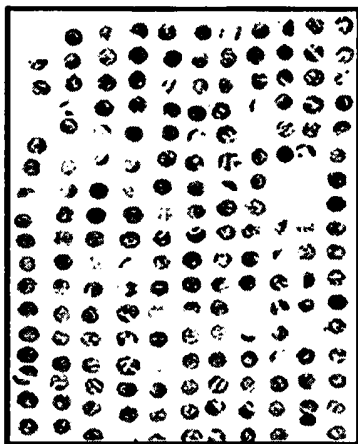


FIG. 1A

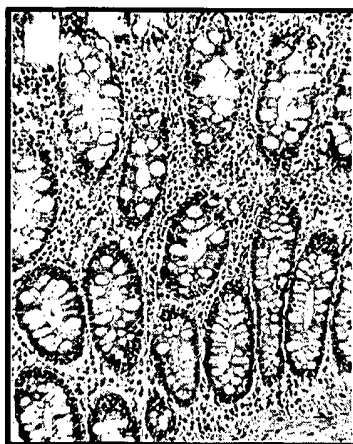


FIG. 1B



FIG. 1C

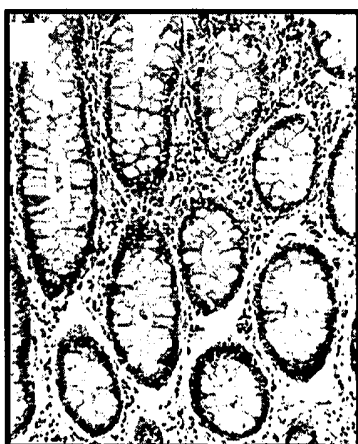


FIG. 1D



FIG. 1E



FIG. 1F

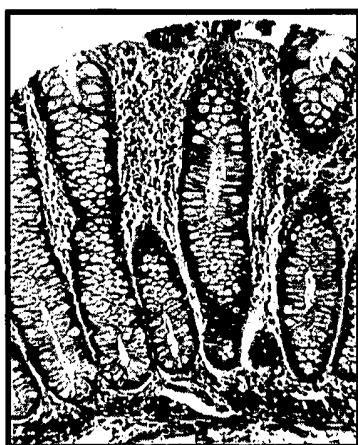


FIG. 1G



FIG. 1H



FIG. 1I

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FIG. 1J

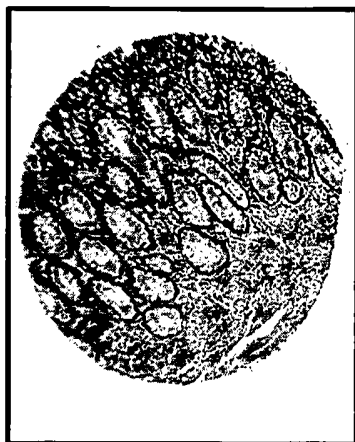


FIG. 1K



FIG. 1L



FIG. 1M



FIG. 1N

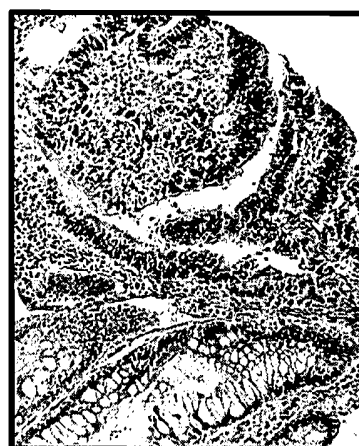


FIG. 1O



FIG. 1P

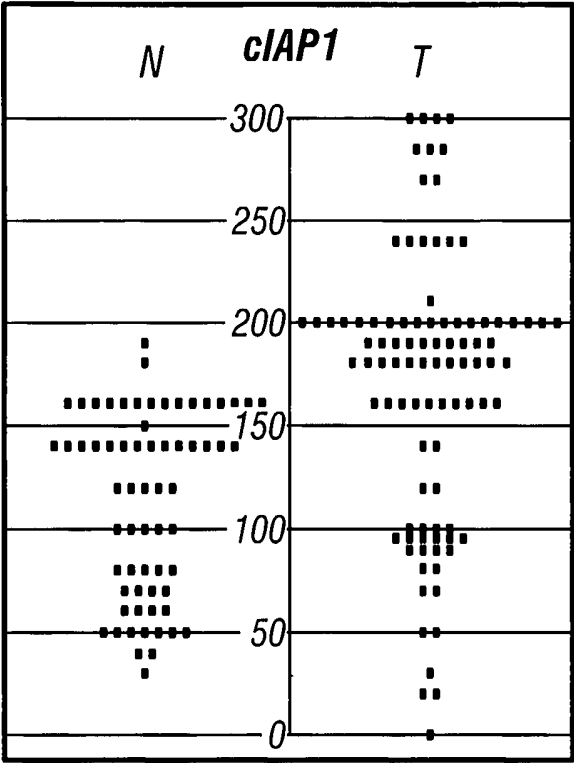


FIG. 2A

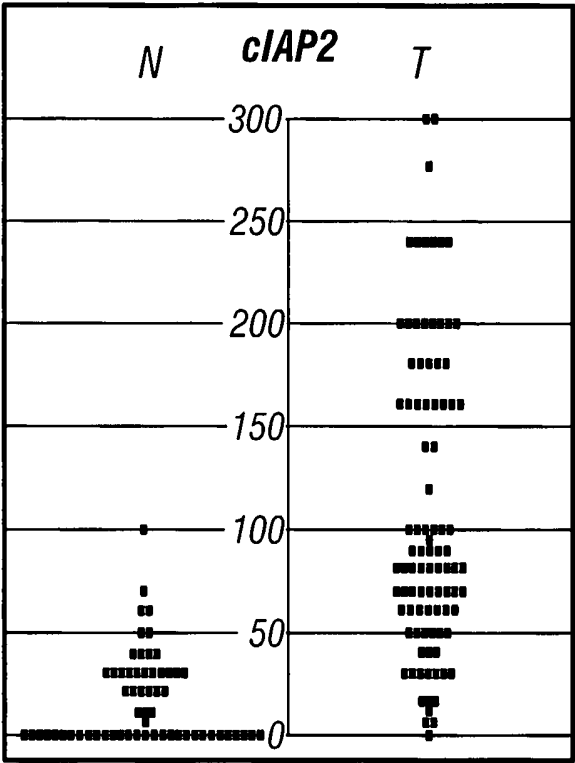


FIG. 2B

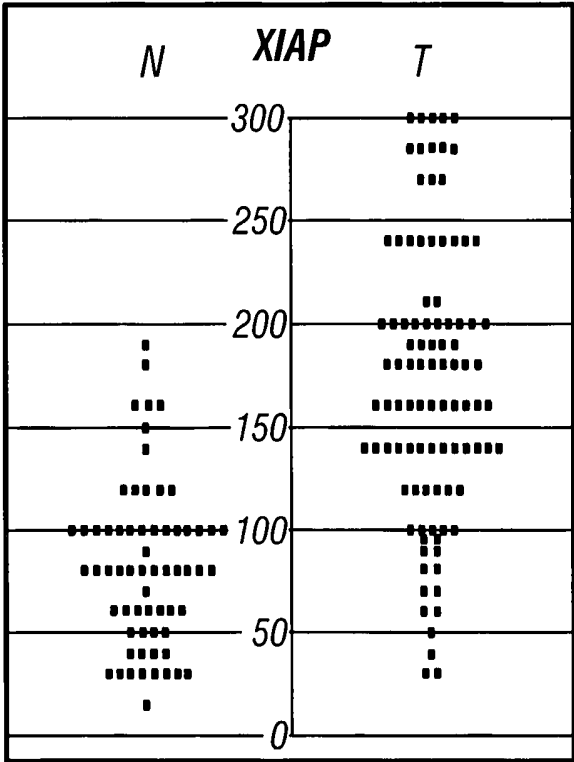


FIG. 2C

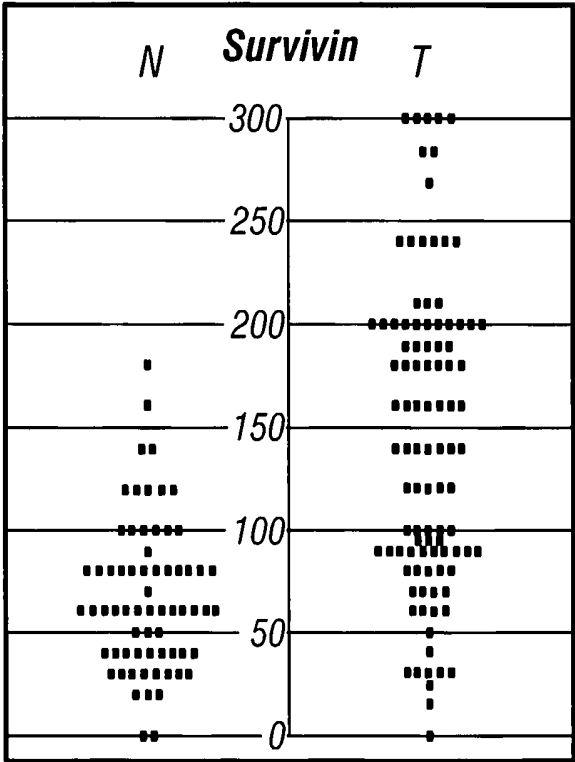


FIG. 2D

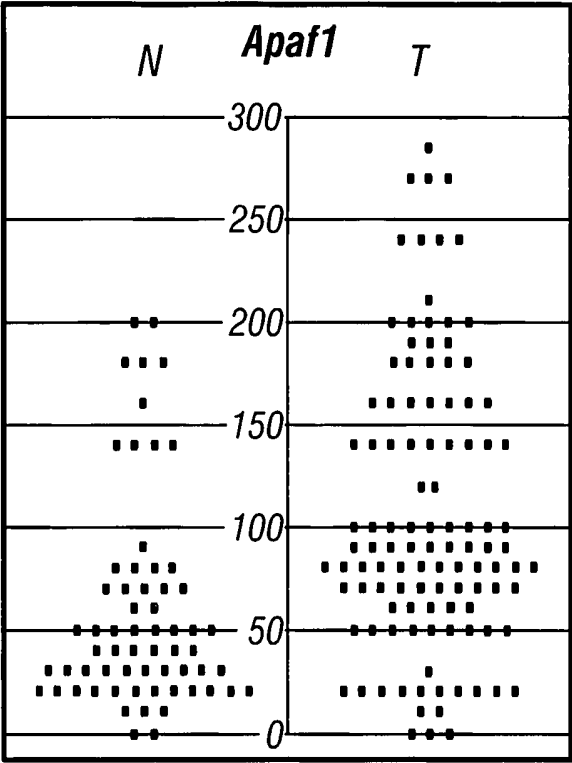


FIG. 2E

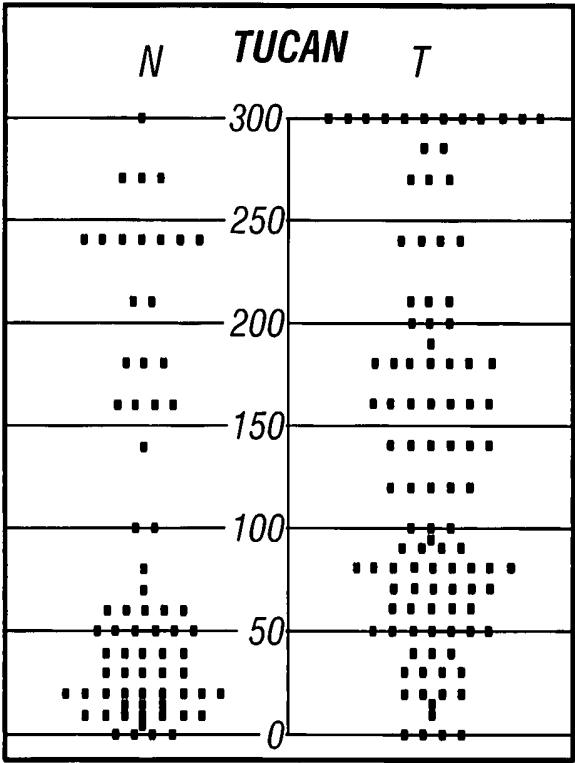


FIG. 2F

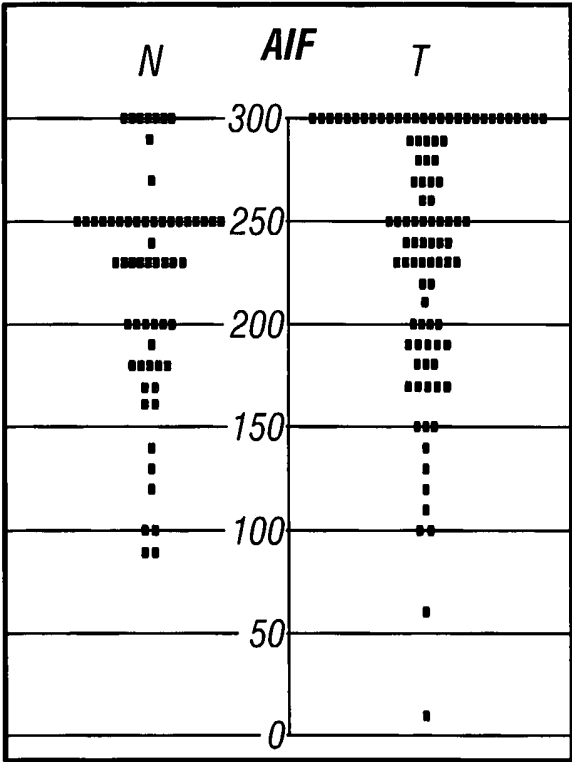


FIG. 2G

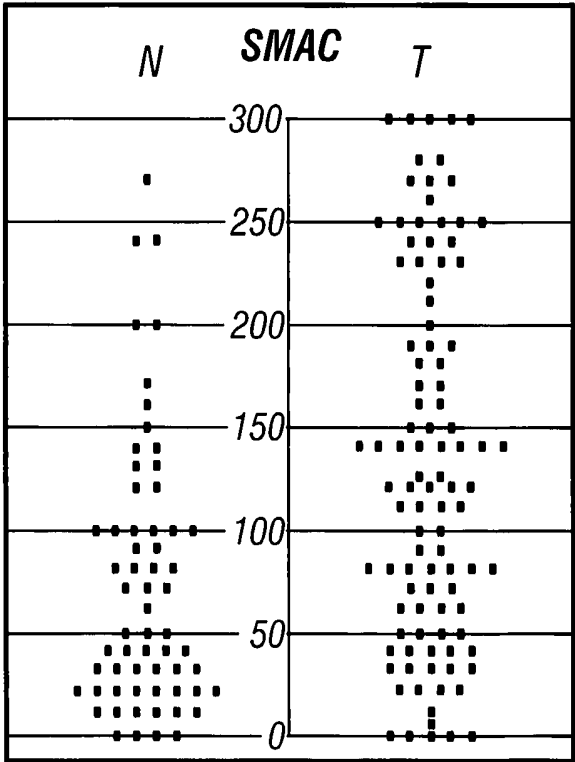


FIG. 2H

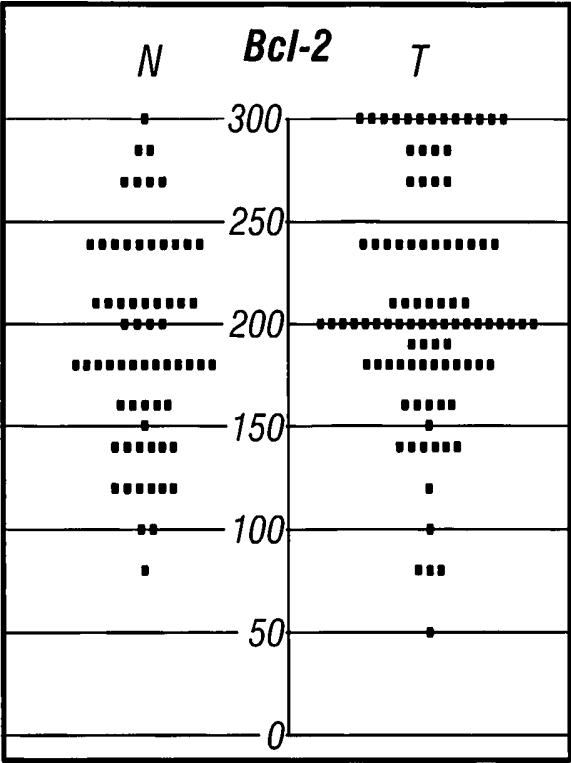


FIG. 2I

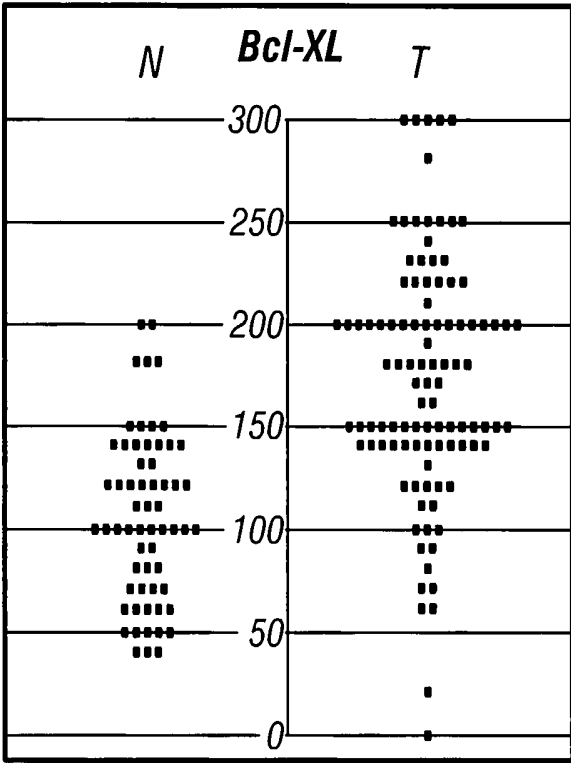


FIG. 2J

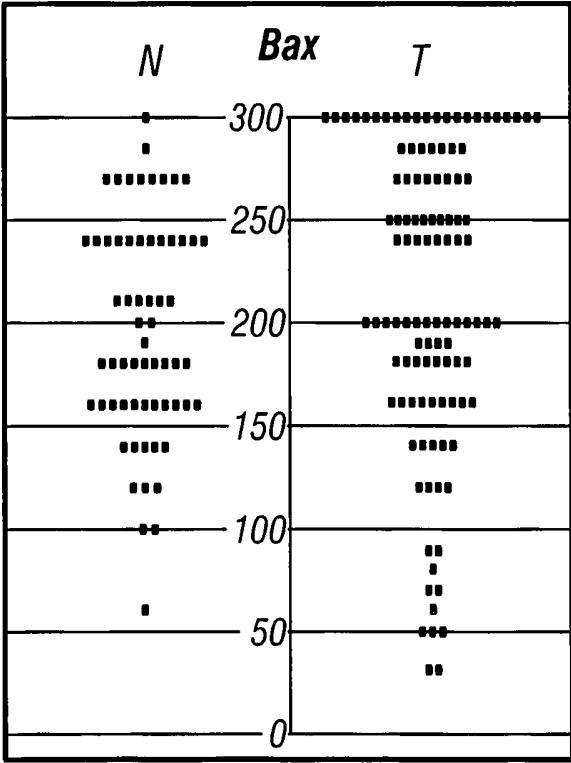


FIG. 2K

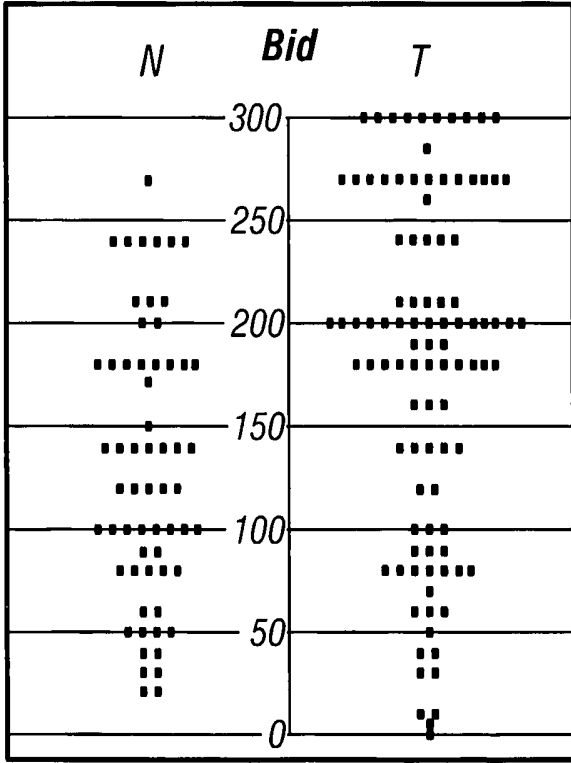


FIG. 2L

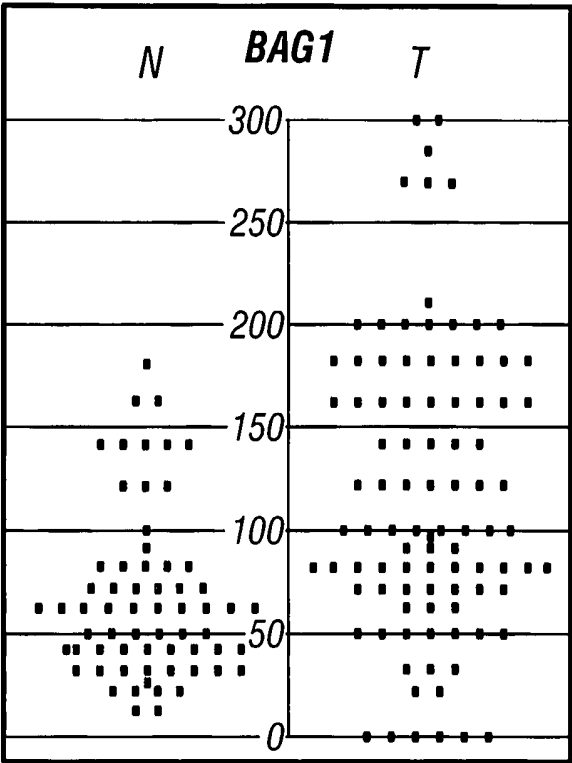


FIG. 2M

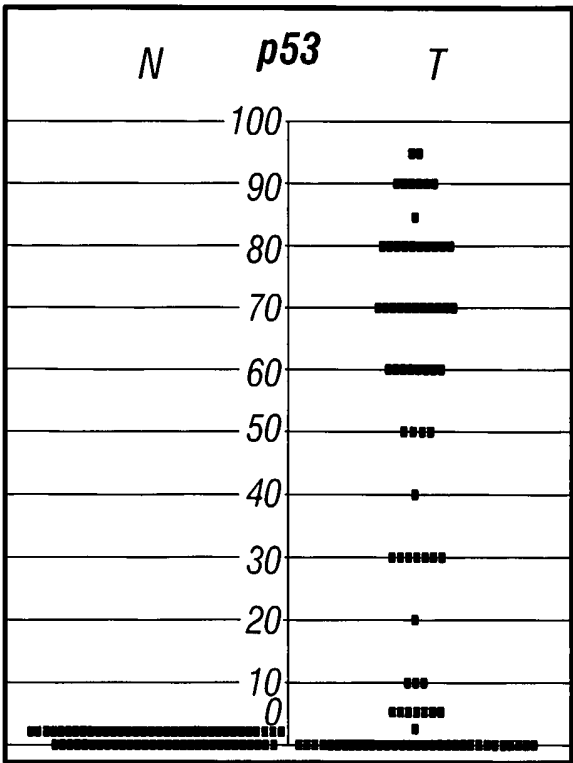


FIG. 2N

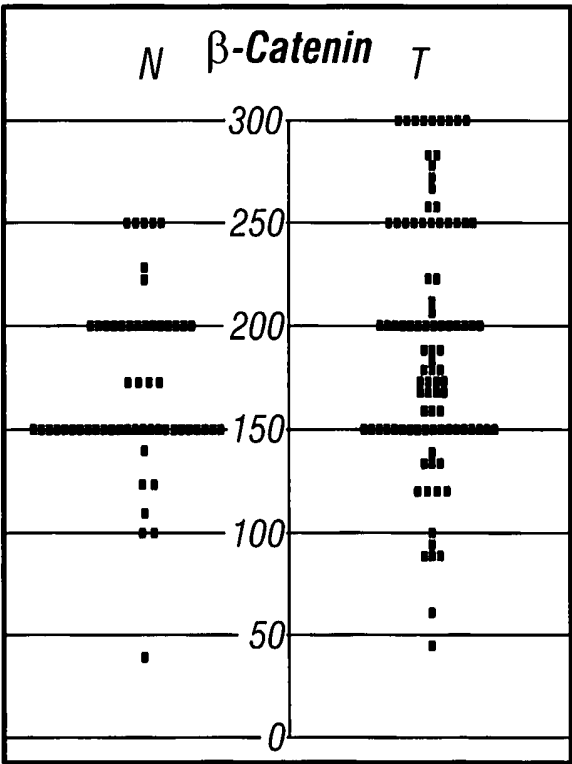


FIG. 2O

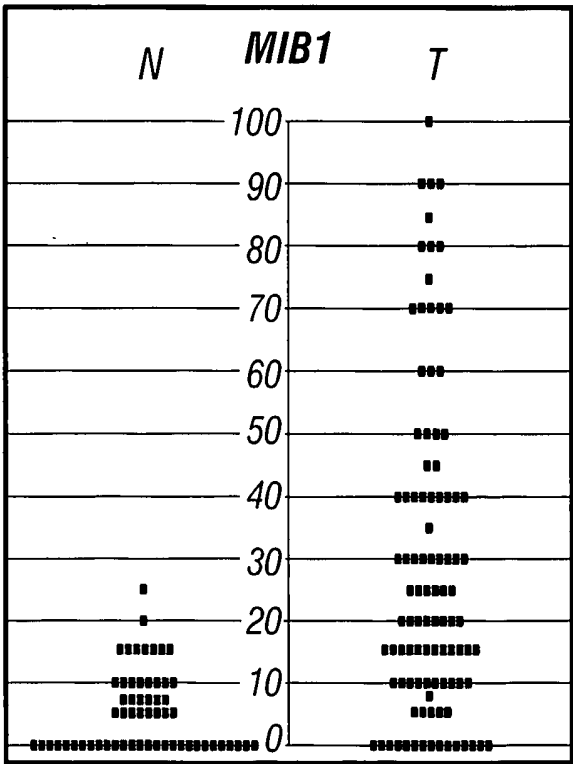


FIG. 2P

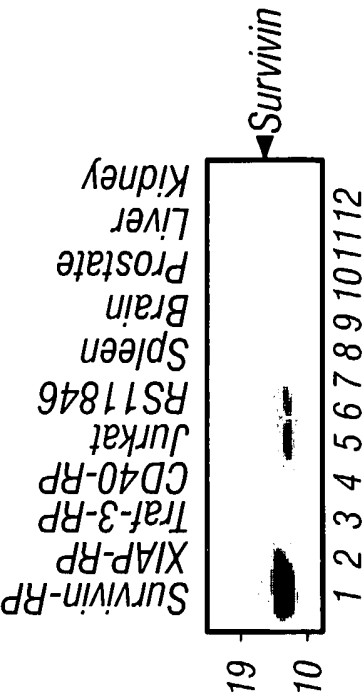


FIG. 3B

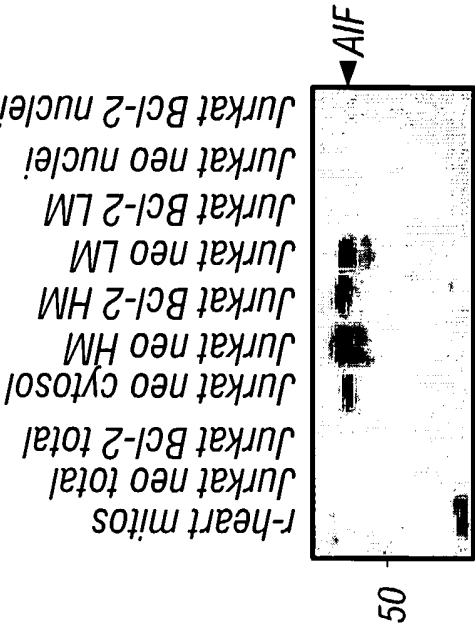


FIG. 3D

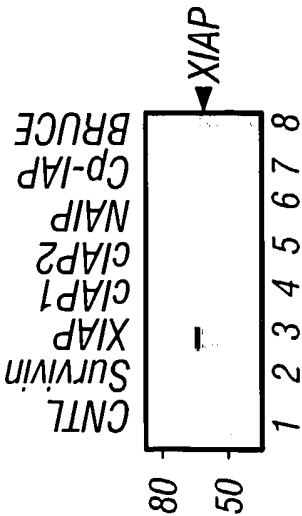


FIG. 3A

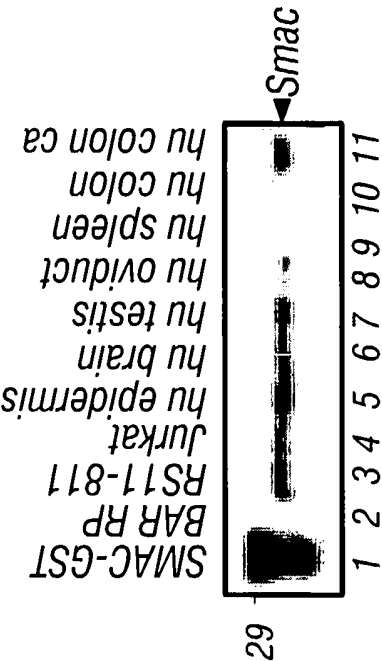


FIG. 3C

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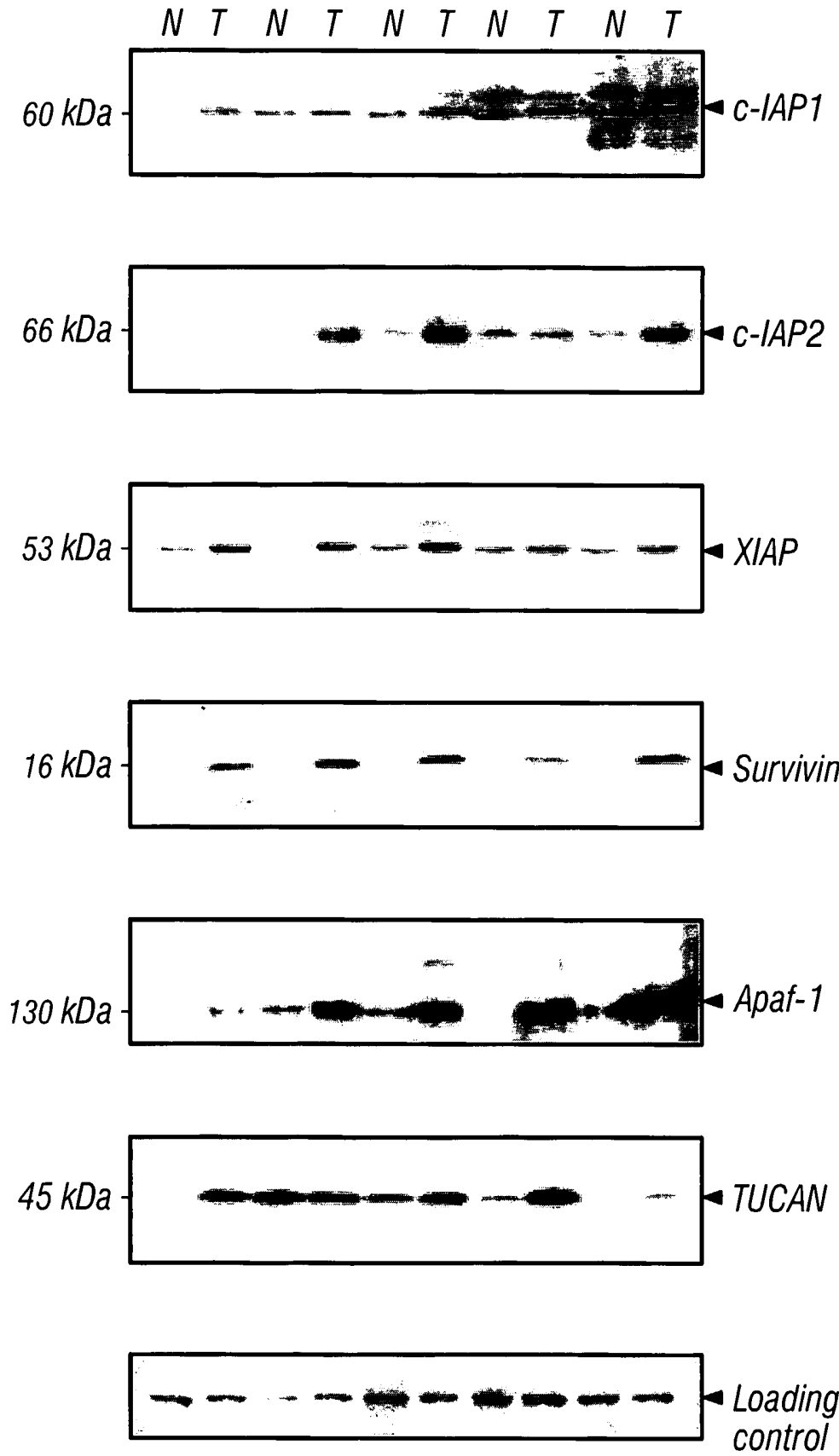


FIG. 3E

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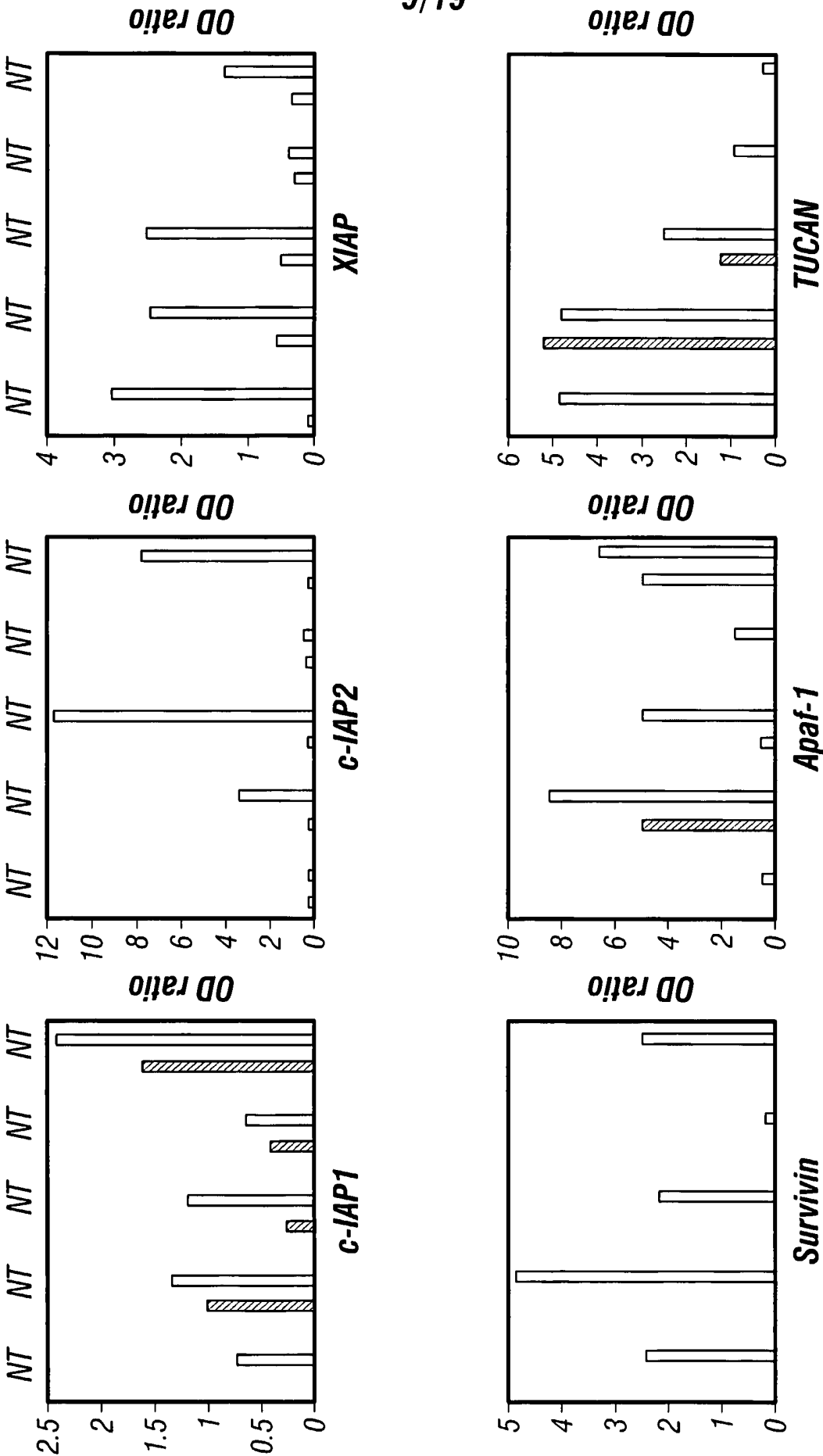


FIG. 3F

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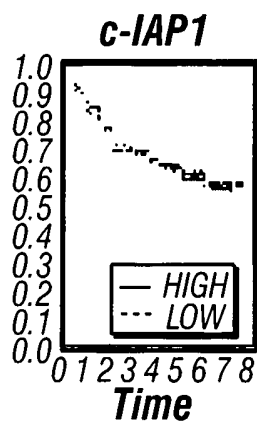


FIG. 4A

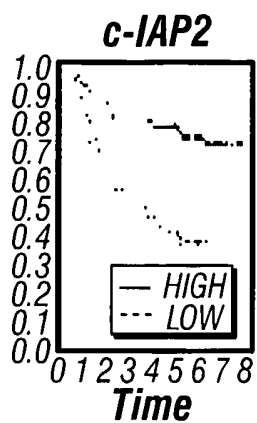


FIG. 4B

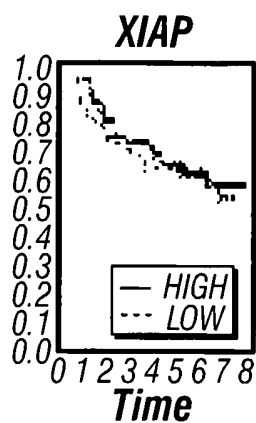


FIG. 4C

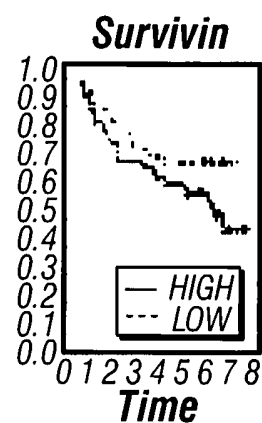


FIG. 4D

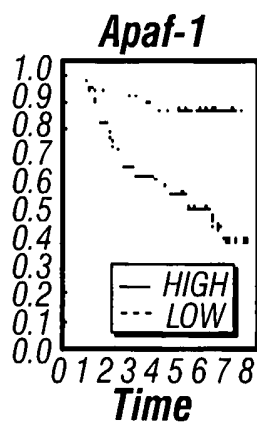


FIG. 4E

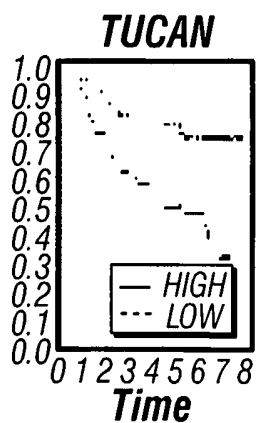


FIG. 4F

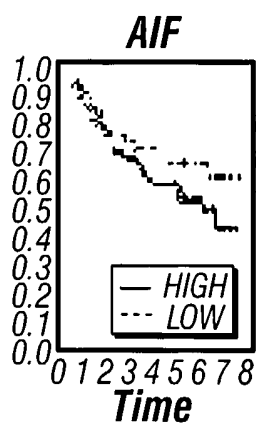


FIG. 4G

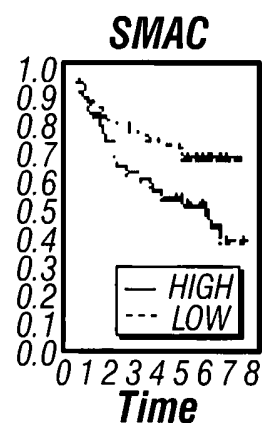


FIG. 4H

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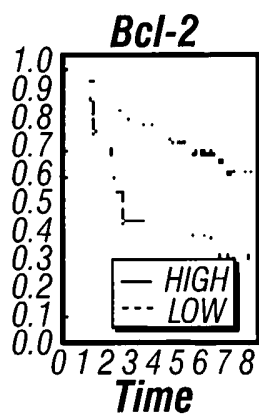


FIG. 4I

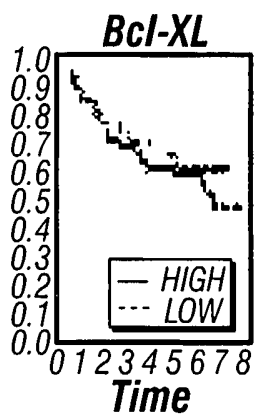


FIG. 4J

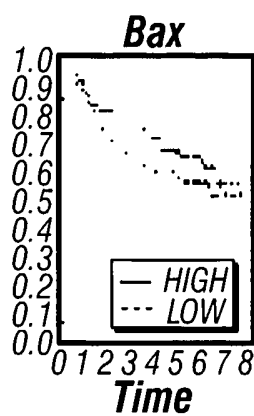


FIG. 4K

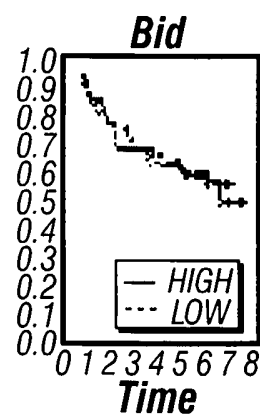


FIG. 4L

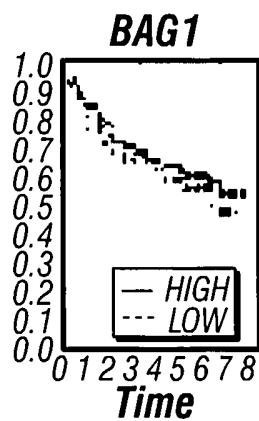


FIG. 4M

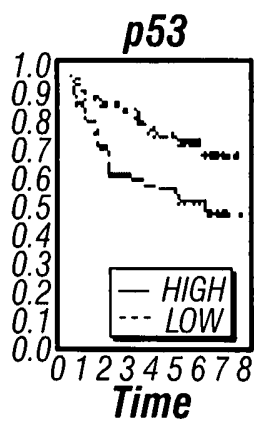


FIG. 4N

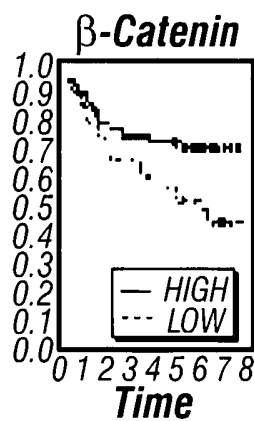


FIG. 4O

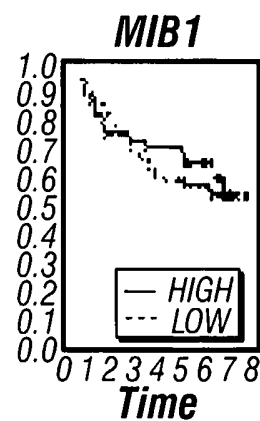


FIG. 4P

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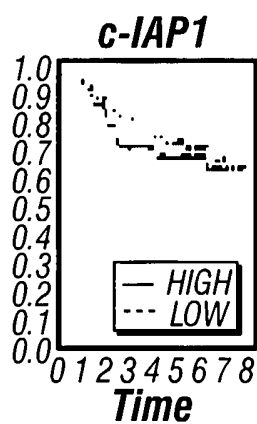


FIG. 4Q

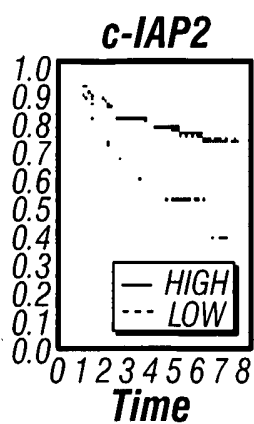


FIG. 4R

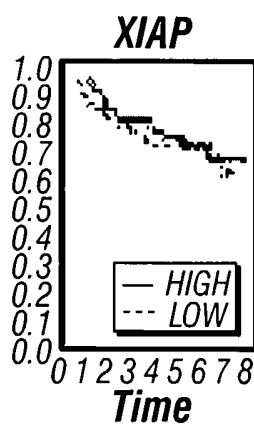


FIG. 4S

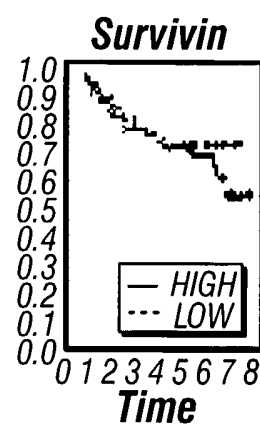


FIG. 4T

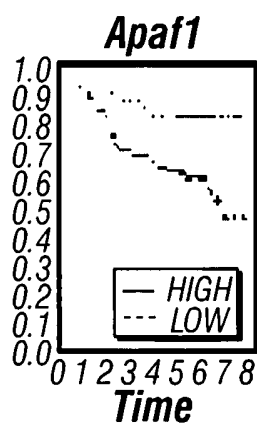


FIG. 4U

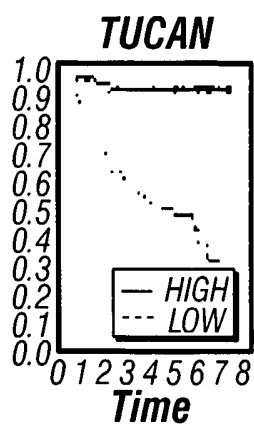


FIG. 4V

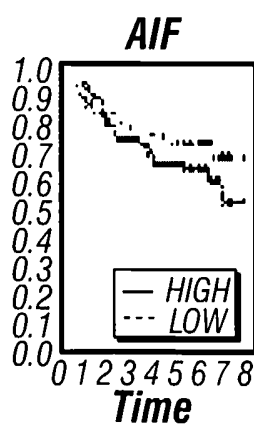


FIG. 4W

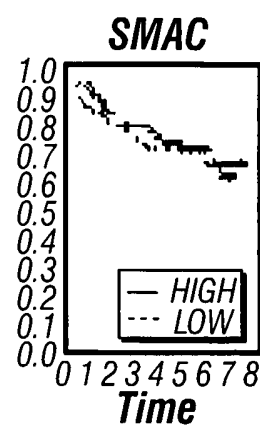


FIG. 4X

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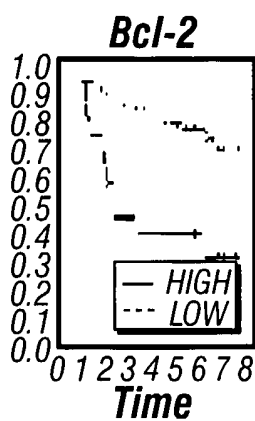


FIG. 4Y

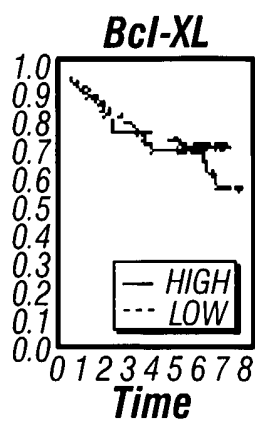


FIG. 4Z

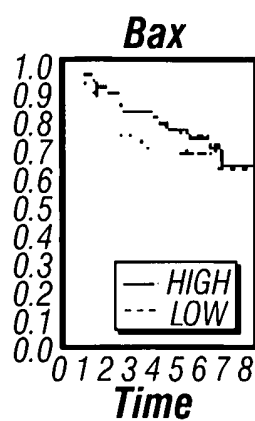


FIG. 4A-1

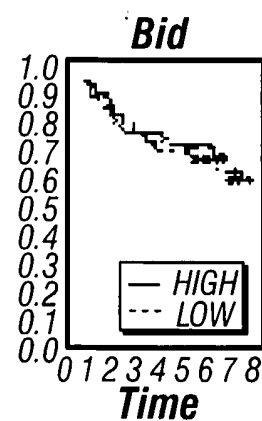


FIG. 4B-1

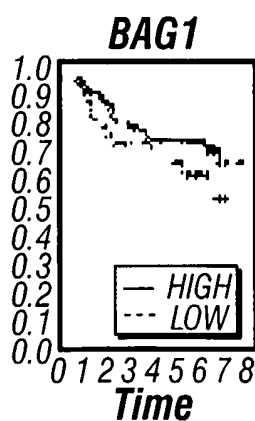


FIG. 4C-1

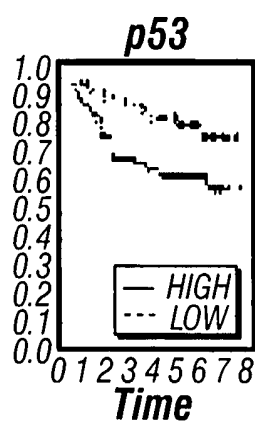


FIG. 4D-1

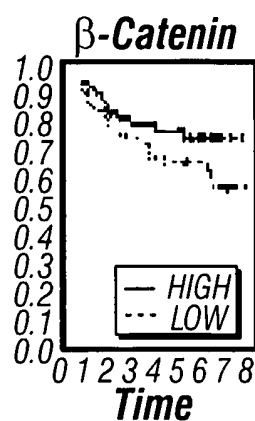


FIG. 4E-1

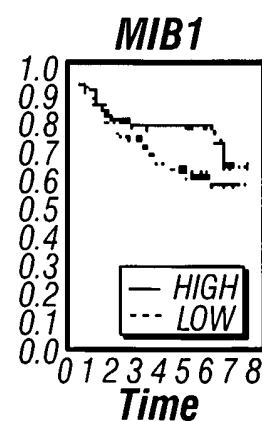


FIG. 4F-1

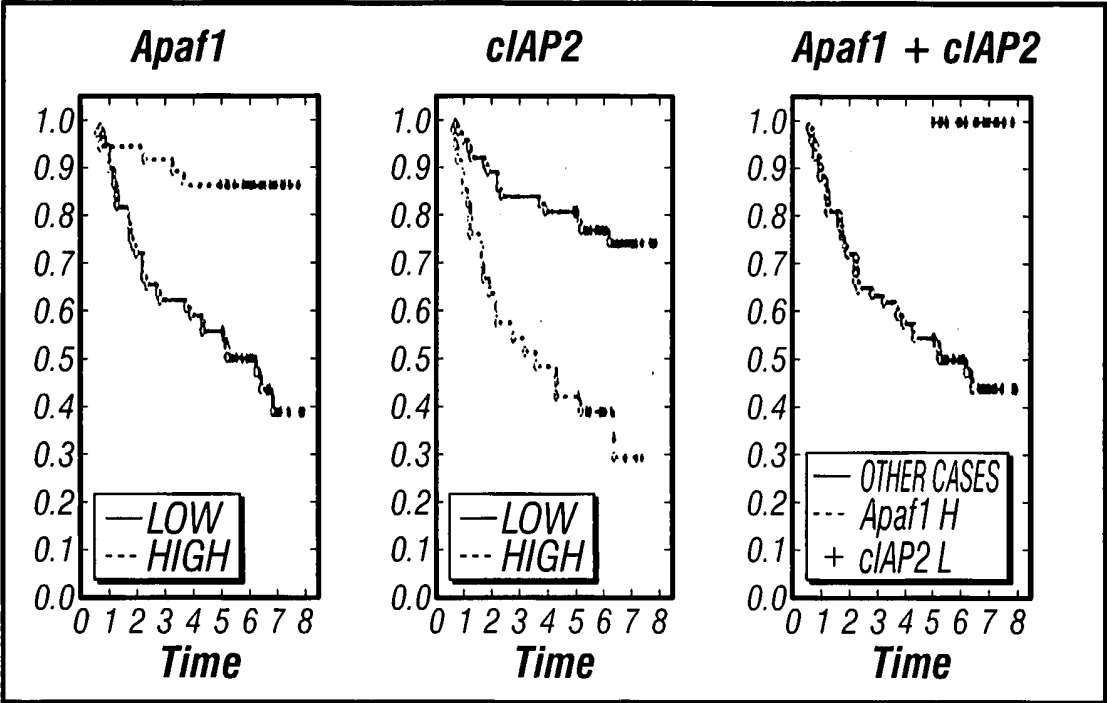


FIG. 5A

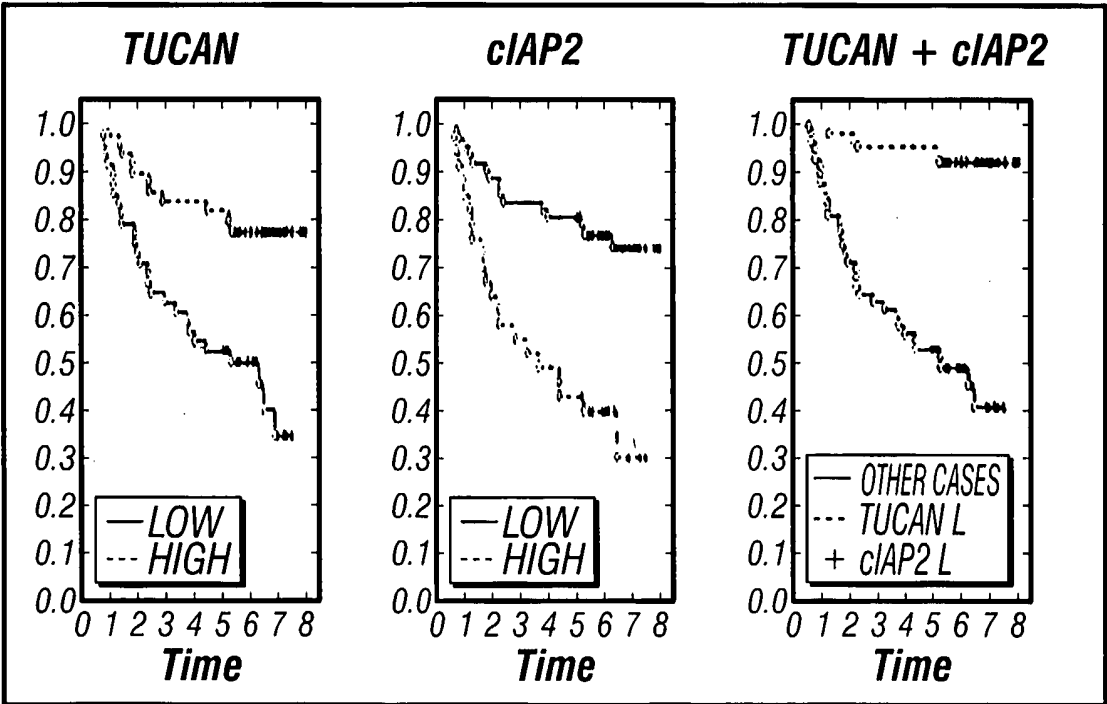


FIG. 5B

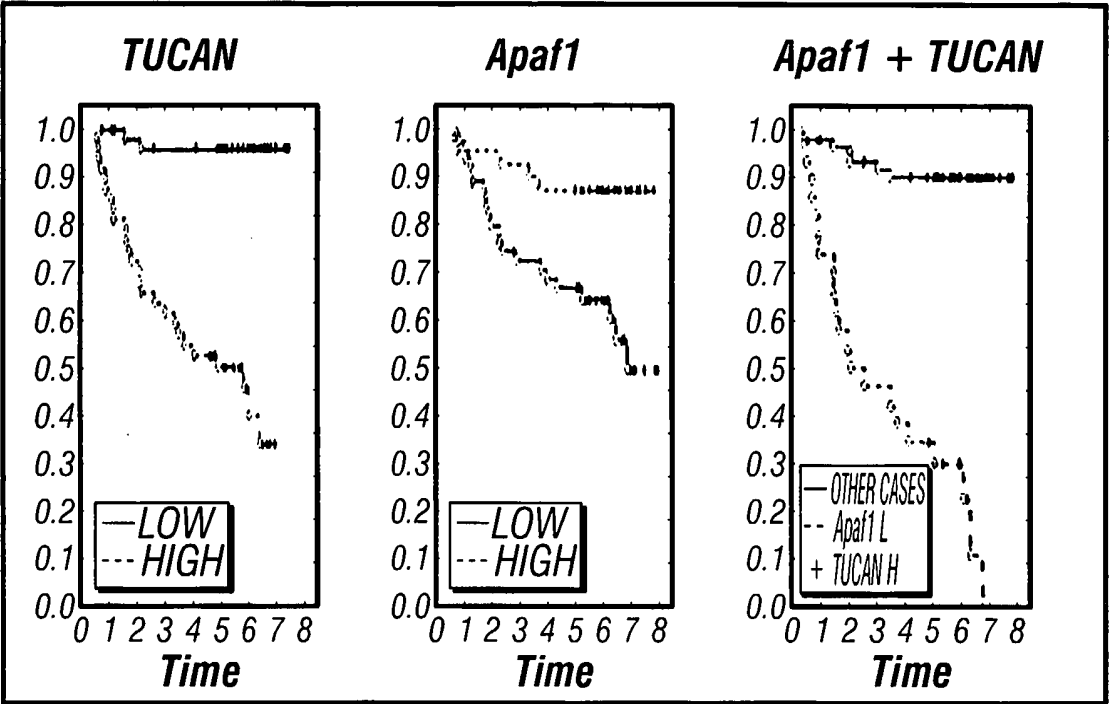


FIG. 5C

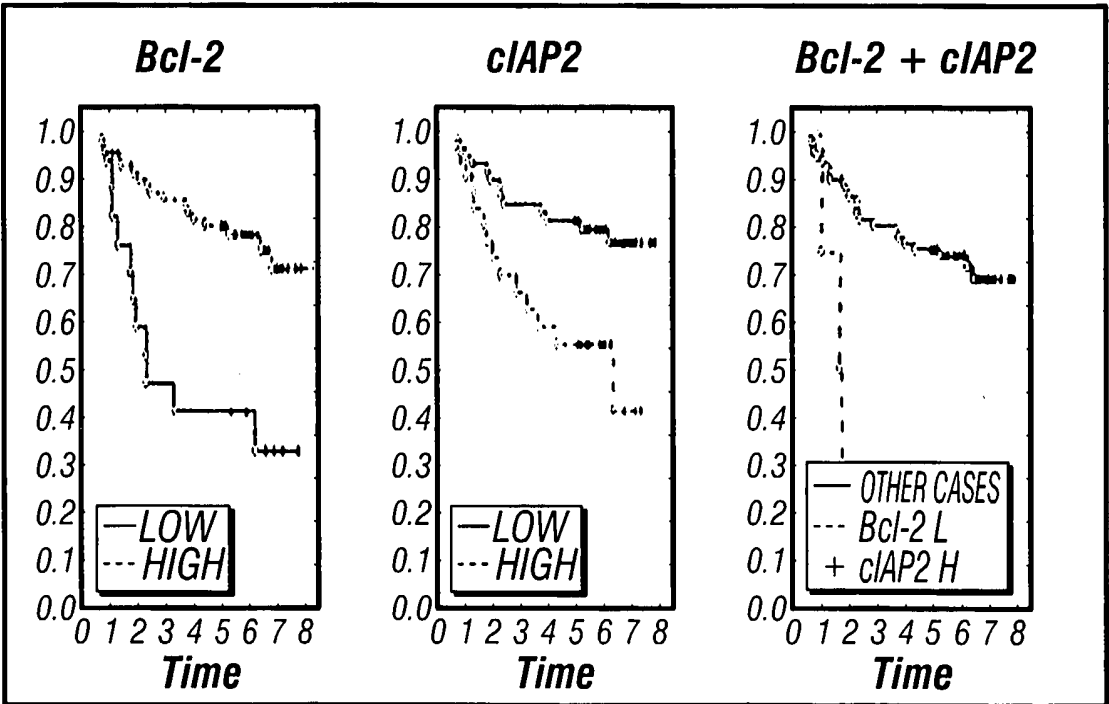


FIG. 5D

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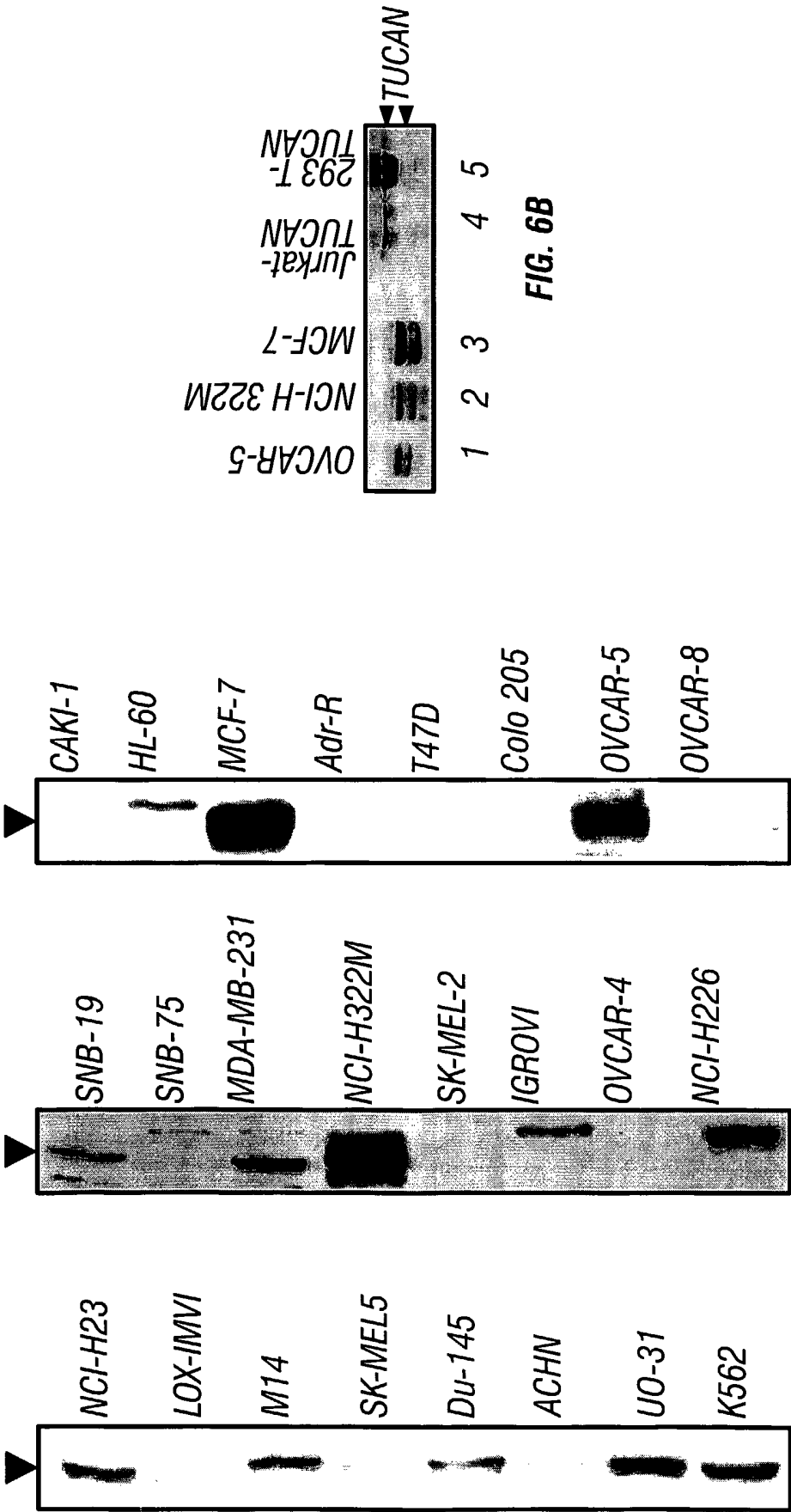


FIG. 6A

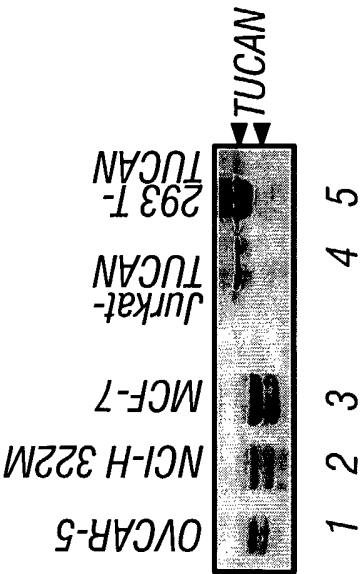


FIG. 6B

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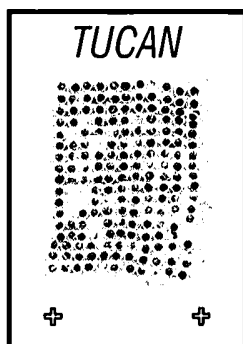


FIG. 7A

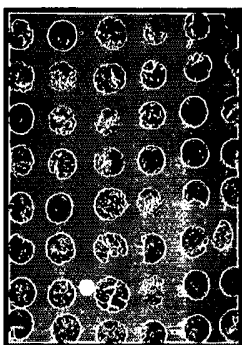


FIG. 7B

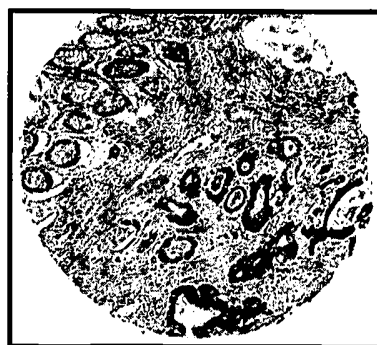


FIG. 7C

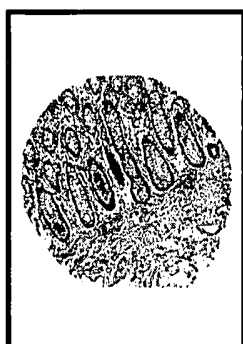


FIG. 7D



FIG. 7E

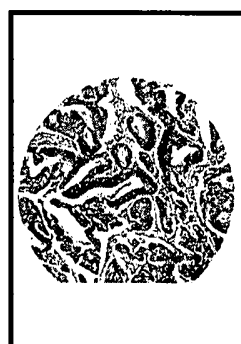


FIG. 7F

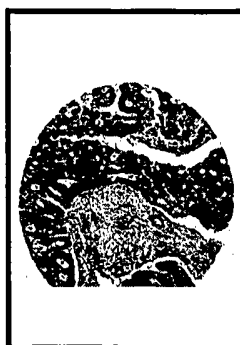


FIG. 7G

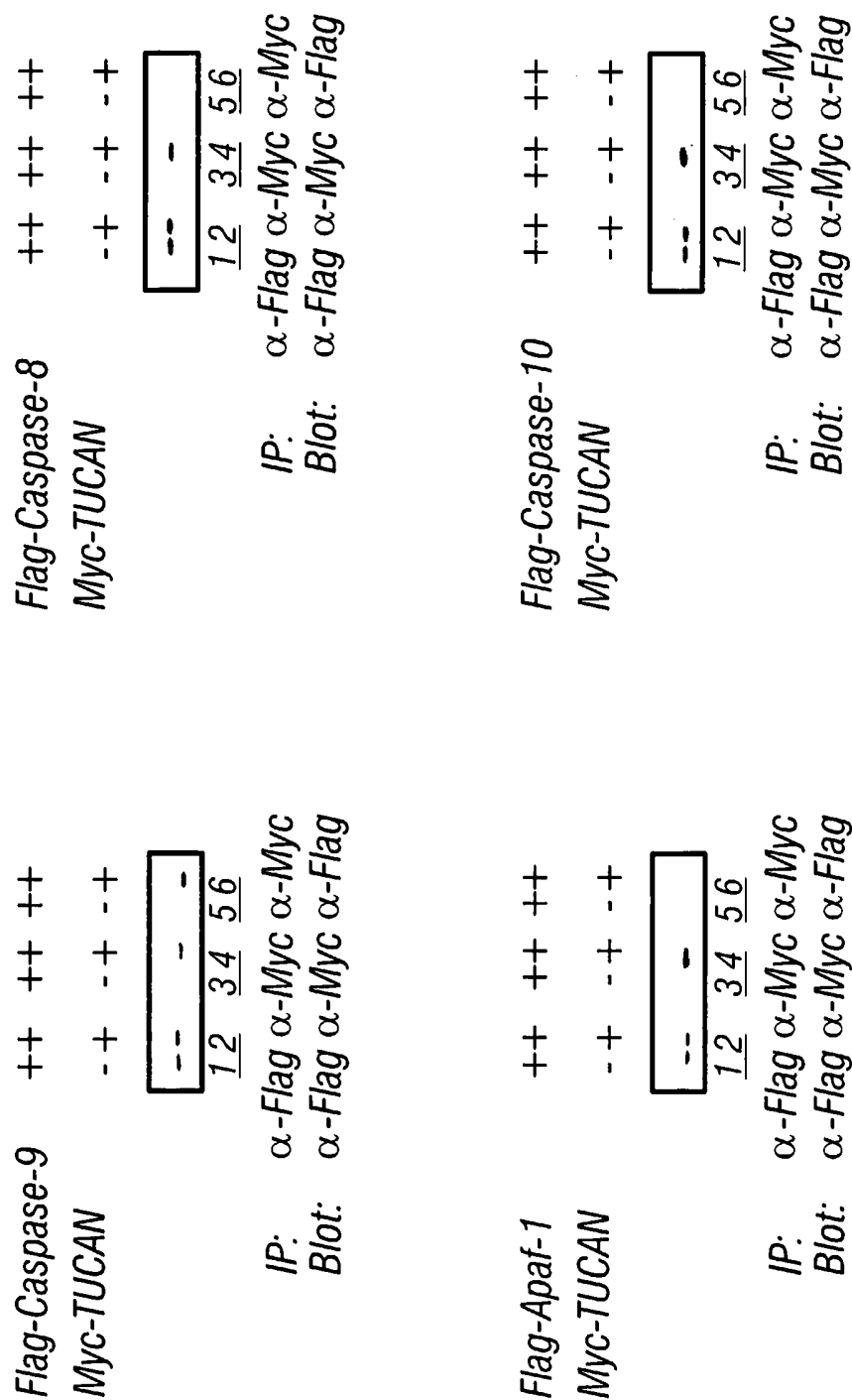


FIG. 8A

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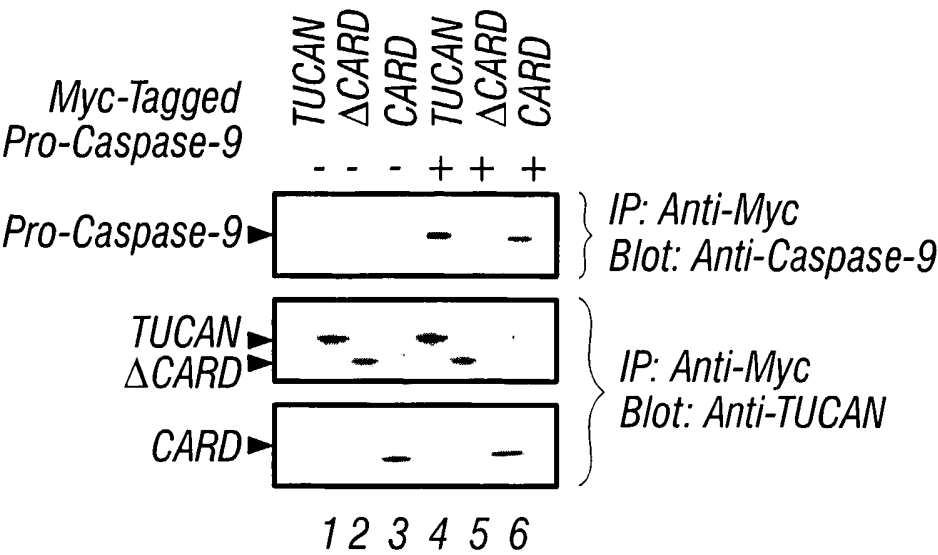


FIG. 8B

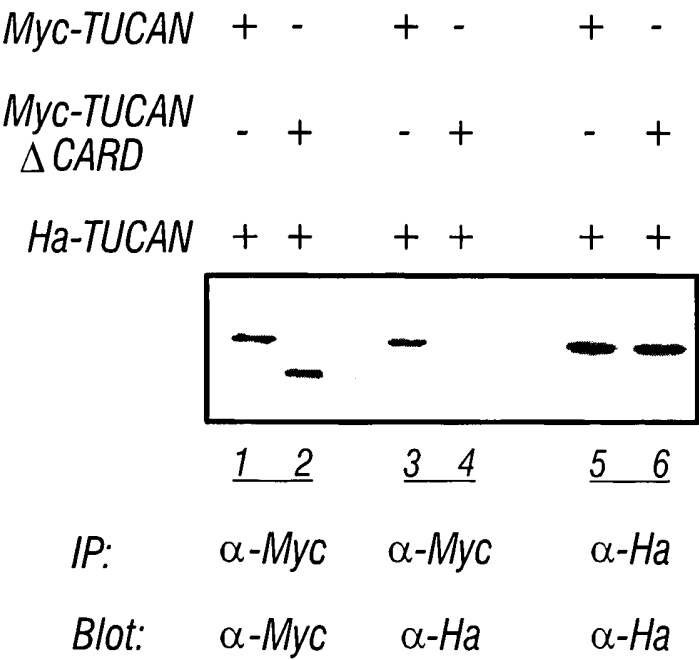


FIG. 8C

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<151> 2001-05-07

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Val Asn Tyr Leu Gly Gly Thr Phe Pro Gly Asp Ile Cys Ser Glu Glu	
20 25 30	
aat caa ata gtt tcc tct tat gct tct aaa gtc tgt ttt gag atc gaa	144
Asn Gln Ile Val Ser Ser Tyr Ala Ser Lys Val Cys Phe Glu Ile Glu	
35 40 45	
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Glu Asp Tyr Lys Asn Arg Gln Phe Leu Gly Pro Glu Gly Asn Val Asp	
50 55 60	
gtt gag ttg att gat aag agc aca aac aga tac agc gtt tgg ttc ccc	240
Val Glu Leu Ile Asp Lys Ser Thr Asn Arg Tyr Ser Val Trp Phe Pro	
65 70 75 80	
act gct ggc tgg tat ctg tgg tca gcc aca ggc ctc ggc ttc ctg gta	288
Thr Ala Gly Trp Tyr Leu Trp Ser Ala Thr Gly Leu Gly Phe Leu Val	

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Arg	Asp	Glu	Val	Thr	Val	Thr	Ile	Ala	Phe	Gly	Ser	Trp	Ser	Gln	His			
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ctg	gcc	ctg	gac	ctg	cag	cac	cat	gaa	cag	tgg	ctg	gtg	ggc	ggc	ccc	384		
Leu	Ala	Leu	Asp	Leu	Gln	His	His	Glu	Gln	Trp	Leu	Val	Gly	Gly	Pro			
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Arg	Val	Glu	Pro	Phe	Tyr	Ala	Val	Leu	Glu	Ser	Pro	Ser	Phe	Ser	Leu			
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Lys	Phe	His	Leu	Tyr	Leu	Val	Pro	Ser	Asp	Ala	Leu	Leu	Thr	Lys	Ala			
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ata	gat	gat	gag	gaa	gat	cgc	ttc	cat	ggt	gtg	cgc	ctg	cag	act	tcg	768		
Ile	Asp	Asp	Glu	Glu	Asp	Arg	Phe	His	Gly	Val	Arg	Leu	Gln	Thr	Ser			
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ccc	cca	atg	gaa	ccc	ctg	aac	ttt	ggt	tcc	agt	tat	att	gtg	tct	aat	816		
Pro	Pro	Met	Glu	Pro	Leu	Asn	Phe	Gly	Ser	Ser	Tyr	Ile	Val	Ser	Asn			
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          325          330          335

tca gcc cct cct cct ttc tca ggt gca gcc ttt gtg aag gag aac cac 1056
Ser Ala Pro Pro Pro Phe Ser Gly Ala Ala Phe Val Lys Glu Asn His
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          355          360          365

ctc cag gac aat gag gtt ctt act gag aat gag aag gag ctg gtg gag 1152
Leu Gln Asp Asn Glu Val Leu Thr Glu Asn Glu Lys Glu Leu Val Glu
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385          390          395          400

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Glu Lys Lys Gly Asp Leu Ala Leu Asp Val Leu Phe Arg Ser Ile Ser
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Glu Arg Asp Pro Tyr Leu Val Ser Tyr Leu Arg Gln Gln Asn Leu
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Glu Asp Tyr Lys Asn Arg Gln Phe Leu Gly Pro Glu Gly Asn Val Asp
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Val Glu Leu Ile Asp Lys Ser Thr Asn Arg Tyr Ser Val Trp Phe Pro

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Leu	Phe	Asp	Val	Thr	Ala	Glu	Pro	Glu	Glu	Ala	Val	Ala	Glu	Ile	His
	130					135					140				
Leu	Pro	His	Phe	Ile	Ser	Leu	Gln	Gly	Glu	Val	Asp	Val	Ser	Trp	Phe
145					150					155					160
Leu	Val	Ala	His	Phe	Lys	Asn	Glu	Gly	Met	Val	Leu	Glu	His	Pro	Ala
			165					170						175	
Arg	Val	Glu	Pro	Phe	Tyr	Ala	Val	Leu	Glu	Ser	Pro	Ser	Phe	Ser	Leu
		180						185					190		
Met	Gly	Ile	Leu	Leu	Arg	Ile	Ala	Ser	Gly	Thr	Arg	Leu	Ser	Ile	Pro
	195					200						205			
Ile	Thr	Ser	Asn	Thr	Leu	Ile	Tyr	Tyr	His	Pro	His	Pro	Glu	Asp	Ile
	210					215						220			
Lys	Phe	His	Leu	Tyr	Leu	Val	Pro	Ser	Asp	Ala	Leu	Leu	Thr	Lys	Ala
225					230					235					240
Ile	Asp	Asp	Glu	Glu	Asp	Arg	Phe	His	Gly	Val	Arg	Leu	Gln	Thr	Ser
			245						250					255	
Pro	Pro	Met	Glu	Pro	Leu	Asn	Phe	Gly	Ser	Ser	Tyr	Ile	Val	Ser	Asn
		260						265					270		
Ser	Ala	Asn	Leu	Lys	Val	Met	Pro	Lys	Glu	Leu	Lys	Leu	Ser	Tyr	Arg
		275					280					285			
Ser	Pro	Gly	Glu	Ile	Gln	His	Phe	Ser	Lys	Phe	Tyr	Ala	Gly	Gln	Met
	290					295					300				
Lys	Glu	Pro	Ile	Gln	Leu	Glu	Ile	Thr	Glu	Lys	Arg	His	Gly	Thr	Leu
305					310					315					320
Val	Trp	Asp	Thr	Glu	Val	Lys	Pro	Val	Asp	Leu	Gln	Leu	Val	Ala	Ala
			325						330					335	
Ser	Ala	Pro	Pro	Pro	Phe	Ser	Gly	Ala	Ala	Phe	Val	Lys	Glu	Asn	His
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Arg	Gln	Leu	Gln	Ala	Arg	Met	Gly	Asp	Leu	Lys	Gly	Val	Leu	Asp	Asp
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Leu	Gln	Asp	Asn	Glu	Val	Leu	Thr	Glu	Asn	Glu	Lys	Glu	Leu	Val	Glu
	370					375					380				
Gln	Glu	Lys	Thr	Arg	Gln	Ser	Lys	Asn	Glu	Ala	Leu	Leu	Ser	Met	Val
385					390					395					400
Glu	Lys	Lys	Gly	Asp	Leu	Ala	Leu	Asp	Val	Leu	Phe	Arg	Ser	Ile	Ser
			405						410					415	
Glu	Arg	Asp	Pro	Tyr	Leu	Val	Ser	Tyr	Leu	Arg	Gln	Gln	Asn	Leu	
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<211> 87

<212> PRT

<213> Homo sapiens

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 35 40 45
 Asn Glu Ala Leu Leu Ser Met Val Glu Lys Lys Gly Asp Leu Ala Leu
 50 55 60
 Asp Val Leu Phe Arg Ser Ile Ser Glu Arg Asp Pro Tyr Leu Val Ser
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 Tyr Leu Arg Gln Gln Asn Leu
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 35 40 45
 Gly Ser Arg Arg Asp Gln Ala Arg Gln Leu Ile Ile Asp Leu Glu Thr
 50 55 60
 Arg Gly Ser Gln Ala Leu Pro Leu Phe Ile Ser Cys Leu Glu Asp Thr
 65 70 75 80
 Gly Gln Asp Met Leu Ala Ser Phe Leu Arg Thr Asn Arg Gln Ala Ala
 85 90 95
 Lys Leu Ser Lys Pro Thr Leu Glu Asn Leu Thr Pro Val Val Leu Arg
 100 105 110
 Pro Glu Ile Arg Lys Pro Glu Val Leu Arg Pro Glu Thr Pro Arg Pro
 115 120 125
 Val Asp Ile Gly Ser Gly Gly Phe Gly Asp Val Gly Ala Leu Glu Ser
 130 135 140
 Leu Arg Gly Asn Ala Asp Leu Ala Tyr Ile Leu Ser Met Glu Pro Cys
 145 150 155 160
 Gly His Cys Leu Ile Ile Asn Asn Val Asn Phe Cys Arg Glu Ser Gly
 165 170 175
 Leu Arg Thr Arg Thr Gly Ser Asn Ile Asp Cys Glu Lys Leu Arg Arg
 180 185 190
 Arg Phe Ser Ser Pro His Phe Met Val Glu Val Lys Gly Asp Leu Thr
 195 200 205
 Ala Lys Lys Met Val Leu Ala Leu Leu Glu Leu Ala Gln Gln Asp His
 210 215 220
 Gly Ala Leu Asp Cys Cys Val Val Val Ile Leu Ser His Gly Cys Gln
 225 230 235 240
 Ala Ser His Leu Gln Phe Pro Gly Ala Val Tyr Gly Thr Asp Gly Cys
 245 250 255
 Pro Val Ser Val Glu Lys Ile Val Asn Ile Phe Asn Gly Thr Ser Cys

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                260                265                270
Pro Ser Leu Gly Gly Lys Pro Lys Leu Phe Phe Ile Gln Ala Cys Gly
                275                280                285
Gly Glu Gln Lys Asp His Gly Phe Glu Val Ala Ser Thr Ser Pro Glu
                290                295                300
Asp Glu Ser Pro Gly Ser Asn Pro Glu Pro Asp Ala Thr Pro Phe Gln
305                310                315                320
Glu Gly Leu Arg Thr Phe Asp Gln Leu Asp Ala Ile Ser Ser Leu Pro
                325                330                335
Thr Pro Ser Asp Ile Phe Val Ser Tyr Ser Thr Phe Pro Gly Phe Val
                340                345                350
Ser Trp Arg Asp Pro Lys Ser Gly Ser Trp Tyr Val Glu Thr Leu Asp
                355                360                365
Asp Ile Phe Glu Gln Trp Ala His Ser Glu Asp Leu Gln Ser Leu Leu
370                375                380
Leu Arg Val Ala Asn Ala Val Ser Val Lys Gly Ile Tyr Lys Gln Met
385                390                395                400
Pro Gly Cys Phe Asn Phe Leu Arg Lys Lys Leu Phe Phe Lys Thr Ser
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<213> Homo sapiens

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ttaaaatagc catcttagaa tcagtgaat atggtaatgt attattttcc tcctttgagt 180
taggtcttgt gctttttttt cctggccact aaatttcaca atttccaaaa agcaaaataa 240
acatattctg aatatttttg ctgtgaaaca cttgacagca gagctttcca ccatgaaaag 300
aagcttcatg agtcacacat tacatctttg gggtgattga atgccactga aacattctag 360
tagcctggag aagttgacct acctgtggag atgcctgcca ttaaattggca tcctgatggc 420
ttaatacaca tcactcttct gtgaagggtt ttaattttca acacagctta ctctgtagca 480
tcatgtttac attgtatgta taaagattat acaaagggtgc aattgtgtat ttcttcctta 540
aaatgtatca gtataggatt tagaatctcc atgttgaaac tctaaatgca tagaaataaa 600
aataataaaa aatttttcat tttggctttt cagcctagta ttaaaactga taaaagcaaa 660
gccatgcaca aaactacctc cctagagaaa ggctagtccc ttttcttccc cattcatttc 720
att atg aac ata gta gaa aac agc ata ttc tta tca aat ttg atg aaa 768
Met Asn Ile Val Glu Asn Ser Ile Phe Leu Ser Asn Leu Met Lys
1 5 10 15

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agc gcc aac acg ttt gaa ctg aaa tac gac ttg tca tgt gaa ctg tac 816
Ser Ala Asn Thr Phe Glu Leu Lys Tyr Asp Leu Ser Cys Glu Leu Tyr
20 25 30

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cga atg tct acg tat tcc act ttt cct gct ggg gtc cct gtc tca gaa 864
Arg Met Ser Thr Tyr Ser Thr Phe Pro Ala Gly Val Pro Val Ser Glu

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agg	agt	ctt	gct	cgc	gct	ggc	ttc	tat	tac	act	ggc	gtg	aat	gac	aag	912
Arg	Ser	Leu	Ala	Arg	Ala	Gly	Phe	Tyr	Tyr	Thr	Gly	Val	Asn	Asp	Lys	
		50					55					60				
gtc	aaa	tgc	ttc	tgt	tgt	ggc	ctg	atg	ctg	gat	aac	tgg	aaa	aga	gga	960
Val	Lys	Cys	Phe	Cys	Cys	Gly	Leu	Met	Leu	Asp	Asn	Trp	Lys	Arg	Gly	
	65					70					75					
gac	agt	cct	act	gaa	aag	cat	aaa	aag	ttg	tat	cct	agc	tgc	aga	ttc	1008
Asp	Ser	Pro	Thr	Glu	Lys	His	Lys	Lys	Leu	Tyr	Pro	Ser	Cys	Arg	Phe	
	80				85					90					95	
gtt	cag	agt	cta	aat	tcc	gtt	aac	aac	ttg	gaa	gct	acc	tct	cag	cct	1056
Val	Gln	Ser	Leu	Asn	Ser	Val	Asn	Asn	Leu	Glu	Ala	Thr	Ser	Gln	Pro	
				100					105					110		
act	ttt	cct	tct	tca	gta	aca	aat	tcc	aca	cac	tca	tta	ctt	ccg	ggc	1104
Thr	Phe	Pro	Ser	Ser	Val	Thr	Asn	Ser	Thr	His	Ser	Leu	Leu	Pro	Gly	
			115					120					125			
aca	gaa	aac	agt	gga	tat	ttc	cgt	ggc	tct	tat	tca	aac	tct	cca	tca	1152
Thr	Glu	Asn	Ser	Gly	Tyr	Phe	Arg	Gly	Ser	Tyr	Ser	Asn	Ser	Pro	Ser	
		130					135					140				
aat	cct	gta	aac	tcc	aga	gca	aat	caa	gat	ttt	tct	gcc	ttg	atg	aga	1200
Asn	Pro	Val	Asn	Ser	Arg	Ala	Asn	Gln	Asp	Phe	Ser	Ala	Leu	Met	Arg	
	145					150					155					
agt	tcc	tac	cac	tgt	gca	atg	aat	aac	gaa	aat	gcc	aga	tta	ctt	act	1248
Ser	Ser	Tyr	His	Cys	Ala	Met	Asn	Asn	Glu	Asn	Ala	Arg	Leu	Leu	Thr	
	160				165					170					175	
ttt	cag	aca	tgg	cca	ttg	act	ttt	ctg	tcg	cca	aca	gat	ctg	gca	aaa	1296
Phe	Gln	Thr	Trp	Pro	Leu	Thr	Phe	Leu	Ser	Pro	Thr	Asp	Leu	Ala	Lys	
				180					185					190		
gca	ggc	ttt	tac	tac	ata	gga	cct	gga	gac	aga	gtg	gct	tgc	ttt	gcc	1344
Ala	Gly	Phe	Tyr	Tyr	Ile	Gly	Pro	Gly	Asp	Arg	Val	Ala	Cys	Phe	Ala	
			195				200					205				
tgt	ggc	gga	aaa	ttg	agc	aat	tgg	gaa	ccg	aag	gat	aat	gct	atg	tca	1392
Cys	Gly	Gly	Lys	Leu	Ser	Asn	Trp	Glu	Pro	Lys	Asp	Asn	Ala	Met	Ser	
		210					215					220				
gaa	cac	ctg	aga	cat	ttt	ccc	aaa	tgc	cca	ttt	ata	gaa	aat	cag	ctt	1440
Glu	His	Leu	Arg	His	Phe	Pro	Lys	Cys	Pro	Phe	Ile	Glu	Asn	Gln	Leu	
	225					230					235					
caa	gac	act	tca	aga	tac	aca	gtt	tct	aat	ctg	agc	atg	cag	aca	cat	1488
Gln	Asp	Thr	Ser	Arg	Tyr	Thr	Val	Ser	Asn	Leu	Ser	Met	Gln	Thr	His	
	240				245					250					255	

gca gcc cgc ttt aaa aca ttc ttt aac tgg ccc tct agt gtt cta gtt	1536
Ala Ala Arg Phe Lys Thr Phe Phe Asn Trp Pro Ser Ser Val Leu Val	
260 265 270	
aat cct gag cag ctt gca agt gcg ggt ttt tat tat gtg ggt aac agt	1584
Asn Pro Glu Gln Leu Ala Ser Ala Gly Phe Tyr Tyr Val Gly Asn Ser	
275 280 285	
gat gat gtc aaa tgc ttt tgc tgt gat ggt gga ctc agg tgt tgg gaa	1632
Asp Asp Val Lys Cys Phe Cys Cys Asp Gly Gly Leu Arg Cys Trp Glu	
290 295 300	
tct gga gat gat cca tgg gtt caa cat gcc aag tgg ttt cca agg tgt	1680
Ser Gly Asp Asp Pro Trp Val Gln His Ala Lys Trp Phe Pro Arg Cys	
305 310 315	
gag tac ttg ata aga att aaa gga cag gag ttc atc cgt caa gtt caa	1728
Glu Tyr Leu Ile Arg Ile Lys Gly Gln Glu Phe Ile Arg Gln Val Gln	
320 325 330 335	
gcc agt tac cct cat cta ctt gaa cag ctg cta tcc aca tca gac agc	1776
Ala Ser Tyr Pro His Leu Leu Glu Gln Leu Leu Ser Thr Ser Asp Ser	
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cca gga gat gaa aat gca gag tca tca att atc cat ttt gaa cct gga	1824
Pro Gly Asp Glu Asn Ala Glu Ser Ser Ile Ile His Phe Glu Pro Gly	
355 360 365	
gaa gac cat tca gaa gat gca atc atg atg aat act cct gtg att aat	1872
Glu Asp His Ser Glu Asp Ala Ile Met Met Asn Thr Pro Val Ile Asn	
370 375 380	
gct gcc gtg gaa atg ggc ttt agt aga agc ctg gta aaa cag aca gtt	1920
Ala Ala Val Glu Met Gly Phe Ser Arg Ser Leu Val Lys Gln Thr Val	
385 390 395	
cag aga aaa atc cta gca act gga gag aat tat aga cta gtc aat gat	1968
Gln Arg Lys Ile Leu Ala Thr Gly Glu Asn Tyr Arg Leu Val Asn Asp	
400 405 410 415	
ctt gtg tta gac tta ctc aat gca gaa gat gaa ata agg gaa gag gag	2016
Leu Val Leu Asp Leu Leu Asn Ala Glu Asp Glu Ile Arg Glu Glu Glu	
420 425 430	
aga gaa aga gca act gag gaa aaa gaa tca aat gat tta tta tta atc	2064
Arg Glu Arg Ala Thr Glu Glu Lys Glu Ser Asn Asp Leu Leu Leu Ile	
435 440 445	
cgg aag aat aga atg gca ctt ttt caa cat ttg act tgt gta att cca	2112
Arg Lys Asn Arg Met Ala Leu Phe Gln His Leu Thr Cys Val Ile Pro	
450 455 460	

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atc ctg gat agt cta cta act gcc gga att att aat gaa caa gaa cat 2160
Ile Leu Asp Ser Leu Leu Thr Ala Gly Ile Ile Asn Glu Gln Glu His
      465                      470                      475

gat gtt att aaa cag aag aca cag acg tct tta caa gca aga gaa ctg 2208
Asp Val Ile Lys Gln Lys Thr Gln Thr Ser Leu Gln Ala Arg Glu Leu
480                      485                      490                      495

att gat acg att tta gta aaa gga aat att gca gcc act gta ttc aga 2256
Ile Asp Thr Ile Leu Val Lys Gly Asn Ile Ala Ala Thr Val Phe Arg
      500                      505                      510

aac tct ctg caa gaa gct gaa gct gtg tta tat gag cat tta ttt gtg 2304
Asn Ser Leu Gln Glu Ala Glu Ala Val Leu Tyr Glu His Leu Phe Val
      515                      520                      525

caa cag gac ata aaa tat att ccc aca gaa gat gtt tca gat cta cca 2352
Gln Gln Asp Ile Lys Tyr Ile Pro Thr Glu Asp Val Ser Asp Leu Pro
      530                      535                      540

gtg gaa gaa caa ttg cgg aga cta caa gaa gaa aga aca tgt aaa gtg 2400
Val Glu Glu Gln Leu Arg Arg Leu Gln Glu Glu Arg Thr Cys Lys Val
      545                      550                      555

tgt atg gac aaa gaa gtg tcc ata gtg ttt att cct tgt ggt cat cta 2448
Cys Met Asp Lys Glu Val Ser Ile Val Phe Ile Pro Cys Gly His Leu
560                      565                      570                      575

gta gta tgc aaa gat tgt gct cct tct tta aga aag tgt cct att tgt 2496
Val Val Cys Lys Asp Cys Ala Pro Ser Leu Arg Lys Cys Pro Ile Cys
      580                      585                      590

agg agt aca atc aag ggt aca gtt cgt aca ttt ctt tca tga 2538
Arg Ser Thr Ile Lys Gly Thr Val Arg Thr Phe Leu Ser *
      595                      600

agaagaacca aaacatcatc taaacttttag aattaattta tttaatgtat tataacttta 2598
actttcatcc taatttggtt tccttaaaat ttttatttat ttacaactca acaaacattg 2658
ttttgtgtaa catatttaat atatgtatct aaaccatatg aacatatatt ttttagaaac 2718
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<211> 604

<212> PRT

<213> Homo sapiens

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Met Ser Thr Tyr Ser Thr Phe Pro Ala Gly Val Pro Val Ser Glu Arg
 35          40          45
Ser Leu Ala Arg Ala Gly Phe Tyr Tyr Thr Gly Val Asn Asp Lys Val
 50          55          60
Lys Cys Phe Cys Cys Gly Leu Met Leu Asp Asn Trp Lys Arg Gly Asp
 65          70          75          80
Ser Pro Thr Glu Lys His Lys Lys Leu Tyr Pro Ser Cys Arg Phe Val
 85          90          95
Gln Ser Leu Asn Ser Val Asn Asn Leu Glu Ala Thr Ser Gln Pro Thr
 100         105         110
Phe Pro Ser Ser Val Thr Asn Ser Thr His Ser Leu Leu Pro Gly Thr
 115         120         125
Glu Asn Ser Gly Tyr Phe Arg Gly Ser Tyr Ser Asn Ser Pro Ser Asn
 130         135         140
Pro Val Asn Ser Arg Ala Asn Gln Asp Phe Ser Ala Leu Met Arg Ser
 145         150         155         160
Ser Tyr His Cys Ala Met Asn Asn Glu Asn Ala Arg Leu Leu Thr Phe
 165         170         175
Gln Thr Trp Pro Leu Thr Phe Leu Ser Pro Thr Asp Leu Ala Lys Ala
 180         185         190
Gly Phe Tyr Tyr Ile Gly Pro Gly Asp Arg Val Ala Cys Phe Ala Cys
 195         200         205
Gly Gly Lys Leu Ser Asn Trp Glu Pro Lys Asp Asn Ala Met Ser Glu
 210         215         220
His Leu Arg His Phe Pro Lys Cys Pro Phe Ile Glu Asn Gln Leu Gln
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Asp Thr Ser Arg Tyr Thr Val Ser Asn Leu Ser Met Gln Thr His Ala
 245         250         255
Ala Arg Phe Lys Thr Phe Phe Asn Trp Pro Ser Ser Val Leu Val Asn
 260         265         270
Pro Glu Gln Leu Ala Ser Ala Gly Phe Tyr Tyr Val Gly Asn Ser Asp
 275         280         285
Asp Val Lys Cys Phe Cys Cys Asp Gly Gly Leu Arg Cys Trp Glu Ser
 290         295         300
Gly Asp Asp Pro Trp Val Gln His Ala Lys Trp Phe Pro Arg Cys Glu
 305         310         315         320
Tyr Leu Ile Arg Ile Lys Gly Gln Glu Phe Ile Arg Gln Val Gln Ala
 325         330         335
Ser Tyr Pro His Leu Leu Glu Gln Leu Leu Ser Thr Ser Asp Ser Pro
 340         345         350
Gly Asp Glu Asn Ala Glu Ser Ser Ile Ile His Phe Glu Pro Gly Glu
 355         360         365
Asp His Ser Glu Asp Ala Ile Met Met Asn Thr Pro Val Ile Asn Ala
 370         375         380
Ala Val Glu Met Gly Phe Ser Arg Ser Leu Val Lys Gln Thr Val Gln
 385         390         395         400
Arg Lys Ile Leu Ala Thr Gly Glu Asn Tyr Arg Leu Val Asn Asp Leu
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Val Leu Asp Leu Leu Asn Ala Glu Asp Glu Ile Arg Glu Glu Glu Arg
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 Glu Arg Ala Thr Glu Glu Lys Glu Ser Asn Asp Leu Leu Ile Arg
 435 440 445
 Lys Asn Arg Met Ala Leu Phe Gln His Leu Thr Cys Val Ile Pro Ile
 450 455 460
 Leu Asp Ser Leu Leu Thr Ala Gly Ile Ile Asn Glu Gln Glu His Asp
 465 470 475 480
 Val Ile Lys Gln Lys Thr Gln Thr Ser Leu Gln Ala Arg Glu Leu Ile
 485 490 495
 Asp Thr Ile Leu Val Lys Gly Asn Ile Ala Ala Thr Val Phe Arg Asn
 500 505 510
 Ser Leu Gln Glu Ala Glu Ala Val Leu Tyr Glu His Leu Phe Val Gln
 515 520 525
 Gln Asp Ile Lys Tyr Ile Pro Thr Glu Asp Val Ser Asp Leu Pro Val
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 Glu Glu Gln Leu Arg Arg Leu Gln Glu Glu Arg Thr Cys Lys Val Cys
 545 550 555 560
 Met Asp Lys Glu Val Ser Ile Val Phe Ile Pro Cys Gly His Leu Val
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<212> DNA

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Met Ala Thr Gln Ala Asp Leu Met Glu Leu Asp

1

5

10

atg gcc atg gaa cca gac aga aaa gcg gct gtt agt cac tgg cag caa 281

Met Ala Met Glu Pro Asp Arg Lys Ala Ala Val Ser His Trp Gln Gln

15

20

25

cag tct tac ctg gac tct gga atc cat tct ggt gcc act acc aca gct 329

Gln Ser Tyr Leu Asp Ser Gly Ile His Ser Gly Ala Thr Thr Thr Ala

30

35

40

cct tct ctg agt ggt aaa ggc aat cct gag gaa gag gat gtg gat acc 377

Pro Ser Leu Ser Gly Lys Gly Asn Pro Glu Glu Glu Asp Val Asp Thr

45	50	55	
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Ser Gln Val Leu Tyr Glu Trp Glu Gln Gly Phe Ser Gln Ser Phe Thr			
60	65	70	75
caa gaa caa gta gct gat att gat gga cag tat gca atg act cga gct			473
Gln Glu Gln Val Ala Asp Ile Asp Gly Gln Tyr Ala Met Thr Arg Ala			
	80	85	90
cag agg gta cga gct gct atg ttc cct gag aca tta gat gag ggc atg			521
Gln Arg Val Arg Ala Ala Met Phe Pro Glu Thr Leu Asp Glu Gly Met			
	95	100	105
cag atc cca tct aca cag ttt gat gct gct cat ccc act aat gtc cag			569
Gln Ile Pro Ser Thr Gln Phe Asp Ala Ala His Pro Thr Asn Val Gln			
	110	115	120
cgt ttg gct gaa cca tca cag atg ctg aaa cat gca gtt gta aac ttg			617
Arg Leu Ala Glu Pro Ser Gln Met Leu Lys His Ala Val Val Asn Leu			
	125	130	135
att aac tat caa gat gat gca gaa ctt gcc aca cgt gca atc cct gaa			665
Ile Asn Tyr Gln Asp Asp Ala Glu Leu Ala Thr Arg Ala Ile Pro Glu			
140	145	150	155
ctg aca aaa ctg cta aat gac gag gac cag gtg gtg gtt aat aag gct			713
Leu Thr Lys Leu Leu Asn Asp Glu Asp Gln Val Val Val Asn Lys Ala			
	160	165	170
gca gtt atg gtc cat cag ctt tct aaa aag gaa gct tcc aga cac gct			761
Ala Val Met Val His Gln Leu Ser Lys Lys Glu Ala Ser Arg His Ala			
	175	180	185
atc atg cgt tct cct cag atg gtg tct gct att gta cgt acc atg cag			809
Ile Met Arg Ser Pro Gln Met Val Ser Ala Ile Val Arg Thr Met Gln			
	190	195	200
aat aca aat gat gta gaa aca gct cgt tgt acc gct ggg acc ttg cat			857
Asn Thr Asn Asp Val Glu Thr Ala Arg Cys Thr Ala Gly Thr Leu His			
205	210	215	
aac ctt tcc cat cat cgt gag ggc tta ctg gcc atc ttt aag tct gga			905
Asn Leu Ser His His Arg Glu Gly Leu Leu Ala Ile Phe Lys Ser Gly			
220	225	230	235
ggc att cct gcc ctg gtg aaa atg ctt ggt tca cca gtg gat tct gtg			953
Gly Ile Pro Ala Leu Val Lys Met Leu Gly Ser Pro Val Asp Ser Val			
	240	245	250
ttg ttt tat gcc att aca act ctc cac aac ctt tta tta cat caa gaa			1001
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Gly Ala Lys Met Ala Val Arg Leu Ala Gly Gly Leu Gln Lys Met Val	
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Ala Leu Leu Asn Lys Thr Asn Val Lys Phe Leu Ala Ile Thr Thr Asp	
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Cys Leu Gln Ile Leu Ala Tyr Gly Asn Gln Glu Ser Lys Leu Ile Ile	
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Leu Ala Ser Gly Gly Pro Gln Ala Leu Val Asn Ile Met Arg Thr Tyr	
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Thr Tyr Glu Lys Leu Leu Trp Thr Thr Ser Arg Val Leu Lys Val Leu	
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Ser Val Cys Ser Ser Asn Lys Pro Ala Ile Val Glu Ala Gly Gly Met	
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Gln Ala Leu Gly Leu His Leu Thr Asp Pro Ser Gln Arg Leu Val Gln	
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Asn Cys Leu Trp Thr Leu Arg Asn Leu Ser Asp Ala Ala Thr Lys Gln	
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Glu Gly Met Glu Gly Leu Leu Gly Thr Leu Val Gln Leu Leu Gly Ser	
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Asp Asp Ile Asn Val Val Thr Cys Ala Ala Gly Ile Leu Ser Asn Leu	
415 420 425	
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Thr Cys Asn Asn Tyr Lys Asn Lys Met Met Val Cys Gln Val Gly Gly	
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Ile Glu Ala Leu Val Arg Thr Val Leu Arg Ala Gly Asp Arg Glu Asp	
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Ile Thr Glu Pro Ala Ile Cys Ala Leu Arg His Leu Thr Ser Arg His	
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Gln Glu Ala Glu Met Ala Gln Asn Ala Val Arg Leu His Tyr Gly Leu	
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Pro Val Val Val Lys Leu Leu His Pro Pro Ser His Trp Pro Leu Ile	
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Lys Ala Thr Val Gly Leu Ile Arg Asn Leu Ala Leu Cys Pro Ala Asn	
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Tyr Ser Pro Ile Glu Asn Ile Gln Arg Val Ala Ala Gly Val Leu Cys	
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Ala Thr Ala Pro Leu Thr Glu Leu Leu His Ser Arg Asn Glu Gly Val	
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Gln Asp Tyr Lys Lys Arg Leu Ser Val Glu Leu Thr Ser Ser Leu Phe	
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gac	ccc	atg	atg	gaa	cat	gag	atg	ggt	ggc	cac	cac	cct	ggt	gct	gac	2441
Asp	Pro	Met	Met	Glu	His	Glu	Met	Gly	Gly	His	His	Pro	Gly	Ala	Asp	
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Asp	Gly	Leu	Pro	Pro	Gly	Asp	Ser	Asn	Gln	Leu	Ala	Trp	Phe	Asp	Thr	
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Asp	Leu	*														
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<212> PRT

<213> Homo sapiens

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Ser	Gly	Ile	His	Ser	Gly	Ala	Thr	Thr	Thr	Ala	Pro	Ser	Leu	Ser	Gly	
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Lys	Gly	Asn	Pro	Glu	Glu	Glu	Asp	Val	Asp	Thr	Ser	Gln	Val	Leu	Tyr	
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Glu	Trp	Glu	Gln	Gly	Phe	Ser	Gln	Ser	Phe	Thr	Gln	Glu	Gln	Val	Ala	
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Gln	Phe	Asp	Ala	Ala	His	Pro	Thr	Asn	Val	Gln	Arg	Leu	Ala	Glu	Pro	
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Asp	Ala	Glu	Leu	Ala	Thr	Arg	Ala	Ile	Pro	Glu	Leu	Thr	Lys	Leu	Leu	

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Asn	Asp	Glu	Asp	Gln	Val	Val	Val	Asn	Lys	Ala	Ala	Val	Met	Val
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Gln	Leu	Ser	Lys	Lys	Glu	Ala	Ser	Arg	His	Ala	Ile	Met	Arg	Ser
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Gln	Met	Val	Ser	Ala	Ile	Val	Arg	Thr	Met	Gln	Asn	Thr	Asn	Asp
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Glu	Thr	Ala	Arg	Cys	Thr	Ala	Gly	Thr	Leu	His	Asn	Leu	Ser	His
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225					230					235				240
Val	Lys	Met	Leu	Gly	Ser	Pro	Val	Asp	Ser	Val	Leu	Phe	Tyr	Ala
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Thr	Thr	Leu	His	Asn	Leu	Leu	Leu	His	Gln	Glu	Gly	Ala	Lys	Met
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Val	Arg	Leu	Ala	Gly	Gly	Leu	Gln	Lys	Met	Val	Ala	Leu	Leu	Asn
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Thr	Asn	Val	Lys	Phe	Leu	Ala	Ile	Thr	Thr	Asp	Cys	Leu	Gln	Ile
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Pro	Gln	Ala	Leu	Val	Asn	Ile	Met	Arg	Thr	Tyr	Thr	Tyr	Glu	Lys
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Leu	Trp	Thr	Thr	Ser	Arg	Val	Leu	Lys	Val	Leu	Ser	Val	Cys	Ser
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His	Leu	Thr	Asp	Pro	Ser	Gln	Arg	Leu	Val	Gln	Asn	Cys	Leu	Trp
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Leu	Arg	Asn	Leu	Ser	Asp	Ala	Ala	Thr	Lys	Gln	Glu	Gly	Met	Glu
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Val	Thr	Cys	Ala	Ala	Gly	Ile	Leu	Ser	Asn	Leu	Thr	Cys	Asn	Asn
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Lys	Asn	Lys	Met	Met	Val	Cys	Gln	Val	Gly	Gly	Ile	Glu	Ala	Leu
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Arg	Thr	Val	Leu	Arg	Ala	Gly	Asp	Arg	Glu	Asp	Ile	Thr	Glu	Pro
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Ile	Cys	Ala	Leu	Arg	His	Leu	Thr	Ser	Arg	His	Gln	Glu	Ala	Glu
465				470					475					480
Ala	Gln	Asn	Ala	Val	Arg	Leu	His	Tyr	Gly	Leu	Pro	Val	Val	Val
			485					490					495	Lys
Leu	Leu	His	Pro	Pro	Ser	His	Trp	Pro	Leu	Ile	Lys	Ala	Thr	Val
	500						505					510		Gly
Leu	Ile	Arg	Asn	Leu	Ala	Leu	Cys	Pro	Ala	Asn	His	Ala	Pro	Leu
	515					520					525			Arg
Glu	Gln	Gly	Ala	Ile	Pro	Arg	Leu	Val	Gln	Leu	Leu	Val	Arg	Ala
	530				535				540					His
Gln	Asp	Thr	Gln	Arg	Arg	Thr	Ser	Met	Gly	Gly	Thr	Gln	Gln	Gln
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			1 5							

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Asn Cys Leu Leu Gln His Arg Glu Ala Leu Glu Lys Asp Ile Lys Thr

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Ser Tyr Ile Met Asp His Met Ile Ser Asp Gly Phe Leu Thr Ile Ser			
25	30	35	
gaa gag gaa aaa gta aga aat gag ccc act caa cag caa aga gca gct			739
Glu Glu Glu Lys Val Arg Asn Glu Pro Thr Gln Gln Gln Arg Ala Ala			
40	45	50	
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Met Leu Ile Lys Met Ile Leu Lys Lys Asp Asn Asp Ser Tyr Val Ser			
55	60	65	70
ttc tac aat gct cta cta cat gaa gga tat aaa gat ctt gct gcc ctt			835
Phe Tyr Asn Ala Leu Leu His Glu Gly Tyr Lys Asp Leu Ala Ala Leu			
75	80	85	
ctc cat gat ggc att cct gtt gtc tct tct tcc agt gta agg aca gtc			883
Leu His Asp Gly Ile Pro Val Val Ser Ser Ser Ser Val Arg Thr Val			
90	95	100	
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Leu Cys Glu Gly Gly Val Pro Gln Arg Pro Val Val Phe Val Thr Arg			
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Lys Lys Leu Val Asn Ala Ile Gln Gln Lys Leu Ser Lys Leu Lys Gly			
120	125	130	
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Glu Pro Gly Trp Val Thr Ile His Gly Met Ala Gly Cys Gly Lys Ser			
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Val Leu Ala Ala Glu Ala Val Arg Asp His Ser Leu Leu Glu Gly Cys			
155	160	165	
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Phe Pro Gly Gly Val His Trp Val Ser Val Gly Lys Gln Asp Lys Ser			
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Gly Leu Leu Met Lys Leu Gln Asn Leu Cys Thr Arg Leu Asp Gln Asp			
185	190	195	
gag agt ttt tcc cag agg ctt cca ctt aat att gaa gag gct aaa gac			1219
Glu Ser Phe Ser Gln Arg Leu Pro Leu Asn Ile Glu Glu Ala Lys Asp			
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Arg Leu Arg Ile Leu Met Leu Arg Lys His Pro Arg Ser Leu Leu Ile			
215	220	225	230

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Cys Gln Ile Leu Leu Thr Thr Arg Asp Lys Ser Val Thr Asp Ser Val	
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Lys Gly Leu Glu Ile Leu Ser Leu Phe Val Asn Met Lys Lys Ala Asp	
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Trp Glu Tyr Tyr Leu Lys Gln Leu Gln Asn Lys Gln Phe Lys Arg Ile	
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Arg Lys Ser Ser Ser Tyr Asp Tyr Glu Ala Leu Asp Glu Ala Met Ser	
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Phe Val Asn Lys Ser Leu Leu Phe Cys Asp Arg Asn Gly Lys Ser Phe	
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Arg Tyr Tyr Leu His Asp Leu Gln Val Asp Phe Leu Thr Glu Lys Asn	
425 430 435	

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Cys Ser Gln Leu Gln Asp Leu His Lys Lys Ile Ile Thr Gln Phe Gln	
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Tyr Trp Tyr Asn Phe Leu Ala Tyr His Met Ala Ser Ala Lys Met His	
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Thr	Cys	Ser	Val	Asp	Lys	Lys	Val	Lys	Ile	Trp	Asn	Ser	Met	Thr	Gly		
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<210> 12

<211> 239

<212> PRT

<213> Homo sapiens

<400> 12

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Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
              35              40              45
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
              50              55              60
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
65              70              75              80
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
              85              90              95
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
              100              105              110
Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
              115              120              125
Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
              130              135              140
Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145              150              155              160
Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
              165              170              175
Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
              180              185              190
Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
              195              200              205
Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
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		1					5					10					
gta	act	tca	ttc	ttc	agg	tac	aga	cag	tgt	ttg	tgt	gtt	cct	gtt	gtg	100	
Val	Thr	Ser	Phe	Phe	Arg	Tyr	Arg	Gln	Cys	Leu	Cys	Val	Pro	Val	Val		
			15					20					25				
gct	aac	ttt	aag	aag	cgg	tgt	ttc	tca	gaa	ttg	ata	aga	cca	tgg	cac	148	
Ala	Asn	Phe	Lys	Lys	Arg	Cys	Phe	Ser	Glu	Leu	Ile	Arg	Pro	Trp	His		
		30				35						40					
aaa	act	gtg	acg	att	ggc	ttt	gga	gta	acc	ctg	tgt	gcg	gtt	cct	att	196	
Lys	Thr	Val	Thr	Ile	Gly	Phe	Gly	Val	Thr	Leu	Cys	Ala	Val	Pro	Ile		
	45				50					55							
gca	cag	aaa	tca	gag	cct	cat	tcc	ctt	agt	agt	gaa	gca	ttg	atg	agg	244	
Ala	Gln	Lys	Ser	Glu	Pro	His	Ser	Leu	Ser	Ser	Glu	Ala	Leu	Met	Arg		
	60				65				70						75		
aga	gca	gtg	tct	ttg	gta	aca	gat	agc	acc	tct	acc	ttt	ctc	tct	cag	292	
Arg	Ala	Val	Ser	Leu	Val	Thr	Asp	Ser	Thr	Ser	Thr	Phe	Leu	Ser	Gln		
				80				85						90			
acc	aca	tat	gcg	ttg	att	gaa	gct	att	act	gaa	tat	act	aag	gct	gtt	340	
Thr	Thr	Tyr	Ala	Leu	Ile	Glu	Ala	Ile	Thr	Glu	Tyr	Thr	Lys	Ala	Val		
			95			100						105					
tat	acc	tta	act	tct	ctt	tac	cga	caa	tat	aca	agt	tta	ctt	ggg	aaa	388	
Tyr	Thr	Leu	Thr	Ser	Leu	Tyr	Arg	Gln	Tyr	Thr	Ser	Leu	Leu	Gly	Lys		
		110				115						120					
atg	aat	tca	gag	gag	gaa	gat	gaa	gtg	tgg	cag	gtg	atc	ata	gga	gcc	436	
Met	Asn	Ser	Glu	Glu	Glu	Asp	Glu	Val	Trp	Gln	Val	Ile	Ile	Gly	Ala		
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aga	gct	gag	atg	act	tca	aaa	cac	caa	gag	tac	ttg	aag	ctg	gaa	acc	484	
Arg	Ala	Glu	Met	Thr	Ser	Lys	His	Gln	Glu	Tyr	Leu	Lys	Leu	Glu	Thr		
140					145					150					155		
act	tgg	atg	act	gca	gtt	ggt	ctt	tca	gag	atg	gca	gca	gaa	gct	gca	532	
Thr	Trp	Met	Thr	Ala	Val	Gly	Leu	Ser	Glu	Met	Ala	Ala	Glu	Ala	Ala		

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tat caa act ggc gca gat cag gcc tot ata acc gcc agg aat cac att			580
Tyr Gln Thr Gly Ala Asp Gln Ala Ser Ile Thr Ala Arg Asn His Ile			
175	180	185	
cag ctg gtg aaa ctg cag gtg gaa gag gtg cac cag ctc tcc cgg aaa			628
Gln Leu Val Lys Leu Gln Val Glu Glu Val His Gln Leu Ser Arg Lys			
190	195	200	
gca gaa acc aag ctg gca gaa gca cag ata gaa gag ctc cgt cag aaa			676
Ala Glu Thr Lys Leu Ala Glu Ala Gln Ile Glu Glu Leu Arg Gln Lys			
205	210	215	
aca cag gag gaa ggg gag gag cgg gct gag tcg gag cag gag gcc tac			724
Thr Gln Glu Glu Gly Glu Arg Ala Glu Ser Glu Gln Glu Ala Tyr			
220	225	230	235
ctg cgt gag gat tga gggcctgagc aactgcccgt gtctccccac tcagtgggga			779
Leu Arg Glu Asp *			

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<210> 14

<211> 239

<212> PRT

<213> Homo sapiens

<400> 14

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20	25 30
Arg Cys Phe Ser Glu Leu Ile Arg Pro Trp His Lys Thr Val Thr Ile	
35	40 45
Gly Phe Gly Val Thr Leu Cys Ala Val Pro Ile Ala Gln Lys Ser Glu	
50	55 60
Pro His Ser Leu Ser Ser Glu Ala Leu Met Arg Arg Ala Val Ser Leu	
65	70 75 80
Val Thr Asp Ser Thr Ser Thr Phe Leu Ser Gln Thr Thr Tyr Ala Leu	
85	90 95
Ile Glu Ala Ile Thr Glu Tyr Thr Lys Ala Val Tyr Thr Leu Thr Ser	
100	105 110

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Leu Tyr Arg Gln Tyr Thr Ser Leu Leu Gly Lys Met Asn Ser Glu Glu
      115                      120                      125
Glu Asp Glu Val Trp Gln Val Ile Ile Gly Ala Arg Ala Glu Met Thr
      130                      135                      140
Ser Lys His Gln Glu Tyr Leu Lys Leu Glu Thr Thr Trp Met Thr Ala
145                      150                      155                      160
Val Gly Leu Ser Glu Met Ala Ala Glu Ala Ala Tyr Gln Thr Gly Ala
      165                      170                      175
Asp Gln Ala Ser Ile Thr Ala Arg Asn His Ile Gln Leu Val Lys Leu
      180                      185                      190
Gln Val Glu Glu Val His Gln Leu Ser Arg Lys Ala Glu Thr Lys Leu
      195                      200                      205
Ala Glu Ala Gln Ile Glu Glu Leu Arg Gln Lys Thr Gln Glu Glu Gly
      210                      215                      220
Glu Glu Arg Ala Glu Ser Glu Gln Glu Ala Tyr Leu Arg Glu Asp
225                      230                      235

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<210> 15

<211> 20

<212> PRT

<213> Homo sapiens

<400> 15

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Cys Gly Pro Lys Tyr Val Val Pro Val Glu Ser Ser Leu Gly Lys Glu
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Lys Gly Leu Glu
      20

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